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Monoclonal antibodies and immune checkpoint inhibitors in the treatment of cancer

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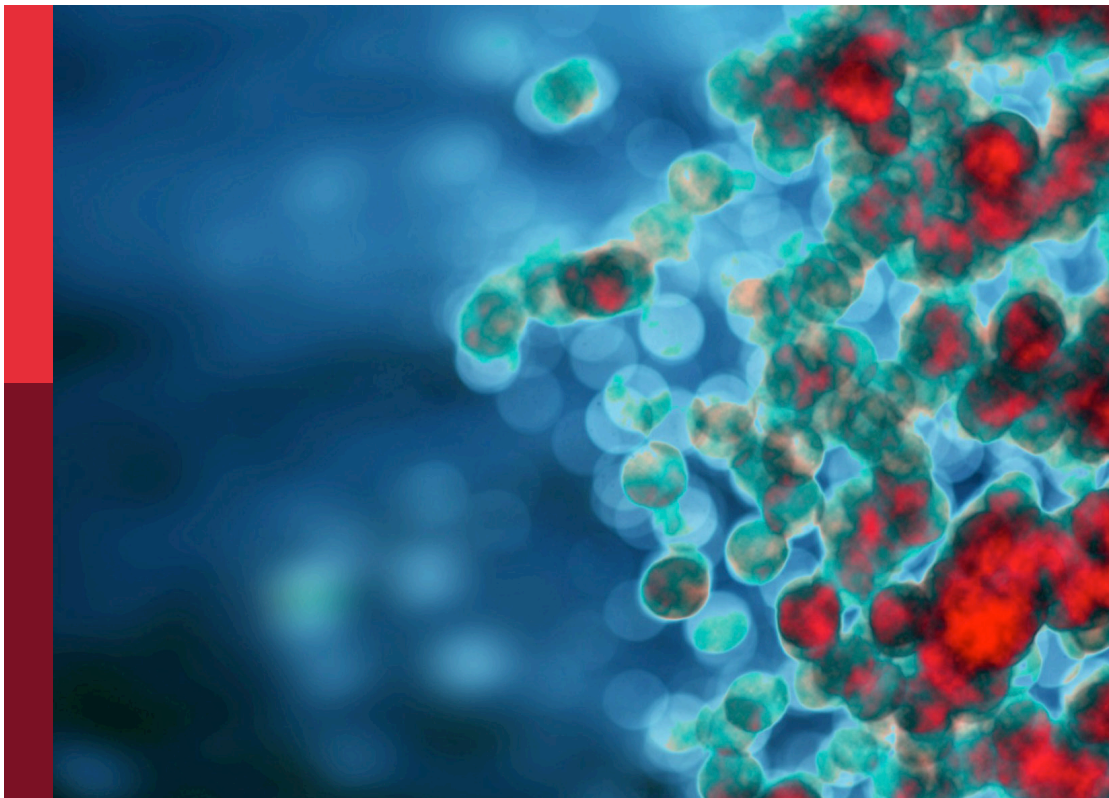

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Monoclonal antibodies and immune checkpoint inhibitors in the treatment of cancer

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Efficacy and safety of first-line immunotherapy plus chemotherapy in treating patients with extensive-stage small cell lung cancer: a Bayesian network meta-analysis

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Background: Despite numerous immunotherapy and chemotherapy regimens available for patients with extensive-stage small cell lung cancer (ES-SCLC), it remains unclear which regimen is the most effective and safest; relative studies comparing such regimens are scarce.

Objective: The aim of this study was to investigate the efficacy and safety of first-line immunotherapy combinations with chemotherapy for patients with extensive-stage small cell lung cancer. In addition, for the first time, comparisons among the first-line systemic regimens on OS and PFS in ES-SCLC by each time node were made.

Methods: Databases including PubMed, Embase, Cochrane Library, Scopus, Google Scholars, and ClinicalTrials.gov, and major international conferences were searched for randomized controlled trials (RCTs) regarding comparing immunotherapy combinations with chemotherapy as first-line treatments for patients with advanced ES-SCLC from inception to 1 November. Hazard ratios (HRs) and odds ratios (ORs) were generated for dichotomous variants by RStudio 4.2.1. The outcomes comprised overall survival (OS), progression-free survival (PFS), objective response rate (ORR), and adverse events of grade 3 or higher (Grade ≥ 3 AEs).

Results: Eventually, a total of nine RCTs reporting 4,352 individuals with nine regimens were enrolled. The regimens were ipilimumabnu (Ipi), atezolizumab (Atez), durvalumab plus tremelimumab (Durv-Trem), durvalumab (Durv), pembrolizumab (Pemb), adebrelimab (Adeb), serplulimab (Serp), atezolizumab plus tiragolumab (Atez-Tira), and nivolumab (Nivo). With regard to OS, serplulimab (HR = 0.63, 95% CI: 0.49 to 0.81) was found to yield the best OS benefit when compared with chemotherapy. Meanwhile, serplulimab had the highest probability (46.11%) for better OS. Furthermore, compared with chemotherapy, serplulimab significantly increased the OS rate from the 6th to the 21st month. With regard to PFS, serplulimab (HR = 0.47, 95% CI: 0.38 to 0.59)

was found to yield the best PFS benefit when compared with chemotherapy. Simultaneously, serplulimab had the highest probability (94.48%) for better PFS. Serplulimab was also a long-lasting first-line regimen in both OS and PFS from a longitudinal perspective. In addition, there was no significant difference among the various treatment options for ORR and grade ≥3 AEs.

Conclusion: Considering OS, PFS, ORR, and safety profiles, serplulimab with chemotherapy should be recommended as the best therapy for patients with ES-SCLC. Certainly, more head-to-head studies are needed to confirm these findings.

Systematic review registration: https://www.crd.york.ac.uk/PROSPERO/, identifier CRD42022373291.

KEYWORDS

extensive-stage small cell lung cancer, immunotherapy, network meta-analysis, efficacy, safety

Background

According to the National Cancer Institute (NCI), the rate of new cases of lung and bronchus cancer was 52.0 per 100,000 persons per year. The death rate was 35.0 per 100,000 persons per year in 2019; there were an estimated 236,740 new cases in 2022 (1). According to the estimates of the National Cancer Center (NCC) of China, approximately 549,800 newly diagnosed lung cancer cases were reported in 2016; 29.7% of all deaths from cancer were ascribed to lung cancer in men and 22.9% in women (2). Small cell lung cancer (SCLC) represents approximately 15% of all lung cancers, which was a high-grade neuroendocrine carcinoma defined by its aggressiveness, poor differentiation, and somber prognosis (3, 4). The veteran's administration lung cancer study categorizes SCLC into limited or extensive-stage disease according to whether the disease is limited to one hemithorax in a field amenable to radiation therapy (5). Despite divergent active treatment, SCLC has a bleak prognosis, with a 5-year survival rate of only approximately 7% due to factors like a high proliferative index, a quick doubling time, and a strong propensity to metastasis (5). Throughout the course of the disease, 50% of patients with SCLC will develop central nervous system (CNS) metastasis (6, 7).

For several decades, platinum drugs (cisplatin or carboplatin) plus etoposide, namely, EP protocol, have been established as the first-line standard treatment protocol for ES-SCLC. However, because of the quick emergence of resistance, the transient benefit of therapy, and the limited efficacy of subsequent lines, the survival outcomes benefit remained poor (8–10). Although some trials in Japan demonstrated that an irinotecan-based regimen as a first-line treatment for ES-SCLC had better PFS, its OS advantage was still vague (11). Thus, the above situation compels physicians and scientists to seek better first-line treatments.

One of the most significant advancements in the treatment of cancer was immunotherapy (IO), particularly immune checkpoint inhibitors (ICIs) that obstruct co-inhibitory molecules such as programmed cell death protein-1 (PD-1) and the associated programmed death ligand 1 (PD-L1) (12–15). Clinical evidence has revealed that anti-PD-L1 monoclonal antibodies like atezolizumab and durvalumab provided additional benefits in both OS and progression-free survival (PFS) when compared with platinum-based chemotherapy as the first-line treatment for patients with ES-SCLC (16–18). The National Comprehensive Cancer Network (NCCN) SCLC panel recommended certain chemotherapy plus immunotherapy regimens as preferred alternatives for patients with ES-SCLC in 2018 (9, 19).

Undoubtedly, randomized controlled trials (RCTs) with placebo are the gold standard for determining the efficacy of novel pharmaceutical treatments (20). Until now, there have been numerous regimens treating ES-SCLC, up to now, simultaneously physicians were trapped with making clinical decisions on which regimen to choose owing to the lack of direct/indirect comparisons among those agents, urgently entailing the launch of relevant studies.

Hence, we conducted a Bayesian network meta-analysis comparing the efficacy and safety of immunotherapy combinations with chemotherapy in treating ES-SCLC to provide more evidence for clinical practice.

Methods

We conducted this meta-analysis in accordance with the Preferred Reporting Items for Systemic Review and Meta-analyses (PRISMA) checklist (Supplementary Table 1). This network meta-analysis (NMA) was performed and reported in accordance with the PRISMA Extension version (PRISMA-NMA) (21). This study

Abbreviations: ES-SCLC, Extensive-stage small cell lung cancer; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; Grade ≥ 3 AEs, adverse events of grade 3 or higher; RCTs, randomized controlled trials; HRs, hazard ratios; ORs, odds ratios; CNS, central nervous system.

protocol has been registered on the international prospective register of systematic review (PROSPERO) (CRD42022373291).

Search strategy

Databases including PubMed, Embase, Cochrane Library, Scopus, Google Scholars, and ClinicalTrials.gov, and major international conferences were searched for RCTs regarding comparing immunotherapy combinations with chemotherapy as first-line treatments for patients with advanced ES-SCLC from inception to 1 November.

The search terms included the following keywords: small cell lung carcinoma, extensive-stage, first-line, immunotherapy, PD-1, PD-L1, CTLA-4, ipilimumab, atezolizumab, durvalumab, pembrolizumab, adebrelimab, serplulimab, tiragolumab, nivolumab, randomized clinical trial, and their related MeSH terms. The detailed strategy is shown in Supplementary Table 2.

Selection and eligibility criteria

Two investigators independently searched and assessed the eligibility of each study by reading the title and abstract or even the full text when necessary.

The inclusion criteria were as follows:

(1) Prospective, randomized, phase 3 or 2, controlled clinical studies.

(2) Eligible patients were newly diagnosed with treatment-naive histologically or cytologically documented ES-SCLC (American Joint Committee on Cancer, 7th edition).

(3) RCTs that used immunotherapy-based combination treatment as first-line treatment settings.

(4) RCTs that used immunotherapy-based combination treatment or placebo treatment as first-line treatment settings.

The exclusion criteria were as follows:

(1) RCTs that were based on overlapping patients.

(2) RCTs with ambiguous clinical outcomes.

Prior to the evaluation of full texts, titles and abstracts were scrutinized to ascertain eligibility. To ensure that the most recent information was included, the abstracts from all the included trials and conferences were double-checked online. Any discrepancies were resolved through discussions with the senior authors.

Data extraction

Essential clinical characteristics extracted from the enrolled studies include the following: trial name, first author, publication sources, year of publication, sample size, patients' age and sex distribution, smoking status, histologic type, PD-L1 expression, and Eastern Cooperative Oncology Group (ECOG) performance status score. The clinical outcomes extracted included hazard ratios (HRs) with corresponding 95% confidence intervals (95% CIs) for OS (randomization to death regardless of any causes) and PFS (randomization to the progression of any causes or death regardless of any causes). Secondary endpoints items consisted of ORR; patients were evaluated as complete response (CR) or partial response (PR) according to the criteria of RECIST version 1.1 or mWHO-best overall response rate (mWHO-BORR, proportion of patients with CR or PR per mWHO), and adverse events of grade 3 or higher (Grade ≥ 3 AEs).

Quality assessment

The quality of the included studies was checked using the Cochrane Risk of Bias Tool in Review Manager 5.3 software (Nordic Cochrane Centre, Copenhagen, Denmark) for RCTs. The data were independently extracted by two investigators (Wenjun Li and Danbei Diwu), and any discrepancies were resolved through discussions with the senior author (Hong Wang).

Statistical analysis

HRs and odds ratios (ORs) were generated for dichotomous variants by using GeMTC (version 0.14.3) and R (version 3.5.3). OS and PFS were reported as HR with an associated 95% CI. ORR and Grade ≥ 3 AEs were reported as OR with an associated 95% CI. As for Rstudio, we set the number of iterations to 300,000 and used the first 20,000 as a burn-in sample (the thinning interval was 10); the surfaces under the cumulative ranking curve (SUCRAs) and matrices were calculated to show pairwise comparisons among regimens on OS, PFS, ORR, and grade≥3 AEs. In addition, the software can calculate the probability that each intervention is rated as the best. Furthermore, trace and density plots as well as convergence plots were generated to determine the degree of convergence. Statistical significance was set as $p < 0.05$.

Results

Baseline characteristics of included studies

We identified a total of 488 records from the databases and 11 additional online records from the conference proceedings during the preliminary literature search. After eliminating the duplicates and non-pertinent articles through abstract screening, 13 articles finally met our eligibility criteria (Figure 1). A total of 4,352 individuals were enrolled to receive the following nine immunotherapy combinations across nine RCT eligible studies: ipilimumab plus etoposide/paclitaxel and platinum (Ipi); atezolizumab plus carboplatin and etoposide (Atez); durvalumab plus tremelimumab plus platinum and etoposide (Durv-Trem); durvalumab plus platinum and etoposide (Durv); pembrolizumab plus etoposide and platinum (Pemb); adebrelimab plus carboplatin and etoposide (Adeb); serplulimab plus carboplatin and etoposide

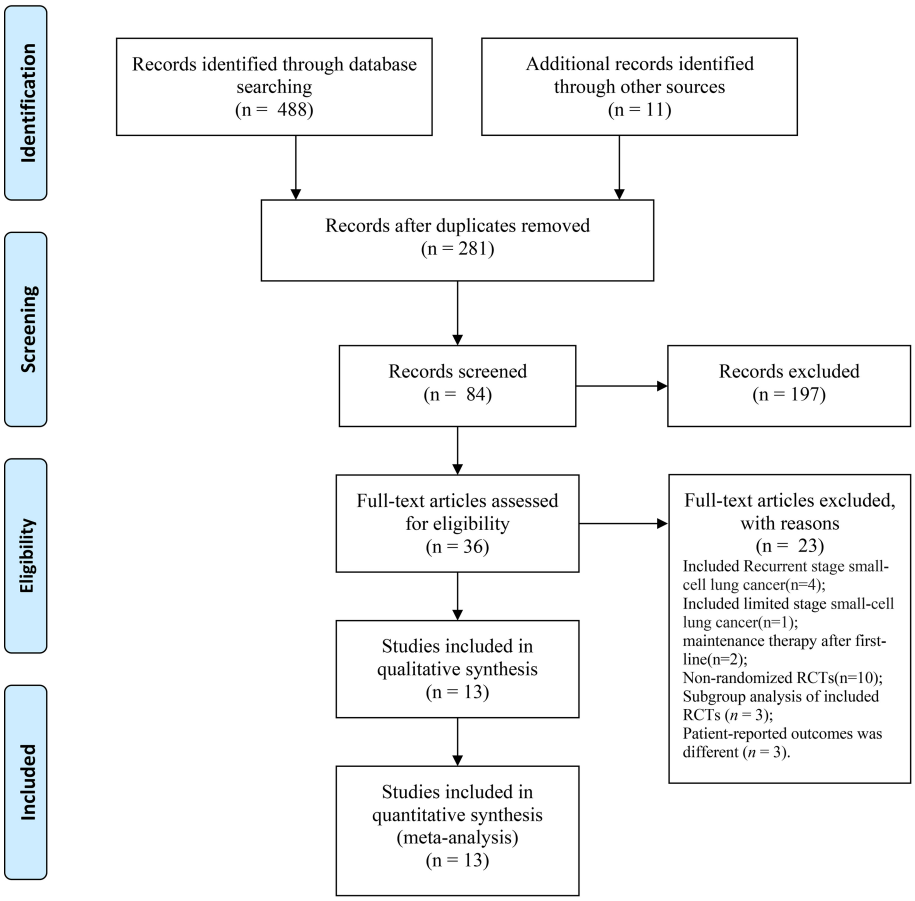

FIGURE 1
PRISMA flowchart illustrating the selection of studies included in our analyses.

(Serp); atezolizumab plus tiragolumab plus carboplatin and etoposide (Atez-Tira); and nivolumab plus carboplatin and etoposide (Nivo). Detailed information on all the included studies is presented in Table 1. All studies' complete outcome reports were achieved, and 10 studies followed the principle of random allocation. All studies were at low risk of bias. The assessment of risk of bias is presented in Supplementary Figure 1. The network plots are depicted in Figure 2.

Comparisons of OS and PFS

Nine studies were randomized studies and provided HR values for PFS and OS (Table 2).

Primary analysis: OS

Regarding OS (Figure 3A), compared with placebo, immunotherapy combined with chemotherapy significantly increased OS except for ipilimumab (HR = 0.92, 95% CI: 0.81 to 1.06) and atezolizumab plus tiragolumab (HR = 1.02, 95% CI: 0.80 to 1.30). Serplulimab (HR = 0.63, 95% CI: 0.49 to 0.81) was found to yield the best OS benefit when compared with placebo. Compared with ipilimumab, serplulimab (HR = 0.68, 95% CI: 0.51 to 0.91) and durvalumab (HR = 0.77, 95% CI: 0.61 to 0.97) significantly increased OS. Compared with atezolizumab plus tiragolumab, serplulimab (HR = 0.62, 95% CI: 0.43 to 0.88), atezolizumab (HR = 0.69, 95% CI: 0.48 to 0.98), durvalumab (HR = 0.70, 95% CI: 0.51-0.94), and adebrelimab (HR = 0.71, 95% CI: 0.51 to 0.98) significantly increased OS. According to Bayesian ranking profiles (Figure 4), serplulimab had the highest probability (46.11%) of ranking first for better OS (Supplementary Table 4).

Regarding the OS for immunotherapy combinations compared to standard chemotherapy, the HRs at the 3rd, 6th, 9th, 12th, 15th, 18th, 21st, and 24th month were examined (Table 2). Compared with placebo, only serplulimab (HR = 2.02, 95% CI: 1.24 to 3.30) significantly increased the 6th month OS rate. Compared with placebo, serplulimab (HR = 1.66, 95% CI: 1.18 to 2.36), durvalumab (HR = 1.71, 95% CI: 1.21 to 2.40), adebrelimab (HR = 1.66, 95% CI: 1.14 to 2.40), atezolizumab (HR = 1.60, 95% CI: 1.08 to 2.38), and nivolumab (HR = 4.03, 95% CI: 1.26 to 12.84) significantly increased the 12th month OS rate. Compared with placebo, serplulimab (HR = 1.72, 95% CI: 1.20 to 2.47), adebrelimab (HR = 1.95, 95% CI: 1.32 to 2.87), and atezolizumab (HR = 1.90, 95% CI: 1.22 to 2.98) significantly increased the 18th month OS rate. However, there was no significant difference in efficacy among all regimens in the 24th month. The first–echelon regimens were

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TABLE 1 Baseline characteristics of studies.

Source	Study	Phase	Treatment	Participants No.	ORR, No./ total No. (%)	PFS, median, m	HR (95% CI)	*p*-value	OS, median, months	HR (95% CI)	*p*-value	Grade ≥ 3 AEs No./ total No. (%)
Reck 2013	CA184-041	III	Concurrent-Ipilimumab plus paclitaxel/carboplatin	43	21/43 (48.83)	5.7 (5.2–6.9)	0.75 (0.48–1.19)	0.11	9.1 (6.7–12.9)	0.95 (0.59–1.54)	0.41	18/42 (43)
					14/43 (32.56)	3.9 (2.9–5.9)	0.93 (0.59–1.45)	0.37				
			Phased-Ipilimumab plus paclitaxel/ carboplatin	42	30/42 (71.43)	6.4 (5.3–7.6)	0.64 (0.40–1.02)	0.03	12.9 (7.9–16.5)	0.75 (0.46–1.23)	0.13	21/42 (50)
					24/42 (57.14)	5.2 (4.14–6.57)	0.93 (0.59–1.48)	0.38				
			Placebo plus paclitaxel/carboplatin	45	24/45 (53.33)	5.3 (4.7–5.7)			9.9 (8.6–11.7)			13/44 (30)
Reck 2016	CA184-156	III	Ipilimumab plus etoposide and platinum	478	297/478 (62.1)	4.6 (4.5–5.0)	0.85 (0.75–0.97)	0.02	11 (10.5–11.3)	0.94 (0.81–1.09)	0.377	231/ 478 (48.3)
			Placebo plus etoposide and platinum	476	196/476 (41.2)	4.4 (4.4–4.6)			10.9 (10–11.5)			214/ 476 (45.0)
Horn 2018	IMpower133	III	Atezolizumab plus carboplatin and etoposide	201	121/201 (60.2)	5.2 (4.4–5.6)	0.77 (0.62–0.96)	0.02	12.3 (10.8–15.9)	0.70 (0.54–0.91)	0.007	115/ 198 (58.1)
			placebo plus carboplatin and etoposide	202	130/202 (64.4)	4.3 (4.2–4.5)			10.3 (9.3–11.3)			113/ 196 (57.7)
Ticiana 2020	EA5161	II	Nivolumab plus cisplatin/carboplatin and etoposide	80	42/80 (52.29)	5.5	0.68 (0.48–1.00)	0.047	11.3	0.73 (0.49–1.1)	0.14	67/75 (89.33)
			cisplatin/carboplatin and etoposide	80	38/80 (47.71)	4.7			9.3			50/70 (71.43)
Paz-Ares 2019-2022	CASPIAN	III	Durvalumab plus tremelimumab plus platinum-etoposide	268	156/267 (58.4)	4.9 (4.7–5.9)	0·84 (0.7–1.01)	NR	10.4 (9.5–12)	0·81 (0·67–0.97)	0·045	196/ 266 (73.68)
			Durvalumab plus platinum-etoposide	268	182/268 (67.9)	5.1 (4.7–6.2)	0·80 (0·66–0.96)	NR	12.9 (11.3–14.7)	0.71 (0.60–0.86)	0.003	171/ 265 (64.53)
			Platinum-etoposide alone	269	156/269 (58.0)	5.4 (4.8–6.2)			10.5 (9.3–11.2)			173/ 266 (65.04)
Rudin 2022	KEYNOTE-604	III	Pembrolizumab Plus Etoposide and Platinum	228	161/228 (70.6)	4.5 (4.3–5.4)	0.75 (0.61–0.91)	0.0023	10.8 (9.2–12.9)	0.80 (0.64–0.98)	0.016	97/223 (43.5)
			Placebo Plus Etoposide and Platinum	225	139/225 (61.8)	4.3 (4.2–4.4)			9.7 (8.6–10.7)			91/223 (40.8)
Wang 2022	CAPSTONE-1	III	Adebrelimab plus carboplatin and etoposide	230	162/230 (70.4)	5.8 (5.6–6.9)	0.67 (0.54–0.83)	<0.0001	15.3 (13.2–17.5)	0.72 (0.58–0.90)	0.0017	197/ 230 (85.65)
			placebo plus carboplatin and etoposide	232	153/232 (65.9)	5.6 (5.5–5.7)			12.8 (11.3–13.7)			197/ 232 (84.91)

(Continued)

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TABLE 1 Continued

Source	Study	Phase	Treatment	Participants No.	ORR, No./ total No. (%)	PFS, median, m	HR (95% CI)	*p*-value	OS, median, months	HR (95% CI)	*p*-value	Grade ≥ 3 AEs No./ total No. (%)
Cheng 2022	ASTRUM-005	III	Serplulimab plus carboplatin and etoposide	389	312/389 (80.2)	5.8 (5.5–6.9)	0.47 (0.38–0.59)	<0.001	15.4 (13.3–NE)	0.63 (0.49–0.82)	<0.01	129/389 (33.2)
			placebo plus carboplatin and etoposide	196	138/196 (70.4)	4.3 (4.2–4.5)			10.9 (10–14.3)			54/196 (27.6)
Rudin 2022	SKYSCRAPER-02	III	Tiragolumab plus atezolizumab + carboplatin + etoposide	243	172/243 (70.8)	5.1 (4.4–5.4)	1.08 (0.89, 1.31)	NR	13.1 (10.9–14.4)	1.02 (0.80, 1.30)	NR	166/239 (69.4)
			atezolizumab + carboplatin + etoposide	247	162/247 (65.6)	5.4 (4.5–5.7)			12.9 (12.1–14.5)			173/246 (70.3)

ES-SCLC, extensive-stage small cell lung cancer; HR, hazard ratio; NR, not reported; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; Grade ≥ 3 AEs, adverse events of grade 3 or higher.

compared to placebo from a longitudinal perspective. With regard to OS, serplulimab, atezolizumab, and durvalumab were first-echelon regimens in the 3rd to 24th month. These data were summarized based on a matrix plot of each pairwise comparison of all regimens on the efficacy across all regimens from the 3rd to 24th months (Supplementary Table 6). Concurrently, it could be seen from the Rank-Heat Plot that each sector was colored according to the surface under the cumulative ranking (SUCRA) value of the corresponding treatment and outcome at each month. Serplulimab has the highest ranking based on its effect compared with the rest of the regimens at each month (Figure 5A).

Primary analysis: PFS

Regarding PFS (Figure 3A), compared with placebo, immunotherapy combined with chemotherapy significantly increased PFS except durvalumab plus tremelimumab (HR = 0.84, 95% CI: 0.70 to 1.01) and atezolizumab plus tiragolumab (HR = 1.08, 95% CI: 0.89 to 1.31). Serplulimab (HR = 0.47, 95% CI: 0.38 to 0.59) was found to yield the best PFS benefit when compared with placebo. Compared with adebrelimab (HR = 0.70, 95% CI: 0.52 to 0.95), pembrolizumab (HR = 0.63, 95% CI: 0.47 to 0.84), atezolizumab (HR = 0.61, 95% CI: 0.45 to 0.83), and durvalumab (HR = 0.59, 95% CI: 0.44 to 0.78), serplulimab significantly increased PFS. According to Bayesian ranking profiles (Figure 4), serplulimab had the highest probability (94.48%) of ranking first for better PFS (Supplementary Table 4).

From the 1st to the 4th month, there was no significant difference in efficacy among all regimens (Table 3). Compared with placebo, serplulimab (HR = 2.67, 95% CI: 1.27 to 5.62) barely significantly increased the 5th month PFS rate. Compared with placebo, serplulimab (HR = 3.31, 95% CI: 2.25 to 4.87), adebrelimab (HR = 1.61, 95% CI: 1.11 to 2.33), pembrolizumab (HR = 1.69, 95% CI: 1.12 to 2.55), and ipilimumab (HR = 1.32, 95% CI: 1.00 to 1.74) significantly increased the 6th month PFS rate. Compared with placebo, adebrelimab and pembrolizumab significantly increased the PFS rate from the 6th to the 12th month. In addition, from the 7th to 11th months, compared with placebo, nivolumab significantly increased the PFS rate. In contrast, the efficacy of atezolizumab plus tiragolumab was poorer than placebo from the 5th to the 12th month. The comparison was made between the first-echelon regimens and placebo from a longitudinal perspective, with regard to PFS, serplulimab, and nivolumab were first-echelon regimens at 1st to 12th month, synchronously, it was the most long-lasting regimen in the first-echelon in PFS. On the other hand, adebrelimab was also a first-echelon regimen compared with placebo at the 1st and the 4th to the 12th month in PFS. These data were summarized based on a matrix plot of each pairwise comparison of all regimens on the efficacy across all regimens from the 1st to 12th months (Supplementary Table 7). Concurrently, it could be seen from the Rank-Heat Plot that serplulimab and nivolumab have a higher ranking based on their effect compared with the rest of the regimens at each month (Figure 5B).

Comparisons of ORR

Regarding ORR (Figure 3B), compared with placebo, except atezolizumab (HR = 1.19, 95% CI: 0.48 to 2.97), immunotherapy combined with chemotherapy non-significantly increased ORR. Here, compared with placebo, atezolizumab plus tiragolumab (HR = 0.79, 95% CI: 0.32 to 1.96) non-significantly increased ORR. According to Bayesian ranking profiles (Figure 4), serplulimab had the highest probability (31.09%) of ranking first for better ORR (Supplementary Table 4).

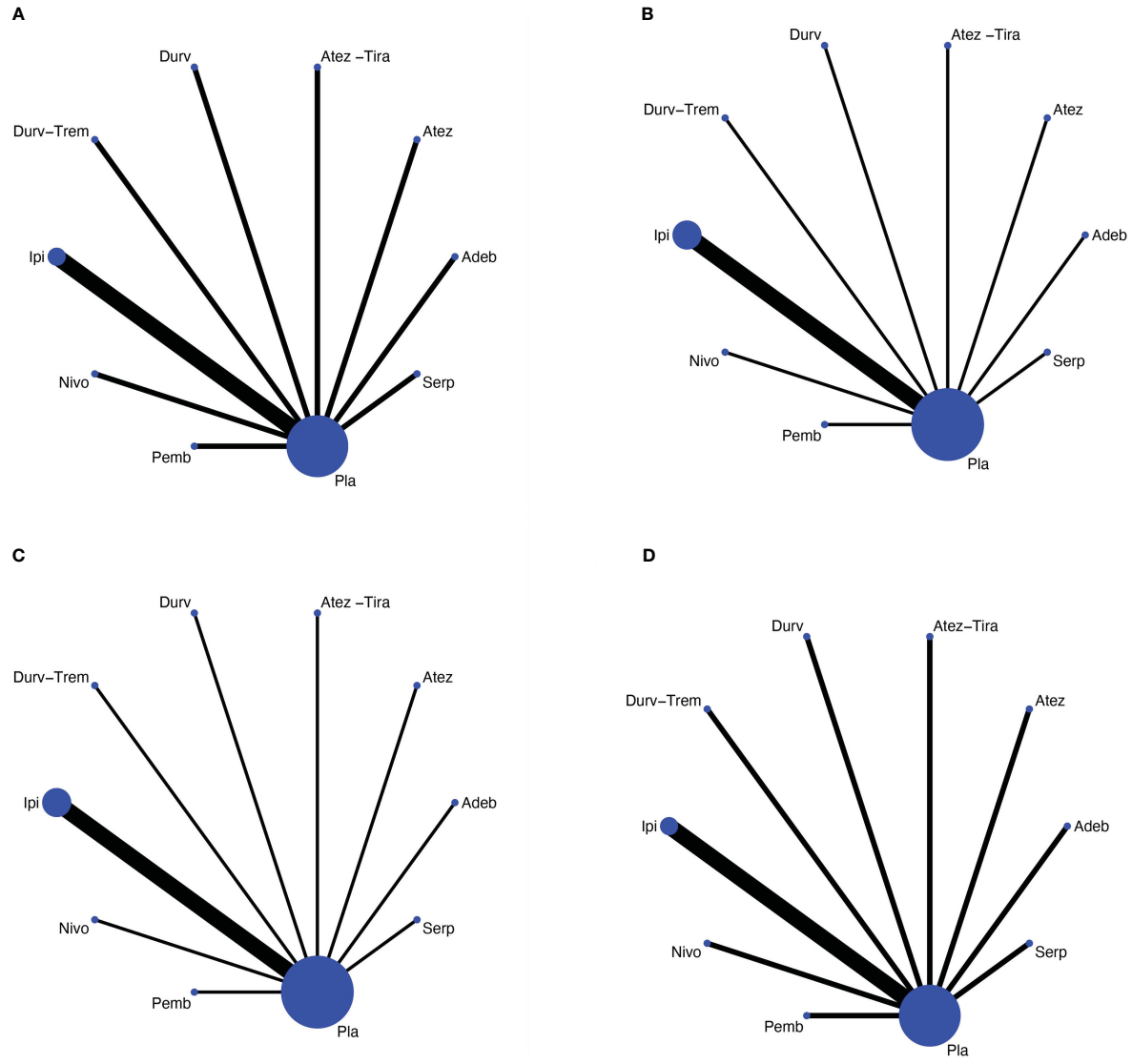

FIGURE 2
Network meta-analysis of comparisons on different outcomes of first-line treatments in different groups of ES-SCLC patients. **(A)** Comparison of overall survival (OS). **(B)** Comparison of progression-free survival (PFS). **(C)** Comparison of objective response rate (ORR). **(D)** Comparison of grade 3 or more adverse events. Direct comparisons are represented by the color lines connecting the treatments. Line width is proportional to the number of trials including every pair of treatments, whereas circle size is proportional to the total number of patients for each treatment in the network. Nivo, Nivolumab; Atez-Tira, Atezolizumab + Tiragolumab; Atez, Atezolizumab; Serp, Serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + Tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, Ipilimumab.

Comparisons of safety and toxicity

Compared with placebo, the immunotherapy combined with chemotherapy elevated the toxicity whereas there was no significant difference between the various treatment options for safety and toxicity (Figure 3B). According to Bayesian ranking profiles, the following analysis was conducted (Figure 4), nivolumab had the highest probability (69.06%) of ranking first of being the most toxicity treatment for patients (Supplementary Table 4). AEs with a grade greater than or equal to 3 that were frequently reported for the immunotherapy combinations included neutropenia, leukopenia, thrombocytopenia, anemia, diarrhea, vomiting, decreased, appetite, nausea, fatigue, rash, pruritus, alopecia, constipation, hypothyroidism, hyperthyroidism, and pneumonitis (Supplementary Table 5). In the chemotherapy plus ipilimumab arm, there were five treatment-related deaths, one from liver toxicity.

Subgroup analysis based on CNS status

Only OS network meta-analysis could be carried out, and it involved eight immunotherapy combinations for patients without CNS metastases at baseline and seven immunotherapy combinations for patients with CNS metastases (Supplementary Table 8). We did not have enough data to perform a meta-analysis on PFS in the subgroup of brain metastases. There was no significant difference

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TABLE 2 HR and 95% CI on 3rd, 6th, 9th, 12th, 15th, 18th, 21st, and 24th month OS for immunotherapy combinations compared to placebo.

Time (months)	Serp	Atez	Durv	Adeb	Nivo	Atez-Tira	Durv-Trem	Pemb	Ipi	Pla
3rd	1.29 (0.22,7.53)	1.31 (0.23,7.65)	1.04 (0.20,5.47)	–	2.61 (0.43,15.98)	1.36 (0.24,7.74)	–	–	–	Reference
6th	**2.02 (1.24,3.30)**	1.34 (0.78,2.29)	1.04 (0.68,1.60)	–	1.74 (0.90,3.36)	1.05 (0.61,1.79)	–	1.07 (0.69,1.64)	1.08 (0.78,1.48)	Reference
9th	**1.72 (1.19,2.50)**	1.31 (0.87,1.96)	1.42 (0.99,2.01)	0.97 (0.63,1.47)	**3.00 (1.23,7.30)**	1.09 (0.71,1.67)	1.06 (0.75,1.49)	1.14 (0.79,1.65)	1.04 (0.81,1.34)	Reference
12th	**1.66 (1.18,2.36)**	**1.60 (1.08,2.38)**	**1.71 (1.21,2.40)**	**1.66 (1.14,2.40)**	**4.03 (1.26,12.84)**	–	1.17 (0.83,1.65)	1.33 (0.92,1.94)	1.17 (0.91,1.50)	Reference
15th	**2.05 (1.44,2.92)**	**1.97 (1.30,2.97)**	**1.53 (1.07,2.18)**	**1.58 (1.09,2.28)**	16.43 (0.92,292.62)	–	1.19 (0.83,1.70)	**1.62 (1.09,2.41)**	1.18 (0.90,1.55)	Reference
18th	**1.72 (1.20,2.47)**	**1.90 (1.22,2.98)**	1.42 (0.98,2.08)	**1.95 (1.32,2.87)**	–	–	1.37 (0.94,2.00)	1.49 (0.97,2.27)	1.22 (0.89,1.67)	Reference
21st	**6.28 (3.68,10.73)**	1.52 (0.89,2.59)	1.43 (0.90,2.29)	**1.97 (1.22,3.17)**	–	–	1.42 (0.89,2.27)	**1.87 (1.10,3.18)**	1.19 (0.53,2.67)	Reference
24th	6.09 (0.68,54.14)	1.38 (0.15,12.28)	1.83 (0.21,16.16)	2.19 (0.25,19.27)	–	–	1.80 (0.20,15.84)	2.35 (0.26,21.16)	2.66 (0.26,27.37)	Reference

Nivo, nivolumab; Atez-Tira, tiragolumab; Atez, atezolizumab; Serp, serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, ipilimumab.
Significant results were in bold.

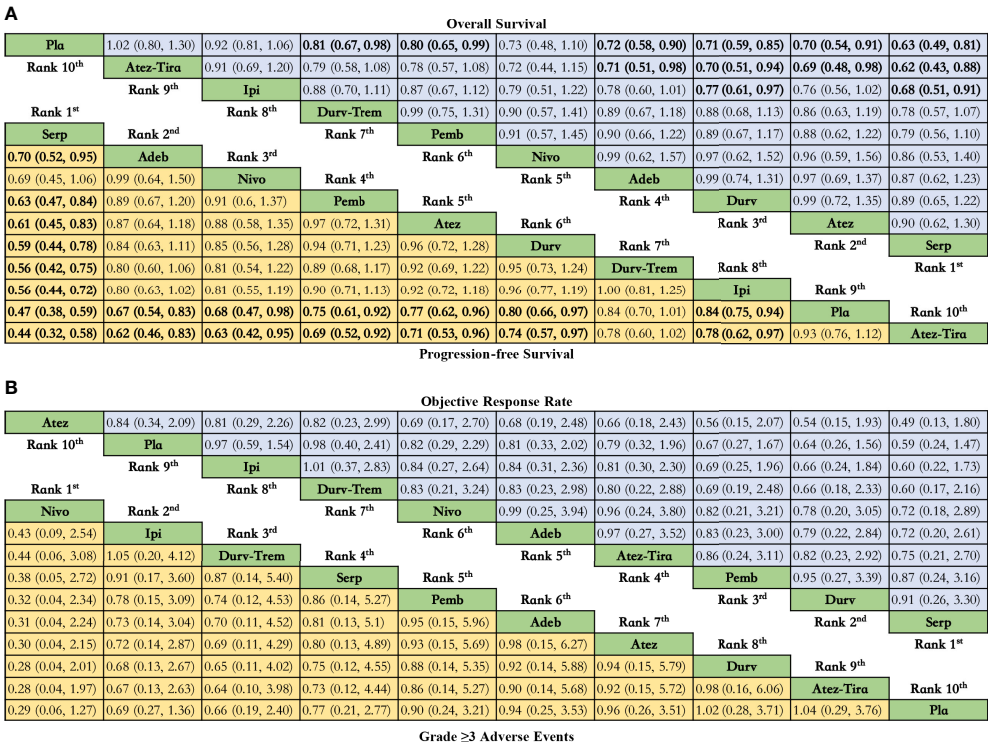

FIGURE 3
Efficacy and safety profiles of the Bayesian network meta-analysis in patients with ES-SCLS. **(A)** HRs and 95% CI for overall survival (upper triangle in blue) and progression-free survival (lower triangle in yellow), and a hazard ratio < 1.00 provides better survival benefits. **(B)** ORs and 95% CI for objective response rate (upper triangle in blue) and grade ≥ 3 adverse events (lower triangle in yellow), and an OR < 1.00 indicates a better efficacy or more toxicity. The results are presented as column-defined treatment versus row-defined treatment. Significant results are in bold. Nivo, Nivolumab; Atez-Tira, Atezolizumab + Tiragolumab; Atez, Atezolizumab; Serp, Serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + Tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, Ipilimumab.

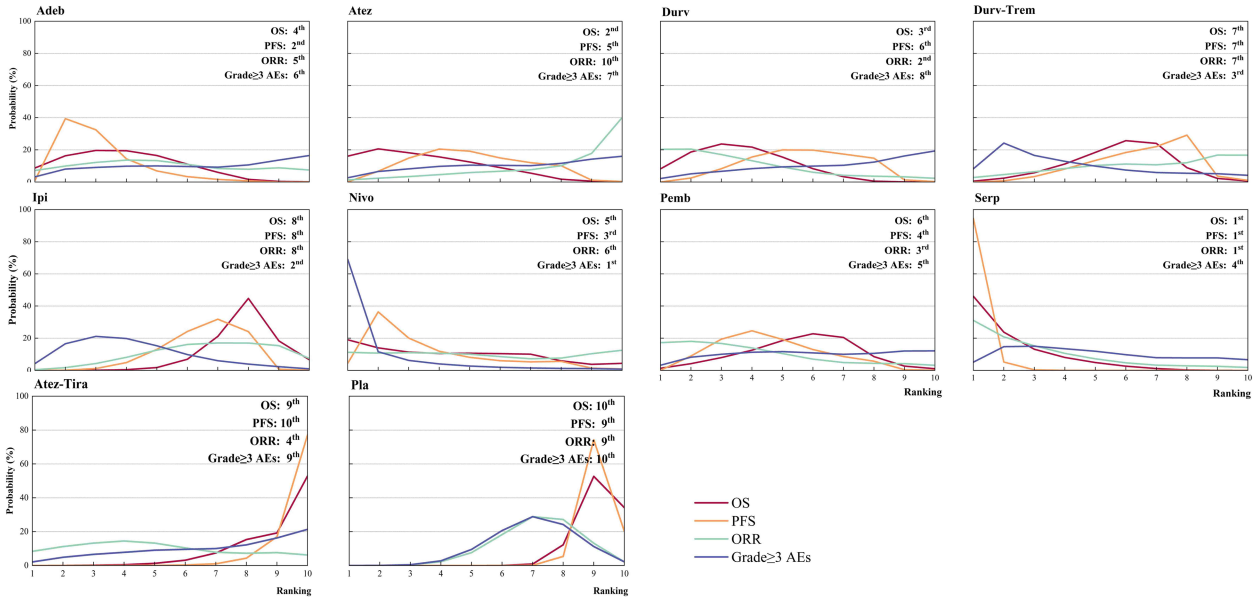

FIGURE 4
Bayesian ranking profiles for immunotherapy combinations on efficacy and safety for patients with ES-SCLC. Ranking plots indicate the probability of each comparable immunotherapy combination being ranked from first to last on OS, PFS, ORR, and grade ≥ 3 AEs. Nivo, Nivolumab; Atez-Tira, Atezolizumab + Tiragolumab; Atez, Atezolizumab; Serp, Serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + Tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, Ipilimumab.

between the various treatment options for patients with CNS metastases. In contrast, compared with placebo, serplulimab (HR = 0.62, 95% CI: 0.47 to 0.82), adebrelimab (HR = 0.68, 95% CI: 0.55 to 0.85), atezolizumab (HR = 0.68, 95% CI: 0.52 to 0.89), durvalumab (HR = 0.71, 95% CI: 0.59 to 0.86), pembrolizumab (HR = 0.75, 95% CI: 0.60 to 0.94), and durvalumab plus tremelimumab (HR = 0.79, 95% CI: 0.65 to 0.95) significantly increased OS for patients without CNS metastases (Figure 6).

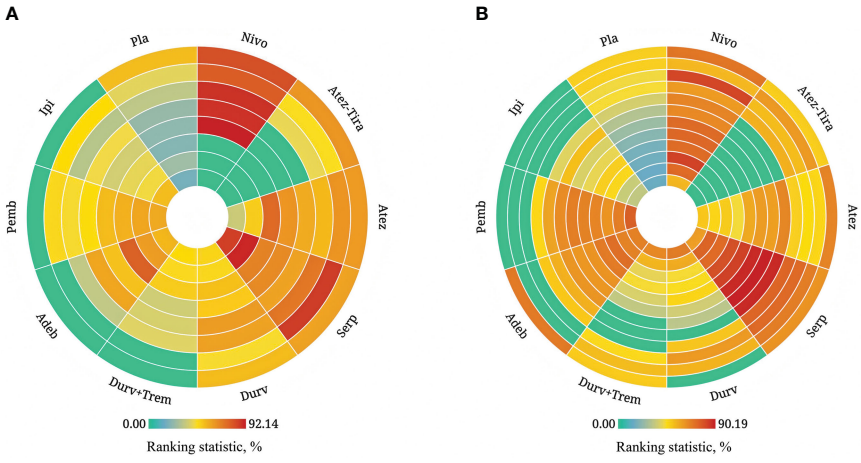

FIGURE 5
Rank-heat plot of multiple therapies in first-line treatment of patients with ES-SCLC. Each sector was colored according to the surface under the cumulative ranking (SUCRA) value of the corresponding treatment and outcome. **(A)** Rank-heat plot based on SUCRA on OS. **(B)** Rank-heat plot based on SUCRA on PFS. Circles from outside to inside refer to SUCRA value of OS on 3rd, 6th, 9th, 12th, 15th, 18th, 21st, and 24th month for immunotherapy combinations compared to chemotherapy, and SUCRA value of PFS on 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, and 12th month. The closer the color is to red, the greater the probability of ranking first, and the closer the color is to green indicates 0% probability of being ranked first. Nivo, Nivolumab; Atez-Tira, Atezolizumab + Tiragolumab; Atez, Atezolizumab; Serp, Serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + Tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, Ipilimumab.

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TABLE 3 HR and 95% CI on 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th, 10th, 11th, and 12th month PFS for immunotherapy combinations compared to placebo.

Time (months)	Serp	Atez	Durv	Adeb	Nivo	Atez-Tira	Durv-Trem	Pemb	Ipi	Pla
1st	1.99 (0.12,31.98)	1.99 (0.18,22.10)	–	1.99 (0.18,22.10)	2.08 (0.50,8.63)	1.77 (0.51,6.14)	–	–	–	Reference
2nd	2.21 (0.02,239.30)	–	1.27 (0.01,134.76)	–	1.22 (0.01,146.78)	1.61 (0.01,177.60)	1.14 (0.01,120.44)	–	–	Reference
3rd	1.96 (0.39,9.89)	1.03 (0.21,5.10)	1.18 (0.12,11.27)	–	2.61 (0.44,15.56)	1.21 (0.24,6.11)	1.06 (0.22,5.09)	–	–	Reference
4th	2.09 (0.28,15.58)	1.08 (0.14,8.05)	1.15 (0.16,8.48)	1.13 (0.15,8.53)	1.50 (0.19,12.07)	1.27 (0.17,9.55)	–	1.04 (0.14,7.72)	–	Reference
5th	**2.67 (1.27,5.62)**	1.54 (0.72,3.30)	–	1.29 (0.60,2.76)	1.57 (0.64,3.89)	–	–	1.35 (0.64,2.87)	1.00 (0.50,1.99)	Reference
6th	**3.31 (2.25,4.87)**	**1.56 (1.00,2.43)**	–	**1.61 (1.11,2.33)**	1.74 (0.90,3.36)	–	–	**1.69 (1.12,2.55)**	**1.32 (1.00,1.74)**	Reference
7th	**3.77 (2.52,5.63)**	**1.81 (1.10,2.96)**	1.14 (0.78,1.67)	**1.81 (1.10,2.96)**	**2.40 (1.15,5.03)**	–	1.08 (0.74,1.59)	**1.81 (1.10,2.96)**	**1.19 (0.88,1.60)**	Reference
8th	**3.92 (2.54,6.05)**	**1.98 (1.16,3.39)**	**1.61 (1.08,2.41)**	**2.26 (1.47,3.46)**	**3.00 (1.23,7.30)**	–	1.34 (0.89,2.02)	**2.48 (1.48,4.16)**	**1.31 (0.95,1.82)**	Reference
9th	**3.56 (2.26,5.61)**	**1.58 (0.90,2.80)**	**1.63 (1.04,2.55)**	**2.68 (1.63,4.41)**	**3.03 (1.19,7.72)**	–	**1.59 (1.02,2.49)**	**2.52 (1.38,4.61)**	**1.59 (1.09,2.32)**	Reference
10th	**3.51 (2.20,5.62)**	**1.98 (1.06,3.72)**	**1.78 (1.11,2.87)**	**3.42 (1.91,6.15)**	**5.13 (1.64,16.02)**	–	**1.66 (1.02,2.68)**	**2.71 (1.44,5.10)**	**1.80 (1.20,2.71)**	Reference
11th	**4.02 (2.42,6.69)**	**2.10 (1.09,4.05)**	**2.64 (1.54,4.55)**	**3.34 (1.85,6.00)**	**4.03 (1.26,12.84)**	–	**2.51 (1.46,4.33)**	**3.34 (1.85,6.00)**	1.47 (0.96,2.23)	Reference
12th	**3.21 (1.92,5.37)**	**2.47 (1.18,5.16)**	**3.97 (2.13,7.40)**	**3.89 (2.07,7.31)**	2.65 (0.89,7.90)	–	**3.68 (1.97,6.87)**	**4.90 (2.11,11.38)**	1.42 (0.88,2.28)	Reference

Nivo, nivolumab; Atez-Tira, tiragolumab; Atez, atezolizumab; Serp, serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, ipilimumab.
Significant results were in bold.

Discussion

To the best of our knowledge, this was the first network meta-analysis to compare the relative efficacy of all current available first-line immunotherapy combinations for ES-SCLC, which is more comprehensive than previously published studies (22, 23). In addition, this was the first network meta-analysis to make comparisons among the first-line systemic regimens on OS and PFS in ES-SCLC by each time node.

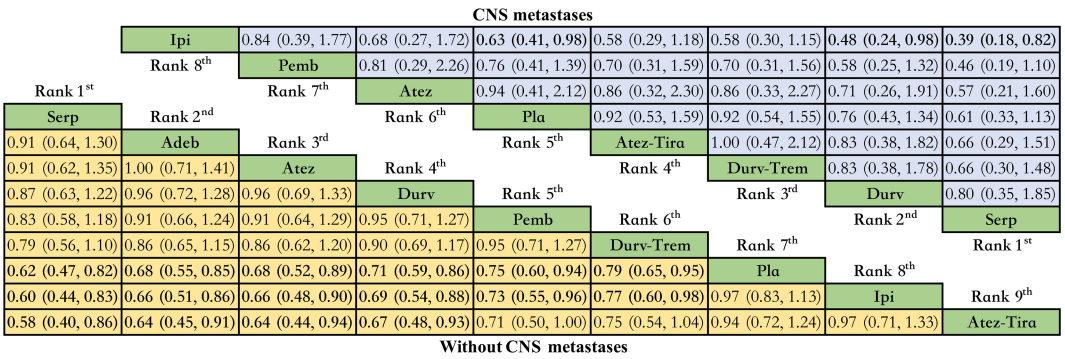

FIGURE 6
Bayesian network meta-analysis in patients with ES-SCLC. Hazard ratios and 95% CIs for patients with the baseline CNS metastases (upper triangle in blue) and for patients without baseline CNS metastases (lower triangle in yellow), and a hazard ratio < 1.00 provides better survival benefits. Hazard ratios less than 1 favor row defining treatment. Significant results are in bold. Nivo, Nivolumab; Atez-Tira, Atezolizumab + Tiragolumab; Atez, Atezolizumab; Serp, Serplulimab; Durv, Durvalumab; Durv-Trem, Durvalumab + Tremelimumab; Pla, Placebo; Adeb, Adebrelimab; Pemb, Pembrolizumab; Ipi, Ipilimumab.

Our analysis results indicated that the immunotherapy–chemotherapy combination strategy showed significant efficacy for OS compared with placebo, except ipilimumab and tiragolumab plus atezolizumab. According to Bayesian ranking profiles, serplulimab had the highest probability for better OS, followed by atezolizumab and durvalumab, with the same results as before (23). In addition, we proved for the first time that among the first–echelon regimens compared to placebo from a longitudinal perspective, serplulimab, atezolizumab, and durvalumab were first-echelon regimens at the 3rd to 24th month on OS. These findings indicated that they may be related to better long-term survival benefits of patients with ES-SCLC. As for PFS, the immunotherapy combinations revealed better PFS than chemotherapy. The only exception was also tiragolumab plus atezolizumab, which was found to have the worst PFS of all treatments. According to Bayesian ranking profiles, serplulimab had the highest probability for better PFS, with the same results as before (23). Furthermore, in our study, serplulimab and nivolumab were first-echelon drugs from the 1st to the 12th month in PFS and had a faster onset of action compared with placebo.

In this study, efficacy and safety were well balanced in the serplulimab group, which ranked first for OS, PFS, and ORR, and fourth for grade greater than or equal to 3 AEs across all immunotherapy combinations. Serplulimab recently became the first anti-PD-1 antibody, when combined with chemotherapy, demonstrates significant improvement in the survival rates of patients with ES-SCLC (24, 25). According to our research results, serplulimab could be a first-echelon regimen because, first, it takes effect sooner and, second, the patients who benefit from it can experience long-lasting effects. Recently, serplulimab received its first approval in China for the treatment of adult patients with advanced unresectable or metastatic microsatellite instability-high (MSI-H) solid tumors that have failed to respond to previous standard treatments (26). Prior to our study, PD-L1 inhibitors might be preferred for patients with ES-SCLC, and atezolizumab and durvalumab were approved by Food and Drug Administration (FDA) as first-line treatment for patients with ES-SCLC based on the primary data from IMpower133 (27, 28) and CASPIAN (29, 30). Our study also found that the addition of atezolizumab to chemotherapy was associated with the best benefit in survival outcomes but not in ORR, with the same results as before (23). The ORR of the atezolizumab and placebo was 60.2% vs. 64.4%, respectively (17). In addition, the 3-year OS rate of durvalumab was 17.6%, which was nearly three times higher than that of chemotherapy, and the long-term survival benefit was significant. The results showed that the combination of durvalumab and EP regimen could significantly improve the OS of patients, while the combination of durvalumab and tremelimumab plus the EP regimen did not further improve the survival prognosis of ES-SCLC (10.4 vs. 10.5 months; HR = 0.81, 95% CI: 0.67 to 0.97) (31). A final analysis of a recent phase 3 clinical trial (CAPSTONE-1) showed that adebrelimab combined with carboplatin and etoposide improved the OS and PFS of ES-SCLC patients (32). In contrast, the experimental results are not very ideal for PD-1 antibody pembrolizumab and nivolumab; in terms of PFS, the efficacy of pembrolizumab and nivolumab was significantly increased compared with chemotherapy, and the secondary endpoint of OS was also improved. However, there was no significant difference between the two groups (33, 34). In terms of safety and toxicity, consistent with expectations, the immunotherapy–chemotherapy combination strategy did not observe unexpected safety events; all adverse events were controllable. A review of the included studies revealed that anti-PD-1/PD-L1 combinations with chemotherapy were relatively safe. However, toxicity increased, but remained tolerable, when anti-CTLA4/TIGIT and chemotherapy were combined (35–38). Furthermore, inclusion of nivolumab may significantly increase AEs according to our results. The PD-1 and PD-L1 antibodies were the most typical inhibitors of immunological checkpoints, with the primary function of the PD-1/PD-L1 pathway being to induce tumor cells to evade immune attacks (27). Preclinical research showed that chemotherapy altered the immune response against tumor cells and increased PD-L1 expression on tumor cells; additionally, while not reducing the number of T cells in the tumor, chemotherapy can lessen the activation and proliferation of T cells in peripheral blood (39). Head-to-head comparisons are still needed to confirm the efficacy of PD-1 and PD-L1 antibodies for patients with ES-SCLC.

According to the result of CA184-041 and CA184-156 studies, ipilimumab could significantly improve the PFS of patients with ES-SCLC; however, it could not significantly improve the OS in our study; this study confirmed the feasibility of immunotherapy combination strategy for ES-SCLC (37, 40). Ipilimumab was a monoclonal antibody (IgG1) that blocks cytotoxic T-lymphocyte-associated protein 4 (CTLA4) through its association with CD28 and enhances the T-cell response (41). SKYSCRAPER-02 evaluated the addition of tiragolumab to atezolizumab plus carboplatin and etoposide (CE), which did not provide an antitumor effect and survival benefits in patients with untreated ES-SCLC with or without brain metastases. Although the remission rate of tiragolumab was higher, it was of little significance and did not meet the prediction of ES-SCLC response rate for first-line treatment (42). Comparing Impower133 and SKYSCRAPER-02, the control arm outperformed expectations in the SKYSCRAPER-02 study, which was likely the cause of negative endpoints, in addition to the fact that an enhanced benefit in the tiragolumab arm was not seen. However, the reason for this is unclear, and further research is needed (43). TIGIT was an inhibitory receptor expressed on CD4+T cells, effector CD8+T cells, and NK cells. TIGIT interacts with CD155 expressed on antigen-presenting cells or tumor cells to downregulate T-cell and natural killer (NK) cell functions; moreover, anti-TIGIT may synergize with other immunotherapies, such as PD-L1/PD-1 inhibitors, and further amplify the immune response to improve clinical outcomes (44). However, increasing only TIGIT antibody does not appear to increase the efficacy in the tumor microenvironment where there are fewer tumor-infiltrating lymphocytes, according to some studies (45). In conclusion, adding immunosuppressive drugs to the immunological checkpoint alone does not appear to be a breakthrough in the treatment of ES-SCLC without the supervision of biomarkers.

For subgroup analysis, single metastatic sites were favorable prognostic factors in patients with ES-SCLC (5). These data support the idea that patients with asymptomatic CNS metastasis can receive first-line systemic treatment (15, 42). More ongoing

clinical trials will shed further light on the safety and efficacy of immunotherapy combination with chemotherapy strategy in patients with CNS metastasis (46, 47).

Immunotherapy combination was the focus of ES-SCLC treatment, and it had higher tumor mutation load (TMB) and higher total immune cell infiltration, suggesting that it may show a greater benefit trend in immunotherapy (48, 49). Whether there were differences in tumor microenvironments between different molecular subtypes of SCLC is also a matter of concern. Recently, some real-world research findings with large sample sizes have further validated the notion that the differential expression of immune genes and predictive biomarkers in various SCLC subtypes might serve as vulnerable areas where rational and personalized treatment strategies can be targeted (50, 51). At the same time, increasing lines of evidence prove that SCLC has different cell origins, suggesting that SCLC was a heterogeneous disease. It might be a feasible strategy to improve the treatment dilemma of SCLC by molecular typing of SCLC through differences in molecular expression, exploring the characteristics of the tumor microenvironment of different molecular subtypes of SCLC, and formulating accurate treatment (50, 52). Therefore, patients with SCLC still urgently need therapeutic drugs with different mechanisms of action. In the realm of future exploration, a crucial direction lies in establishing an organic connection between key factors of SCLC molecular typing and tumor evolution. This could be accomplished through comprehensive multi-group research, aiming to identify targeted treatment strategies.

Limitation

First, we came up with a very comprehensive search strategy; however, regrettably, publication bias limitations could have resulted from missing unpublished literature.

Second, owing to the limited number of studies that met our inclusion criteria, the inclusion of eligible studies with small sample sizes presumably increased the overall uncertainty of our results.

Third, patients were not stratified according to factors like race, which might modify treatment benefits, and the efficacy of immunotherapy combined with chemotherapy in the Asian population may differ from that in the Western population. Subsequent studies should investigate the relative treatment efficacy according to these clinical characteristics.

Conclusion

According to our findings from this research, serplulimab combined with standard chemotherapy appears to be the best course of treatment. More head-to-head clinical trials are needed to confirm these findings.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

TZ wrote the manuscript and contributed to the data analysis and interpretation. WL and DD extracted data and ensured the accuracy of the data analysis. LC and XC contributed to the data interpretation and critical revision of the manuscript for important intellectual content. HW obtained funding and approved the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1197044/full#supplementary-material

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Bispecific antibody targeting TGF-β and PD-L1 for synergistic cancer immunotherapy

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The PD-1/PD-L1 signaling pathway plays a crucial role in cancer immune evasion, and the use of anti-PD-1/PD-L1 antibodies represents a significant milestone in cancer immunotherapy. However, the low response rate observed in unselected patients and the development of therapeutic resistance remain major obstacles to their clinical application. Accumulating studies showed that overexpressed TGF-β is another immunosuppressive factor apart from traditional immune checkpoints. Actually, the effects of PD-1 and TGF-β pathways are independent and interactive, which work together contributing to the immune evasion of cancer cell. It has been verified that blocking TGF-β and PD-L1 simultaneously could enhance the efficacy of PD-L1 monoclonal antibody and overcome its treatment resistance. Based on the bispecific antibody or fusion protein technology, multiple bispecific and bifunctional antibodies have been developed. In the preclinical and clinical studies, these updated antibodies exhibited potent anti-tumor activity, superior to anti-PD-1/PD-L1 monotherapies. In the review, we summarized the advances of bispecific antibodies targeting TGF-β and PD-L1 in cancer immunotherapy. We believe these next-generation immune checkpoint inhibitors would substantially alter the cancer treatment paradigm, especially in anti-PD-1/PD-L1-resistant patients.

KEYWORDS

cancer immunotherapy, immunotherapy resistance, immune checkpoint inhibitor, bispecific antibody, TGF-β, PD-L1

Abbreviations: APC, antigen presentation cell; BiTE, bispecific T-cell engager; BsAb, bispecific antibody; BTK, Bruton's tyrosine kinase inhibitor; CAF, carcinoma-associated fibroblast; CRS, cytokine release syndrome; CRI, cancer-related inflammation; DC, dendritic cell; IL-2, interleukin-2; MAPK, mitogen-activated protein kinase; MDSC, myeloid-derived suppressor cell; NK, natural killer; NSCLC, non-small cell lung cancer; ORR, objective response rate; PD-1, programmed cell death protein 1; PI3K-AKT, phosphoinositide-3-kinase-serine/threonine kinase; scFv, single-chain fragment variable; TGF-β, transforming growth factor-beta; TGFβRI, TGF-β type I receptor; TGFβRII, TGF-β type II receptor; TME, tumor microenvironment; Treg, regulatory T cell; TandAb, Tandem diabody.

1 Background

Programmed cell death 1 (PD-1) is a crucial signaling pathway that inhibits the immune response and helps maintain immune homeostasis (1). However, when overactivated in the tumor microenvironment, this pathway hinders host immune surveillance and clearance of tumor cells (2). Monoclonal antibodies targeting PD-1 or its ligand PD-L1 can restore the activity of exhausted immune cells and enhance the killing effect on tumor cells by blocking this immunosuppressive signaling (3–5). While anti-PD-1/PD-L1 monoclonal antibodies have been clinically approved for treating multiple malignancies and have exhibited promising anti-tumor effects in some patients, the low objective response rate of patients remains a challenge (6). In fact, the cancer-immunity cycle model suggests that in addition to the highly activated PD-1/PD-L1 pathway, multiple factors may become rate-limiting steps that restrict the anti-tumor immune response. Several studies have demonstrated that the activity of the TGF-β pathway in immunotherapy-resistant tumor is significantly increased (7, 8). The highly expressed TGF-β in the tumor microenvironment is also involved in cancer immune escape (9). The immunosuppressive mechanisms of TGF-β and PD-1 pathways are independent and complementary to each other, jointly promoting tumors to escape from host immune surveillance (10).

Highly expressed TGF-β in tumor tissues is primarily secreted by tumor cells and stromal cells. Highly expressed TGF-β not only promotes the epithelial-mesenchymal transition of tumor cells, but also regulates multiple tumor-infiltrating immune cells, leading to the formation of the immunosuppressive tumor microenvironment (11–14). On the one hand, TGF-β suppresses the functions of $CD8^+$ T cells and natural killer cells (NK), and on the other hand, upregulates the numbers of regulatory T cells (Treg), M2-like macrophage, and myeloid-derived suppressor cells (MDSCs) (15–18). In addition, it has been confirmed that the high TGF-β tumor microenvironment can improve the activity of tumor-associated fibroblasts (CAFs) and promote the generation of collagen fibers in tumor stroma. The thickened collagen fibers around the tumor tissue are not conducive to immune cell infiltration, eventually forming the immune-excluded tumor type (7). Commonly, this type of tumor does not respond to anti-PD-1/PD-L1 monoclonal antibodies, while blocking TGF-β signaling can significantly reverse the therapeutic resistance of PD-1/PD-L1 blockade therapy and enhance anti-tumor immunotherapy effects (19–22). Theoretically, agents simultaneously blocking TGF-β and PD-1/PD-L1 pathways might have superior anti-tumor activity, relative to anti-PD-1/PD-L1 monoclonal antibodies.

Currently, Merck reported a bifunctional antibody called M7824 that simultaneously blocks PD-L1 and TGF-β (23). M7824 combines PD-L1 antibody with a trap structure targeting TGF-β, acting as a neutralizing receptor for TGF-β. Phase I clinical data indicate that the side effects of M7824 treatment are manageable and therapeutic effects have been observed in multiple types of cancers (24). Later, more bispecific antibodies (BsAbs) such as YM101 and BiTP are developed, which also exhibit potent anti-tumor activities in preclinical and clinical studies (25, 26). BsAbs targeting both PD-1/PD-L1 and TGF-β represent a significant breakthrough and an upgrade to current PD-1/PD-L1 monoclonal antibodies. By synergistically blocking both PD-1/PD-L1 and TGF-β inhibitory signals, these antibodies can effectively promote the transformation from immune-excluded tumors into immune-inflamed tumors. This can improve the efficacy of current PD-1/PD-L1 monoclonal antibodies and broaden their anti-tumor effects spectrum. In this review, we provide a summary of the recent advances in anti-TGF-β/PD-L1 BsAb development. Additionally, we discuss both the advantages and disadvantages of this next-generation immune checkpoint inhibitor.

2 The present status of PD-1/PD-L1 blockade

PD-1 is a pivotal immune regulation signal molecule distributed in a wide breadth of immune cells, including DC, T cells, B cells and natural killer cells (NK), and activated monocytes or macrophages (27). PD-1, together with its ligands, PD-L1 and PD-L2, was found to take immunosuppressive effects in the antiviral inflammation and the tumor microenvironment (28, 29). Contemporarily, monoclonal antibodies targeting PD-1/PD-L1 have been widely utilized in clinical settings and exhibited remarkable therapeutic efficacy against various malignancies, particularly advanced and refractory tumors.

2.1 The biogenesis and biological pathway of PD-1/PD-L1

PD-1 was initially discovered to play a role in immune suppression of inflammation by serving as a negative feedback regulator. The two ligands, PD-L1 and PD-L2, activate and transmit inhibitory signals to target cells. Relative to PD-L2, PD-L1 is more widely distributed, particularly on tumor cells (27). Recent research has shed light on the elaborated and intriguing expression patterns of PD-L1, which is now known to be present not only on cell membrane, but on various cellular compartments and secreted in extracellular vesicles (30). Specifically, PD-L1 has been found to localize endocellularly on endosomes, the Golgi apparatus's membrane, and endocytic vesicles. PD-L1 has also been detected in extracellular vesicles, which are involved in intercellular communication and the exchange of biological material between cells. These findings offer fascinating new prospects and possibilities for the development of novel therapeutic strategies targeting PD-L1 in cancer therapy (31). The PD-1/PD-L1 pathway exerts immune regulatory effects via recognition of effector T cells in the inflammatory context, with persistently high expression on activated T cells. Cytokines across the extracellular interval of tissue cells induce and modulate the expression of PD-L1, blunting the activation of T cells, and consequently resulting in immune homeostasis that the immune system eliminates exogenous microbiota while attenuating damage on normal tissue cells

simultaneously. The most important inductive cytokine is IFN-γ, mainly derived from Th1 cells (32). PD-1 is persistently expressed at a high level in the circumstance of ongoing inflammation. As a result, persistently high expression of PD-1 triggers T cell exhaustion or inactivation (33).

Mechanistically, the immune system relies on a complex network of interactions between different cells and molecules to mount an effective response against invading pathogens or endogenous abnormalities, including tumor cells. One crucial aspect of this system is the ability of T cells to recognize and respond to antigens presented by other cells. In this bioprocess, MHC II molecules on antigen presentation cells (APCs) or MHC I molecules on all the karyocytes present fragments of antigens on cell membrane, which can be recognized by the T cell receptor (TCR) (34). This interaction activates T cells and triggers a cascade of signaling events that lead to the proliferation and differentiation of T cell, as well as the secretion of cytokines and other effector immune molecules. However, to prevent excessive immune activation and tissue damage, the immune system could dampen or terminate immune responses. Simultaneously with TCR-induced cascade, PD-1 begins to be expressed on the activated T cells. PD-1 and PD-L1 are brought into close proximity to each other in the microscale spatial structure. Subsequently, Immunoreceptor Tyrosine-based Inhibitory Motif (ITIM) and Immunoreceptor Tyrosine-based Switch Motif (ITSM) domain of PD-1 receptor are phosphorylated (35). The phosphorylated domains recruit tyrosine phosphatases, SHP-2 and SHP-1, which are capable of impeding critical factors in TCR signaling (36–38).

Consequently, T cell activation and function are blunted, restraining the degree and duration of the immune response. In addition to signaling repression, PD-1 can also interfere with the recognition of tumor cells by directly dampening the trimeric interaction between the TCR, pMHC, and CD8 molecules (39). This further contributes to immune evasion and tolerance by tumor cells and highlights the importance of PD-1/PD-L1 blockade as a promising immunotherapy strategy for cancer treatment.

2.2 The present approved PD-1/PD-L1 blockade therapies

As of March 2023, 21 PD-1/PD-L1 blockade drugs are available worldwide (Figure 1) (40–48). Herein, Nivolumab and Pembrolizumab are the most widely used PD-1/PD-L1 blockers and have received approval for the most indications. Notably, Cadonilimab is the first approved anti-PD-1/CTLA-4 BsAb for cervical cancer (44). More drugs targeting these immune checkpoints are in development, and clinical trials are underway to explore new indications for existing drugs. New drugs need to be tested against drugs already on the market to show better efficacy or performance. However, primary or acquired treatment resistance of PD-1/PD-L1 blockades has become an interactive conundrum for both physicians and tumor patients (49). Combination therapy regimens containing PD-1/PD-L1 inhibitors and other agents might be promising strategies for overcoming treatment resistance (50–52).

2.3 The challenges of PD-1/PD-L1 blockades in cancer therapy

Although PD-1/PD-L1 blockers have achieved an unprecedented breakthrough in cancer therapy, there have been increasing concerns about their disadvantages and insufficiency in the clinic (53, 54). Besides the adverse events and hyper progression associated with PD-1/PD-L1 blockade therapies (55–57), there is a

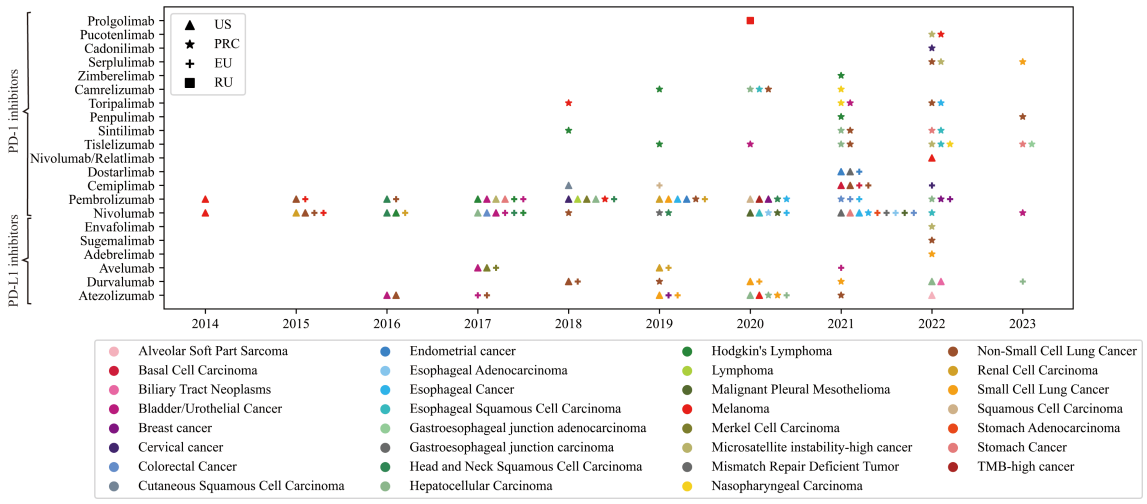

FIGURE 1
The details of approved PD-1/PD-L1 blockades in the United States of America, European Union, People's Republic of China, and Russian Federation. Triangle symbol indicates that the corresponding indication of the drug has been approved by the United States Food and Drug Administration (FDA); Stellated symbol indicates that the corresponding indication of the drug has been approved by the National Medical Products Administration (NMPA) of People's Republic of China. Similarly, other symbols represent drug approvals by corresponding regulatory agencies in European Union and Russian Federation. The abscissa represents the time of the approval. The color of the icon represents the approved indication.

prevalent concern regarding the limited response rate among cancer patients (58). Commonly, the therapeutic effect of anti-PD-1/PD-L1 antibodies mainly depends on the PD-L1 expression status, such as tumor proportion score (TPS), combined positive score (CPS), and immune cell proportion score (IPS) (59). However, a universe evaluation criterion is absent (60). Moreover, recent investigations suggest that single PD-L1 status is not a reliable indicator of predicting the response of patients to PD-1/PD-L1 blockade (61). Nevertheless, for patients without sufficient histopathologic evidence, the response rate to PD-1/PD-L1 blockades is less than 17% (62). Other responding assessing systems intervene to mount the accuracy, which hinges on the genomic instability, which is defined by tumor mutational burden (TMB) and microsatellite instable (MSI) (63–65). However, there are more or less deficiencies with these systems (66). The low response is partially attributed to the discrepancies in immune cell infiltration phenotype. In theory, the tumor immune microenvironment is classified histopathological as three phenotypes, inflamed, immune-excluded, and immune-desert, respectively. They are inextricably intertwined with immune cytokines level, which includes IFN-γ and TGF-β, fatty acid metabolism, neuroendocrine features, and EMT phenotype (67). Only the first tumor immune type benefits from the immune checkpoint inhibitors (68), but the proportion is less than 50% (67). The immune-excluded type takes up 30 to 50 percent of colorectal and ovarian cancer (69, 70). Herein, TGF-β exerts essential effects for hindrance of immune surveillance and tumor elimination in the immune-excluded type (7). The exploitation of an immunotherapeutic strategy that combines PD-1/PD-L1 blockades and TGF-β inhibiting or trap medicine is a promising direction.

3 The effects of TGF-β on anti-tumor immunity

3.1 TGF-β signaling

TGF-β superfamily consists of more than 40 members, mainly classified as four subtypes, the TGF-β subtype, the bone morphogenetic protein-growth differentiation factor (BMP-GDM), activin-inhibin-nodal and others, orchestrates in the biological processes of carcinoma initiation, progression, and immune elimination (71–77). TGF-β, the most classic subtype, is a highly conserved and distributed breadth of the organism in the mammal. Three isoforms, TGF-β1, β2, and β3, are highly conserved with 80% of the same amino sequence, despite being encoded by separate genes. However, they still exhibit slight discrepancies in structural and bio-functional aspects, which can be recapitulated that TGF-β1 is more tendentious to immune regulation (78). It is secreted into the extracellular matrix, initially exists in an inactive form of latent precursor, and readily exerts biological functions in the tumor microenvironment via autocrine and paracrine (79). TGF-β is transcribed into a polypeptide, which is then cleaved by the Furin proteinase into two subunits. These subunits are further reassembled into an inactivated form that is secreted out of the cells. The extracellular inactivated TGF-β exists as a large complex, with the dimeric regulatory subunit called the long latency-associated peptide (LAP) forming the peripheral compound. The initial segment of LAP is a short signal peptide called the arginyl-glycyl-aspartic acid site (RGD). The bioactive catalytic subunit is ensconced internally, noncovalently combined with and wrapped around by the dimeric LAPs (72). In most cases, the cage-like complex could anchor via a disulfide bond to a compound of extracellular matrix, namely latent TGFβ-binding protein (LTBP), for stabilization. Additionally, the complex could also bind to transmembrane milieu proteins, particularly glycoprotein A repetitions predominant (GARP) on Treg and negative regulator of reactive oxygen species (NRROS) on microglia or macrophage (80–82). In the extracellular space, physical and chemical perturbance or serine protease would cleave and separate the dimeric LAPs from the complex to release the bioactive subunit. But integrin complexes capable of transmitting force derived from cytoskeleton across the cytomembrane, play the most prominent activated executor role (83). β-αv integrin heterodimer, particularly, recognizes the RGD motif, further interdigitates with the LAP, and consequently, changes autologous conformation. The allosterism tightens and gradually tears the "sleeve" of the latent TGF-β off and exposes the internal bioavailable TGF-β (83). Nevertheless, a unique kind of integrin is able to transduce the signaling without tearing LAP off and releasing the internal bioactive TGF-β (84). The free activated TGF-β or integrin αvβ8-latent TGF-β complex is capable of attaching to their three isoforms of the specific receptor, namely TGFBR1, 2, and 3, in divergent degrees of affinity (85). TGFBR3 doesn't possess kinase activity like the others but can bind with all types of TGF-β with a high affinity therein. Therefore, it was previously believed to sequester and hinder the redundant TGF-β signaling (86). Recent studies recover its crucial roles in cellular signaling transduction in TGF-β dependent or independent manners (87, 88).

Bioavailable TGF-β could bind with TGFBR2 on the plasm membrane of specific cells. TGFBR1, subsequently, is recruited by the signaling complex, and together companies into a transmembrane heterotetrameric signaling complex. The endo-domain of the allosteric complex is phosphorylated. Then, the signaling cascade is triggered off. There are two signaling processes after the TGF-β-TGFBR1/2 tetrameric complex phosphorylated (10).

3.1.1 Canonical downstream signaling pathway

The Smad2/3 are firstly recruited by the transmembrane signaling and ulteriorly phosphorylated. The phosphorylation induces the binding of Smad4. Then, phosphorylated-Smad2/3/4 (Phospho-Smad complex) is assembled and translocates into the nucleus. As a result, the ultimate signaling complex induces downstream alterations in gene expression (89).

3.1.2 Noncanonical downstream signaling pathway

The redundantly initial transmembrane signaling could cross the recognition of Smad2/3, and directly activate the downstream

signaling, such as phosphoinositide-3-kinase-serine/threonine kinase (PI3K-AKT) and mitogen-activated protein kinase (MAPK). The PI3K-AKT and MAPK signaling ulteriorly cause respective downstream cascades, which regulate the physiologic and pathologic alterations (90).

3.2 TGF-β signaling around the development of cancer

The smoldering cancer-related inflammation (CRI) accompanied by the development of cancer, with multitudinous kinds of immune cells and immune regulatory cytokines and chemokines pervading across mesenchyme is the persistent intrinsic characteristic of the tumor microenvironment (91). TGF-β, as a pleiotropic cytokine, exerts nuanced, complicated, and even contradictory biological regulatory functions with the development of cancer. In general, TGF-β stimulates the proliferation, transformation, and motility of mesenchyme-originated cells, while inhibiting proliferation and promoting differentiation of epithelium-originated cells and hemopoietic cells (92, 93). In the physiologic condition and early stage of cancer, TGF-β across mesenchyme delicately induces cell cycle arrest and inhibits cell proliferation through the canonical pathway. In the progression of malignancy, the loss of function mutations across the TGF-β pathway and rewiring TGF-β bioprocess make TGF-β a mutineer against tumor suppression signal network, to elicit tumor unconfinedly growing (94). The stimulation of TGF-β, the other cytokines, and chemokines in the tumor microenvironment transforms normal fibroblasts into CAF (95, 96). CAF can prompt tumor progression versatilely (97). In this condition, the tumor cells and cancer-associated fibroblasts excessively secret the amount of TGF-β to the extracellular matrix (97, 98). TGF-β also induces tumor cell EMT, a critical biologic process for migration and invasion, and biological features robustly associated with metastasis (99, 100). Besides, TGF-β can enhance angiogenesis which is beneficial to tumor growth and metastasis through either intracellular pathways or indirectly mediating EMT (101, 102). Apart from the aforementioned direct effects, TGF-β also assumes the paramount role in tumor immunity, indirectly influencing tumor cells throughout the tumor initiation and progression (Figure 2).

3.2.1 TGF-β signaling in the tumor immune system

TGF-β is a widely distributed signaling molecule involved in the regulation of almost all kinds of immune cells in the tumor microenvironment. Foxp3 positive Treg, particularly terminally differentiated effector Treg cells, plays a crucial role in tumor immune evasion, suppressing immune recognition and diminishment and ensuring tumor development and metastasis (103). With the development of genetic tracing and multiple-colors flow cytometer analysis, the lineage derivation of Treg has been demonstrated as dual origins, thymic Treg (tTreg) or natural Treg (nTreg), and peripheral Treg (pTreg) or alias-induced Treg (iTreg) (104–106). tTreg, which constitutes 80% of the total Treg repertoire, is derived from CD4-positive T cells that are induced by moderately robust co-stimulation of the TCR and a series of soluble cytokines, such as interleukin-2 (IL-2), IL-7, and IL-15, secreted by other autoreactive immunocytes in the thymus medulla. These cells express Foxp3 more stably and strongly, which is the major component of immune regulation (107–109). The pTreg, despite minor proportion, plays a comprehensive and cryptic role in local immune regulation and immune homeostasis in a Foxp3-independent way (16, 110). TGF-β plays nuanced, complicated, and pleiotropic roles in Tregs of both origins. In thymus medulla, it is highly enriched. The activated TGF-β participates in promoting the differentiation of Treg and the negative selection of neonatal T cells (108). Nevertheless, it is reported when TGF-β signaling is depleted, medullary thymic epithelial cells are stimulated, acting as "caregivers" of Treg cells and ultimately increasing the number of Treg cells (111). It suggests the multiplicity of TGF-β signaling on tTreg.

In the induction of pTreg, TGF-β accompanied with other immune regulatory signals, is capable of boosting Foxp3 expression on mature CD4 positive T cells in the peripheral. In the tumor microenvironment, excessive TGF-β from tumor cells and CAF not only suppresses the proliferation of other normal epithelial cells and conventional immune cells, but facilitates the transformation of pTreg (11). An aforesaid TGF-β anchored transmembrane milieu protein, GARP, is located on Treg, which is essential to extracellular latent TGF-β stabilization. Besides, the GARP on Treg could interact with integrin αvβ8, which also widely distributes on the Treg (112, 113). Both transmembrane proteins orchestrate more efficiently in the signaling transduction on Treg without breaking latent TGF-β off (113). Therefore, the increased pTreg in the local tumor context accelerates the transmission of TGF-β through the GARP pathway. To summarize, TGF-β prompts the activation of Treg of both origins, through whose pathway cancer cells trigger immune evasion and immunotherapy resistance.

The understanding of tumor-infiltrating B cells and their regulatory molecules remains vague. A regulatory type of B cells is identified as IgA positive and capable of inhibiting $CD8^+$ T cell activation in colorectal tumors (114). Breg can also secret TGF-β, contributing to the apoptosis of effector T cells in the tumor microenvironment (115).

TGF-β can inhibit immunological surveillance and conventional effector immune cells in the tumor-developing stage. On the one hand, TGF-β directly dampens immunological surveillance by targeting cytotoxic lymphocytes (CTL) (15). On the other hand, it can prevent mature inflammation dendritic cells (DC) infiltration and induces the tolerogenic DC to indirectly deceive the surveillance (116, 117). Although tumor antigen bypasses or breaks through the first immunosuppression barrier to prime and activate the antigen immunity, TGF-β can suppress igniting CRI by decreasing total effector immune cells repertoire by reducing IL-2 secreted (118). Moreover, it precludes the transformation from naïve T cells to Th1/Th2 cells (119–121), but prompts the transformation to anti-inflammation Th17 cells (122, 123). In the natural immunity of tumor context, TGF-β polarizes tumor-associated macrophages to M2 (124), and reprogram tumor-associated neutrophil (125), both of which are detrimental to tumor immune elimination (126). Additionally, TGF-β inhibits the activation and functions of natural killer cells by suppressing the mTOR pathway (81, 127).

4 Advances of BsAb

BsAb is a powerful tool for improving the limited sensitivity and effectiveness of traditional antibodies in immunotherapy (128, 129). BsAb can broaden the range of applications by targeting two different molecules and enhancing the anti-tumor effects (130–132). There are two primary types of BsAbs, depending on the presence of the Fc region. The first type is the Fc-containing BsAb, also known as the IgG-like molecule. This type of antibody exerts Fc-mediated effects, such as antibody-dependent cellular cytotoxicity or phagocytosis, and complement-dependent cytotoxicity (133, 134). IgG-like molecules also have a longer half-life than fragment-based molecules (135). Alternatively, the antigen-binding site can be directly connected by a peptide, which

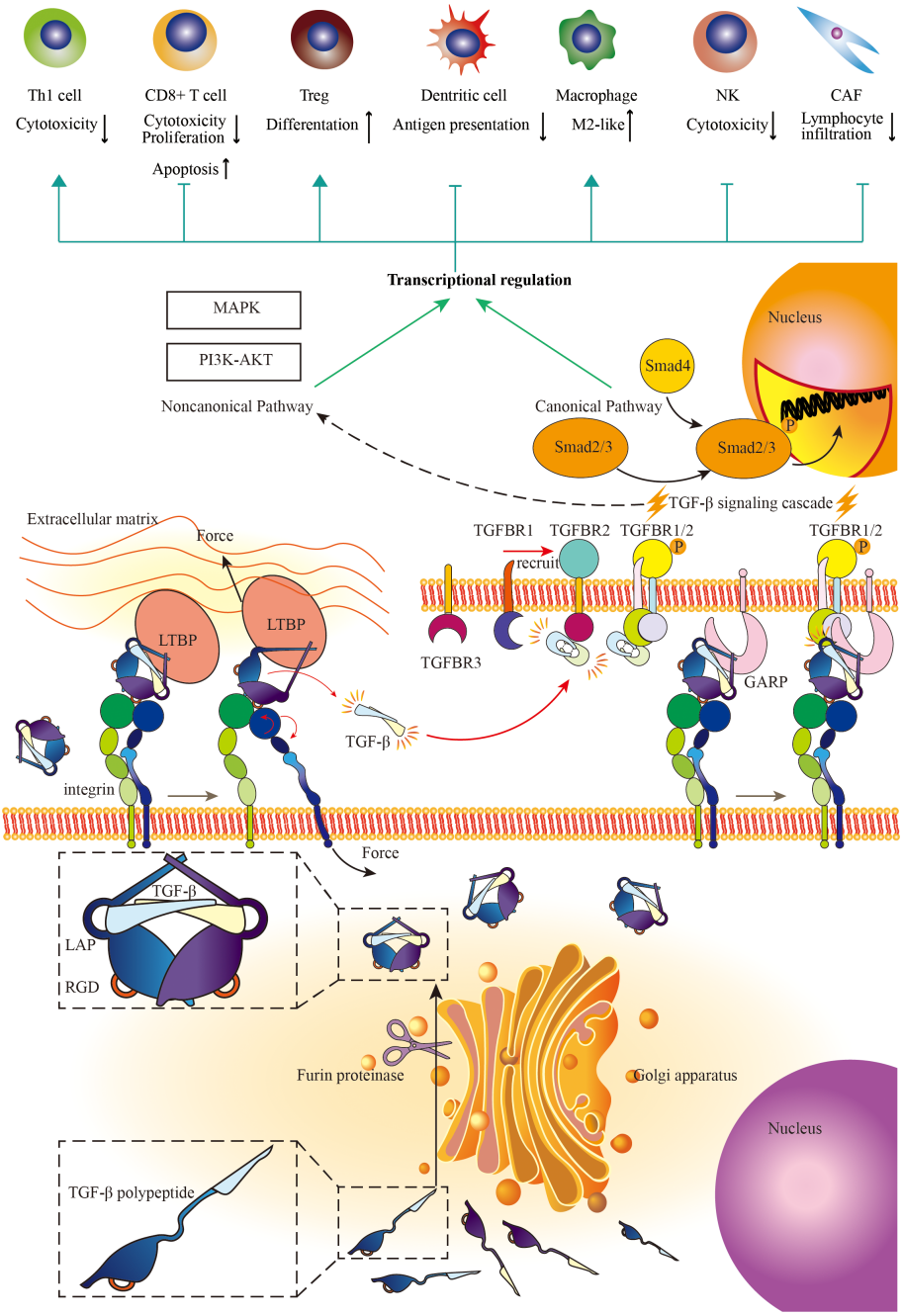

FIGURE 2
The mechanisms of TGF-β signaling and the immunosuppressive effects of TGF-β signaling on the immune response. Cells with yellow nuclei refer to immune cells and CAFs regulated by TGF-β signaling pathway. Cells with purple nuclei refer to TGF-β-producing cells (mainly secreted by fibroblast). TGF-β is a widely distributed signaling molecule involved in the regulation of almost all kinds of immune cells in the tumor microenvironment. On the one hand, TGF-β limits the functions of T cell, NK, and dendritic cell (DC). On the other hand, TGF-β promotes the differentiation towards Treg and M2-like macrophage. Besides, TGF-β enhances cancer-associated fibroblast (CAF) to generate collagen, which hampers immune cell infiltration. Adapted from Yi et al, 2022 (10).

lacks the Fc region, known as the fragment-based molecule. This type of antibody has demonstrated flexibility in targeting tumor cells. Additionally, fragment-based molecules have promising potential to develop multi-specific antibodies (136). BsAbs can be developed using one of three methods: genetic or protein engineering, chemical conjugation, and quadroma (137). With these manufacturing methods, a vast array of BsAbs has been developed and tested in clinical trials.

The targeted antigens of BsAb are diverse. To summarize, the main targets include EpCAM, CEA, PSMA, ErbB, GPC3, immune checkpoints like PD-1 and CTLA-4, DLL4, and VEGF (138). The therapeutic effect of BsAb mainly regulates tumor immune response, which can be divided into two parts: immune cell redirection and anti-tumor immunity enhancement. Physiologically, immune cells, especially $CD8^+$ T cells, detect and kill potential tumor cells. During tumorigenesis, multiple dysfunctions of T cells result in cancer immune escape (139).

Some BsAbs have two types of binding antigens: a specific tumor-associated antigen (TAA) and an extracellular CD3 subunit located on the T cell surface. This kind of antibody is called T cell-engaging BsAbs (TCE). Thus, TAAs direct T cells to targeted tumor cells. TCE was first introduced in 1985 and has rapidly developed at the beginning of the 21st century (140). Catumaxomab and blinatumomab are representatives of TCE. The TAAs of the two antibodies are EpCAM and CD19 (141, 142). Additionally, other TAAs such as CD20, CEA, gpA33, EGFR variant III, PSMA, MUC-1, glypican-3, P-cadherin, B7-H3, and even intracellular antigens can be TAA of TCE (143–151). Another mechanism of BsAb is anti-tumor immunity enhancement. To achieve this effect, this kind of antibody mainly blocks immune checkpoints. The blockade of innate immune checkpoints like CD47 can disrupt the antiphagocytic signals expressed by tumor cells and enhance phagocytosis by macrophages (152–154). Immune checkpoints of T cell activity can also be blocked to enhance adaptive immunity. PD-1/PD-L1, CTLA-4, LAG3, and TIM3 are receptors of coinhibitory immune checkpoints. The blockade of these molecules resuscitates the function of tumor-infiltrating T cells in various kinds of cancers in 10%-30% of patients (155). Molecules in the TNF receptor superfamily and glucocorticoid-induced TNFR-related protein are receptors of costimulatory immune checkpoints. Using an agonist to activate the receptors can reverse the suppression of CTL and promote tumor cell death (156). The combination of blockade of coinhibitory molecules and activation of costimulatory molecules also achieves promising anti-tumor effects *in vivo* (40, 157, 158). The immune cell redirection and anti-tumor immunity enhancement can also be combined to achieve a robust anti-tumor effect *in vivo* (159).

However, there are some adverse effects of BsAb that cannot be eliminated at present. Since BsAb primarily regulates the immune response through signaling pathways and cytokines, the most common adverse effect is cytokine release syndrome (CRS) (160, 161). CRS is caused by a large number of cytokines, such as IFN-γ secreted by activated T cells (162, 163). They can suppress the overstimulated inflammatory reactions without significantly reducing the anti-tumor effect (147). Cytokine receptor antagonism can block the receptors of overexpressed cytokines and relieve the symptoms of CRS (164, 165). However, to prevent the potential immune suppressive effect of corticosteroids, small molecule inhibitors have been developed. This type of inhibitor specifically targets the signaling molecules involved in CRS induction. Since most cytokines promote inflammation via the JAK/STAT pathway in CRS, preclinical studies of JAK1/2 inhibitors have shown promising efficacy in preventing CRS (166). In addition, Bruton's tyrosine kinase inhibitors (BTK) can directly bind to B cells to reduce the overexpression of cytokines caused by BTK signaling, preventing CRS without affecting anti-tumor efficacy (167).

5 Anti-TGF-β/PD-L1 bsAb (including bifunctional protein)

Given the high expression levels and specific enrichment of both PD-L1 and TGF-β in the TME, reagents that target them simultaneously could provide more precise targeting of cancerous lesions while sparing normal tissues. As a result, bispecific antibodies (BsAbs) may accumulate in the TME, reducing side effects and improving tumoral precision therapy.

5.1 YM101 and BiTP

YM101 is the world's first publicly reported anti-TGF-β/PD-L1 bsAb (25). Although fusion protein M7824 has been published before, it is the first time to target these two molecules by bsAb technology. YM101 is the first molecule developed based on the Check-BODY™ technology platform (Figure 3) (25). The results showed that YM101 could effectively antagonize the biological effects of TGF-β and PD-1/PD-L1 pathways. In addition, *in vivo* experiments showed that the anti-tumor activity of YM101 was superior to that of anti-TGF-β and anti-PD-L1 monotherapies. Investigations into the TME found that YM101 promoted the formation of inflamed tumor: increased the number and activity of TIL and DC, and increased the ratio of M1/M2 macrophages. Additionally, hyperactive TGF-β signaling in CAF leads to thickened peritumoral collagen, which hampers immune cell infiltration and limits the efficacy of anti-PD-L1. However, YM101 suppressed the functions of CAFs and undermined the peritumoral barrier by neutralizing TGF-β in the TME. As a result, YM101 promoted T cell infiltration and relieved anti-PD-L1 resistance (25). Moreover, the combination therapy of Mn^{2+} and YM101 has been shown to have a synergistic anti-tumor effect, effectively reversing immunotherapy resistance in non-inflamed tumors (168). It has been validated that that Mn^{2+} activates the STING pathway, promotes DC maturation, and cooperates with YM101 to promote T cell activation. Moreover, in multiple mouse tumor models, the combination of Mn^{2+} and YM101 treatment has exhibited durable anti-tumor effects and prolonged the survival of tumor-bearing mice (168). Compared to monotherapy, the combination of Mn^{2+} and YM101 has a stronger anti-tumor effect

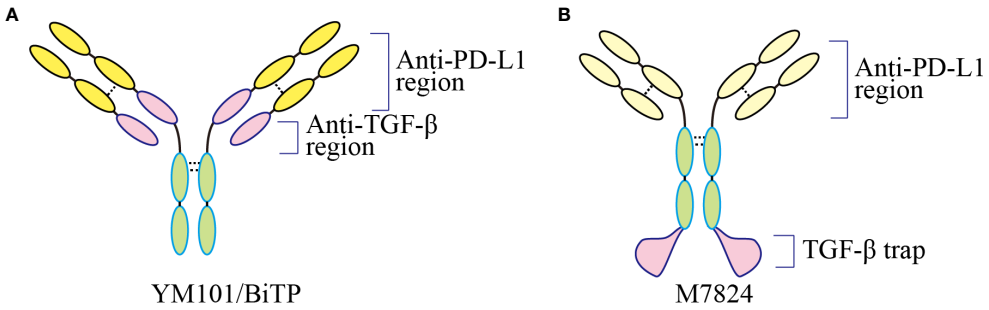

FIGURE 3
The structure of bispecific and bifunctional antibodies targeting TGF-β and PD-L1. **(A)** The structure of YM101 and BiTP. YM101 and BiTP contain anti-TGF-β and anti-PD-L1 domains in Fab region. **(B)** The structure of M7824. M7824 contains an anti-PD-L1 domain in Fab region and a TGF-β trap in Fc region. Adapted from Yi et al, 2022 (40).

and a broader anti-tumor spectrum. Mechanistically, this strategy (168). Further single-cell transcriptome analysis demonstrated that STING agonist combined with YM101 simultaneously regulates multiple components of anti-tumor immunity, promoting the transition from immune-exclude or immune-desert to inflamed tumors. This novel combined approach has the potential to be a general treatment for both inflamed and non-inflamed tumors (74). Encouraged by the positive preclinical data, the alternative molecule BiTP was constructed for further clinical trials (26). With a similar structure to YM101, BiTP is created by Check-BODY™ as well. The results of murine triple-negative breast cancer models showed that BiTP decreased peritumoral collagen generation and promoted T cell infiltration (26). A phase I clinical trial (NCT05028556) is also on recruiting to explore the optimal dose, efficacy, and safety of BiTP. There has been no observation of serious immune-related adverse events in the trial.

5.2 M7824

As a novel bifunctional fusion protein targeting TGF-β and PD-L1, M7824 contains an anti-PD-L1 domain in Fab region and a TGF-β trap in Fc region (23). In murine cancer models, M7824 showed potent anti-tumor efficacy and significantly prolonged the survival of tumor-bearing mice (23). Further investigations showed that M7824 substantially reshaped the tumor immune microenvironment: upregulating the numbers and activities of tumor-killing effectors and decreasing the ratio of immunosuppressive subsets such as MDSC, M2-like macrophage, and Treg (23). Also, M7824 led to tumor matrix remodeling, which might contribute to improved immune cell infiltration (23). Notably, preclinical data indicated that radiotherapy or chemotherapy might enhance the anti-tumor effect of M7824 (23, 169). The successful *in-vivo* studies and animal studies inspire researchers to conduct clinical trials associated with M7824 as listed in Table 1. In the phase 1 trial NCT02517398, the response rate was 87.5% in patients with PD-L1 high NSCLC (170). Up to now, M7824 has undergone 19 clinical trials, with 4 completed, 1 actively not recruiting, 12 terminated and 2 withdrawn, according to the ClinicalTrials database (Supplementary File: Table S1).

5.3 Other novel antibodies

The triumph of M7824 has stimulated the exploration of novel fusion protein endeavors, among which is SHR-1701, a monoclonal anti-PD-L1 domain fused with an N-terminal-truncated domain of TGFβRII that bears a resemblance to M7824 in structure (171). The linked TGFβRII domain serves as a trap and neutralizes TGF-β in the tumor microenvironment, while the Fab segment of the antibody blocks PD-L1. This dual blockade overcomes anti-PD-1 resistance in murine tumor models (172). In advanced tumors, SHR-1701 showed anti-tumor activity with objective response rate (ORR) of over 20% (173). In recurrent and metastatic cervical cancer, the ORR of SHR-1701 reached 15.6% (174). Also, fusion protein BR102 contains anti-PD-L1 antibody and TGFβRII ectodomain (175). Further animal studies confirmed the anti-tumor activity of BR102 in murine tumor models (176).

6 Perspective and Conclusion

For advanced cancers, TGF-β changes from a tumor suppressor to a tumor promoter. In cancer immunology, TGF-β substantially undermines immune surveillance and immune clearance by limiting the activities of antigen-presenting cells and cytotoxic T cells. Therefore, TGF-β blockade is a promising approach to improve immunotherapy performance. Although the enhanced anti-tumor effect of TGF-β and PD-L1 dual blockade has been validated in several clinical studies, the combination therapy of two antibodies indeed complicates grouping in clinical trials.

Based on BsAb or fusion protein technology, multiple BsAbs have been developed, which could simultaneously counteract PD-1 and TGF-β signaling pathways. Commonly, these BsAbs exhibit more potent anti-tumor activities and effectively reshape the immunosuppressive microenvironment. Notably, the therapeutic effect of anti-TGF-β/PD-L1 BsAb is even superior to anti-TGF-β plus anti-PD-L1 treatment, which might be attributed to the high tumor specificity brought by BsAb structure. We believe anti-TGF-β/PD-L1 BsAb has a significant advantage in treatment effect, especially in TGF-β-driven immune-excluded tumors.

TABLE 1 Clinical trials of M7824.

NCT number	Cancer type	Phase	Primary Measure Outcomes	Status
NCT03833661	Biliary tract cancer, Cholangiocarcinoma, Gallbladder cancer	2	ORR	Completed
NCT03524170	Breast cancer	1	Safety	Completed
NCT03840915	NSCLC	1/2	Safety	Completed
NCT02699515	Solid tumors	1	Safety	Completed
NCT04066491	Biliary tract cancer, Cholangiocarcinoma, Gallbladder cancer	2/3	Safety and OS	Completed
NCT02517398	Solid tumors	1	Safety	Completed
NCT04489940	TNBC	2	ORR	Completed
NCT04246489	Uterine cervical neoplasms	2	ORR	Completed
NCT04220775	HNSCC	1/2	Safety and PFS	Completed
NCT04551950	Cervical cancer	1	Safety	Completed
NCT04501094	Urothelial cancer	2	ORR	Terminated
NCT03840902	NSCLC	2	PFS	Terminated
NCT03451773	Pancreatic cancer	1/2	Safety and ORR	Terminated
NCT04327986	Pancreatic cancer	1/2	PR2D, Safety, and ORR	Terminated
NCT04560686	NSCLC	2	ORR	Terminated
NCT04428047	HNSCC	2	ORR	Terminated
NCT04727541	Cholangiocarcinoma	2	ORR	Terminated
NCT04971187	NSCLC	2	ORR and PFS	Terminated
NCT04417660	Thymic cancer	2	ORR	Recruiting
NCT05005429	Mesothelioma and lung cancer	2	PFS	Recruiting
NCT04303117	Kaposi sarcoma	1/2	Safety	Recruiting
NCT04432597	HPV+ cancer	1/2	Safety, PR2D, and CD3+ TIL	Recruiting
NCT03554473	SCLC	1/2	ORR	Recruiting
NCT03493945	Prostate cancer	1/2	Clinical benefit	Recruiting
NCT05012098	Olfactory neuroblastoma	2	ORR	Recruiting
NCT03315871	Prostate cancer	2	PSA	Recruiting
NCT04708470	Solid tumors	1/2	ORR and PR2D	Recruiting
NCT03427411	Solid tumors	2	ORR	Active, not recruiting
NCT03631706	NSCLC	3	PFS and OS	Active, not recruiting
NCT03436563	Colorectal cancer, MSI-H solid tumors	1/2	ORR, ctDNA	Active, not recruiting
NCT05061823	Lung cancer	3	Safety	Active, not recruiting
NCT04247282	Head and neck cancer	1/2	ORR	Active, not recruiting
NCT04574583	Solid tumors	1/2	ORR	Active, not recruiting
NCT04491955	Small bowel cancer, colorectal cancer	2	ORR	Active, not recruiting
NCT04287868	Solid tumors	1/2	ORR	Active, not recruiting
NCT04789668	Solid tumors	1/2	ORR, PR2D, Safety, and OS	Active, not recruiting
NCT04396535	NSCLC	2	PFS	Active, not recruiting

ORR, objective response rate; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; TNBC, triple negative breast cancer; OS, overall survival; HNSCC, head and neck squamous cell carcinoma; PFS, progression-free survival; PR2D, recommended phase II dose; TIL, tumor-infiltrating lymphocyte; MSI-H, microsatellite instability-high; ctDNA, circulating tumor DNA.

However, in a head-to-head phase III clinical study with pembrolizumab, M7824 failed to achieve the expected endpoints in patients with non-small cell lung cancer and cholangiocarcinoma. Although the reasons for the large discrepancy between the results of the phase III trial and the phase I trial have not been published, the lack of precise molecular markers to select suitable patients may be one reason for the failure of the phase III trial. For immune-desert tumors, both TGF-β and PD-1 pathways are not primary rheostats for the cancer-immunity cycle. In this case, combination therapy with agents stimulating antigen release or improving antigen-presenting cell functions is essential to overcome immunotherapy resistance. It has been confirmed that anti-TGF-β/PD-L1 BsAb combined with STING agonist effectively conquers anti-PD-1/PD-L1 resistance in immune-desert and immune-exclude tumors. Hereto, anti-TGF-β/PD-L1 BsAb-involved combination therapy might effectively broaden the anti-tumor spectrum of immunotherapy in the future.

Author contributions

TL and XW performed the selection of literature, drafted the manuscript and prepared the figures. MN, MW, and JZ collected the related references and participated in discussion. MY and KW designed this review and revised the manuscript. All authors contributed to this manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1196970/full#supplementary-material

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Current status of clinical trial research and application of immune checkpoint inhibitors for non-small cell lung cancer

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Immunotherapy has emerged as a hot topic in the treatment of non-small cell lung cancer (NSCLC) with remarkable success. Compared to chemotherapy patients, the 5-year survival rate for immunotherapy patients is 3-fold higher, approximately 4%–5% versus 15%–16%, respectively. Immunotherapies include chimeric antigen receptor T-cell (CAR-T) therapy, tumor vaccines, immune checkpoint inhibitors, and so forth. Among them, immune checkpoint inhibitors are in the spotlight. Common immune checkpoint inhibitors (ICIs) currently in clinical use include programmed death receptor-1(PD-1)/programmed death ligand-1(PD-L1) and cytotoxic T lymphocyte-associated antigen 4(CTLA-4). This article focuses on monotherapy and combination therapy of CTLA-4 and PD-1/PD-L1 immune checkpoint inhibitors. In particular, the combination therapy of ICIs includes the combination of ICIs and chemotherapy, the combination therapy of dual ICIs, the combination of ICIs and anti-angiogenic drugs, the combination of ICIs and radiotherapy, and the combination of ICIs inhibitors and tumor vaccines and so forth. This article focuses on the combination therapy of ICIs with chemotherapy, the combination therapy of dual ICIs, and the combination therapy of ICIs with anti-angiogenic drugs. The efficacy and safety of ICIs as single agents in NSCLC have been demonstrated in many trials. However, ICIs plus chemotherapy regimens offer significant advantages in the treatment of NSCLC with little to no dramatic increase in toxicity, while combined dual ICIs significantly reduce the adverse effects (AEs) of chemotherapy. ICIs plus anti-angiogenic agents regimen improves anti-tumor activity and safety and is expected to be the new paradigm for the treatment of advanced NSCLC. Despite some limitations, these agents have achieved better overall survival rates. In this article, we review the current status and progress of research on ICIs in NSCLC in recent years, aiming to better guide the individualized treatment of NSCLC patients.

KEYWORDS

non-small cell lung cancer, immune checkpoint inhibitors, CTLA-4, PD-1, PD-L1

1 Introduction

Lung cancer is the second most common cancer worldwide and the leading cause of cancer deaths, accounting for 11.4% of new cancers and 18% of cancer-related deaths in 2020 (1). According to statistics, NSCLC accounts for 80%–90% of all lung cancer diagnoses (2). Surgical resection is the main treatment modality for early-stage NSCLC; however, the prognosis and 5-year survival rate of patients after surgery remain unsatisfactory. In addition, approximately two-thirds of patients have already developed local or distant metastases at the time of detection and lost the opportunity for surgery (3). NSCLC is characterized by rapid proliferation, which will multiply rapidly during the radiotherapy stage, usually starting to multiply in 3-4 weeks of radiotherapy, and is also the main factor leading to the failure of radiotherapy, so the overall effect of conventional radiotherapy in treating NSCLC is unsatisfactory (4). Platinum-based two-drug chemotherapy is the standard first-line treatment for advanced negative mutation-driven NSCLC. However, the median overall survival (OS) is only 7.9 months, and chemotherapy-related side effects are not well tolerated by many patients (5). At present, numerous clinical studies have shown that immune checkpoint inhibitors (ICIs) are safer and more effective than conventional treatments, such as radiotherapy and chemotherapy. ICIs have better guidance for the clinical treatment of patients with advanced NSCLC.

This article reviews the mechanism of action of programmed death receptor-1 (PD-1)/programmed death ligand-1 (PD-L1) and cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) ICIs, including their clinical applications and related clinical trials, focusing on clinical trials related to ICIs to provide ideas for treatment options for NSCLC patients.

2 Mechanisms of ICIs for NSCLC

According to immunology, in tumorigenesis and regression, the body resists tumorigenesis through acquired immunity, while tumor cells evade recognition and attack by the body's immune system through various mechanisms, which then grow and metastasize free of immune killing effects. The immune response begins with antigen uptake, processing, and presentation by antigen-presenting cells (APC), which bind to the major histocompatibility complex molecules through the APC to the T cell surface receptors. The T cell cluster of differentiation (CD)28 receptor then binds to the CD80/CD86 ligand on the APC, and the two signals together activate the T cell (6). Ligands of immune checkpoints bind to receptors, thereby inhibiting the activation of CTLs, which is one of the key causes of tumor immune escape (7). Tumor cells can upregulate the molecular expression of cell surface immune checkpoints and use the immune checkpoint pathway to evade the host immune system, thereby suppressing immune cell function (Figure 1A) (8).

2.1 Anti-CTLA-4 antibody

CTLA-4 is a suppressor receptor expressed only by T cells and is used to inhibit T cell activity. Although CTLA-4 and CD28 are homologous analogs, they produce different effects. CD28 exerts positive regulation of the immune response, while CTLA-4 exerts negative regulation of the immune response. CTLA-4 has a higher affinity for CD80/CD86 than CD28, thus CTLA-4 pre-emptively binds to CD80/CD86 through competitive action. In addition, CTLA-4 can downregulate CD80/CD86 expression on APC or remove it through cellular cytokinesis, blocking the B7-CD28 signaling pathway in T-cell activation by competing with CD28, and thus blocking T-cell activation (Figure 1B) (9). CTLA-4 plays a negative regulatory role in T cell activation and activation of the immune response, and anti-CTLA-4 antibodies block inhibitory signals, induce T cell activation and proliferation, and restore their function. In addition, CTLA-4 induces the development and function of regulatory T cells (Treg), and CTLA-4 deficiency impairs Treg suppressor function *in vivo* and *in vitro* (Figure 1C) (10).

2.2 Anti-PD-1/PD-L1 antibody

PD-1 (also known as CD279) is an important immune checkpoint protein, mainly expressed in activated T cells and is associated with specific ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-H2, CD273) (11). Both can competitively bind to the PD-1 interaction. The PD-L2/PD-1 interaction has a higher affinity for cancer cells than the differentiated PD-L1/PD-1 interaction and activation; however, the tumor expression and activity of differentiated PD-L2 in regulatory cancer cells is much less than that of PD-L1 (12). PD-L1 is induced to be expressed by immune cells and epithelial cells, and PD-L2 is induced to be expressed by APC. Physiologically, PD-1 interacts with PD-L1 and PD-L2 on the surface of APC to inhibit T-cell overactivation and maintain immune homeostasis (Figure 1D). When tumor cells expressing PD-L2 bind to PD-1 (CD279) on T cells for synergistic signaling, it induces dephosphorylation of binding protein tyrosine phosphatases and affects downstream signaling pathways such as PI3K/Akt, Ras/ERK, PLCγ, and VAV, leading to immunosuppression and cancer progression by inhibiting T cell activation, proliferation, survival, and cytolytic functions in the tumor microenvironment (Figure 1E) (11). PD-1/PD-L1 monoclonal antibody disrupts tumor immune tolerance by specifically blocking PD-1/PD-L1 interaction, restoring the killing function of tumor-specific T cells and achieving tumor clearance (Figure 1F) (13).

3 Research progress of ICIs

3.1 CTLA-4 ICIs

Ipilimumab is a CTLA-4 immunoglobulin G1 (IgG1) monoclonal antibody, which enhances T cell activation and proliferation for anti-tumor effects. A randomized phase II study showed that ipilimumab in combination with chemotherapy improved outcomes more significantly than chemotherapy alone in patients with driver-negative squamous NSCLC (sq-NSCLC) (14). In a subsequent phase III trial, patients with advanced sq-NSCLC who had not received chemotherapy were randomized to

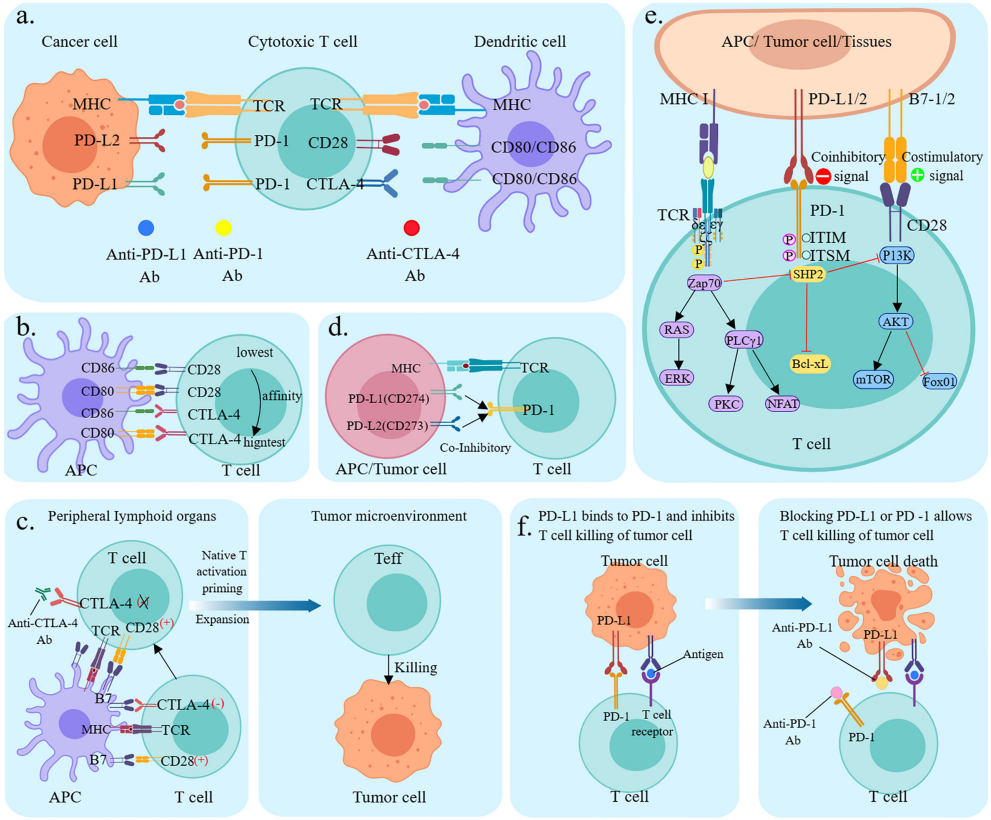

FIGURE 1
Mechanism of immune checkpoint inhibitors. **(A)** Mechanism of T cell activation and mechanism of action of immune checkpoint inhibitors. TCR presents antigen in MHC molecules on APC, CD80/CD86 ligands on APC co-stimulate interaction with CD28 receptors on T cells. cTLA-4 competitively binds CD80/CD86, PD-1 binds to its ligand PD-L1/PD-L2. Anti-CTLA-4, anti-PD-1, and anti-PD-L1 antibodies block the corresponding immune checkpoint pathways and restore T-cell activity; **(B)** Relative ligand affinity of CTLA-4 and CD28. **(C)** Classical CTLA-4 immunosuppressive mechanism of action; **(D)** PD-L1, PD-L2 competitively combined with PD-1; **(E)** Signaling pathways **(F)** Mechanism of action of PD-1/PDL1 immunosuppressants.

either the ipilimumab +paclitaxel+ carboplatin(I+CP)or placebo in combination with chemotherapy groups and found no significant difference in OS between the two groups (median OS:13.4 months vs12.4 months), with a median progression-free survival (PFS) of 5.6 months in both groups. The results suggest that I+CP did not improve OS in patients with advanced sq-NSCLC and resulted in a higher incidence of treatment-related adverse events (15). It has been hypothesized that Ipilimumab, which stimulates early T-cell activation in the lymphoid region, may not produce a sufficiently strong anti-tumor response in SCLC if there is no corresponding effector T-cell stimulation in the local tumor microenvironment. This explanation may also apply to sq-NSCLC (15) Nivolumab, a fully human anti-PD-1 antibody, and ipilimumab, a fully human anti-CTLA4 antibody, are ICIs with distinct but complementary mechanisms of action. Ipilimumab induces T-cell proliferation and *de-novo* anti-tumor T-cell responses, including in memory T cells, whereas nivolumab restores the function of existing anti-tumor T cells (16–18). 2020 FDA approval for Nivolumab+ipilimumab +chemotherapy first line for advanced or relapsed NSCLC.

3.2 PD-1 ICIs

3.2.1 PD-1 ICIs monotherapy

Pembrolizumab is a monoclonal antibody that targets PD-1 and binds to the PD-L1 receptor, blocking its interaction with PD-L1 and PD-L2. KEYNOTE-001 was the first phase Ib study to evaluate pembrolizumab in patients with advanced NSCLC, showing an objective response rate (ORR) of 27%, median OS of 22.1 months, and PFS of 6.2 months. Among patients with PD-L1 tumor proportion score (TPS) ≥50%, ORR was 45.2% (19). Based on the findings in KEYNOTE-001, KEYNOTE-024 further investigated the efficacy of first-line pembrolizumab monotherapy in advanced NSCLC patients with PD-L1 TPS ≥50% and compared it with chemotherapy (20). The study found that the pembrolizumab group had significantly better PFS and ORR than the chemotherapy group; however, OS had not been reached. Based on the KEYNOTE-001/024 study, the US Food and Drug Administration (FDA) announced the approval of pembrolizumab as the first-line treatment for patients with advanced NSCLC with high PD-L1

expression (21). To further expand the population for first-line immunotherapy, the KEYNOTE-042 study was created. This study demonstrated that immunotherapy was more effective than chemotherapy in patients with PD-L1 TPS ≥1%, with lower rates of side effects and an extended OS of nearly 8 months. Furthermore, the study included a Chinese population for the first time, thus allowing the results to be more relevant to Chinese patients as well (22). Based on the results of the KEYNOTE-042 study, The National Medical Products Administration approved pembrolizumab as a single-agent first-line treatment for advanced NSCLC with PD-L1 TPS ≥1% in September 2019. The results of the KEYNOTE024 and KEYNOTE042 studies showed that people with high PD-L1 expression benefitted more from immune monotherapy (20, 22).

Nivolumab, an IgG4 monoclonal antibody that binds to the PD-1 receptor, blocks the interaction of PD-1 with PD-L1 and PD-L2 and relieves PD-1 pathway-mediated suppression of the immune response. It is the first FDA-approved humanized IgG4-type monoclonal antibody against PD-1 as the second-line treatment for advanced or metastatic NSCLC, with a high safety profile and durable efficacy (23, 24). The CheckMate017 study showed that in patients with advanced sq-NSCLC cancer, the (median OS: 9.2 months vs 6.0 months), (median PFS: 3.5 months vs 2.8 months) and response rates were significantly better in the nivolumab group than in the docetaxel group, regardless of PD-L1 expression levels (25). Check Mate-063 results showed an ORR of 14.5% and a 1-year OS rate of 39%, demonstrating the significant benefits of nivolumab in relapsed refractory sq-NSCLC (26). Subsequent Check Mate-012 results showed a 23% ORR and 74% 1-year OS rate for nivolumab monotherapy in advanced NSCLC, confirming a significant prolongation of the duration of response (DoR) for nivolumab monotherapy in first-line treatment of advanced NSCLC (24). In the CheckMate 078 and 057 trials, nivolumab had a significant improvement in patient survival and a significantly lower incidence of AEs compared to docetaxel, and the benefit was also more pronounced in patients with low PD-L1 expression (27, 28).

3.2.2 PD-1 ICIs in combination with chemotherapy

Tislelizumab is a humanized IgG4 monoclonal antibody with high affinity and specificity for PD-1, which rarely binds to FcγR on macrophages, thus eliminating antibody-dependent phagocytosis, T-cell clearance mechanisms, and potential resistance to anti-PD-1 therapy (29, 30). A study revealed that tislelizumab was well tolerated in patients with advanced solid tumors, regardless of PD-1 expression, and anti-tumor activity was observed in NSCLC (31). RATIONALE304, a phase III clinical trial in nonsquamous NSCLC (nsq-NSCLC), showed that PFS was significantly longer in stage IIIB or IV nsq-NSCLC patients treated with tislelizumab + platinum + pemetrexed(T+PP) as compared to platinum + pemetrexed (PP)(median PFS:9.7 months vs 7.6 months). Furthermore, the main adverse effect (AE) of this regimen is decreased neutrophil count. In addition, the combination therapy had a higher response rate and longer response time, and the best PFS benefit was observed in patients with ≥50% PD-L1 expression (32). RATIONALE 307 is one of the first phase 3 trials of a PD-1 inhibitor in combination with chemotherapy for sq-NSCLC.Tislelizumab+carboplatin+nab-paclitaxel/paclitaxel (T+CnP/T+CP) dramatically improved PFS and ORR and provided evidence of stable safety/tolerability compared to carboplatin+nab-paclitaxel/paclitaxel(CnP/CP). The study also fully demonstrates the clinical benefit of tislelizumab in combination with chemotherapy as a first-line treatment for sq-NSCLC (33).

Sintilimab is a potent and selective anti-PD-1 antibody that inhibits the interaction between PD-1 and its ligands. Compared to nivolumab and pembrolizumab, sintilimab has a different binding epitope and greater PD-1 binding affinity (34). Platinum and gemcitabine (GP) are the most common inter-standard chemotherapy regimens for sq-NSCLC in Asia. ORIENT12 is the first study to use GP as a backbone combination to assess the benefit of adding an anti-PD-1 antibody to first-line sq-NSCLC chemotherapy in Asia (35). The results showed that the addition of sintilimab+GP (S+GP) standard chemotherapy significantly prolonged PFS in previously untreated patients with advanced or metastatic sq-NSCLC, and the greatest benefit was observed in the subgroup with PD-L1TPS >50%. Furthermore, this regimen could be used as first-line treatment for locally advanced or metastatic sq-NSCLC. ORIENT11 showed that in patients with previously untreated, locally advanced, or metastatic nsq-NSCLC, the addition of sintilimab+pemetrexed+platinum (S+PP) significantly prolonged PFS (median PFS:8.9 months vs 5.0 months) with a manageable safety profile compared to chemotherapy alone. Thus, the combination regimen may provide a new treatment option for this patient population (36).

Camrelizumab (SHR-1210), a humanized Ig G4-k monoclonal antibody against PD-1, exhibits anti-tumor activity and tolerability in lung cancer (37). In the phase 3 CameL trial, camrelizumab + pemetrexed + platinum (C+PP) significantly prolonged PFS compared to chemotherapy (PFS:11.3 months vs 8.3 months). The main AEs of this regimen are decreased white blood cell and neutrophil counts and anemia. The regimen is identified as the standard first-line therapy for Chinese patients with advanced nsq-NSCLC without EGFR mutations or ALK translocations (38). In the CAMEL-SQ study, first-line camrelizumab + carboplatin+ paclitaxel (C+CP) showed stable and durable clinical benefit in patients with advanced sq-NSCLC (median PFS:8.5 months vs 4.9 months) (39). Although the OS had not been reached, the survival benefit was consistent across all PD-L1 TPS subgroups, with an ORR of 64.8% versus 36.7%, DoR of 13.1 versus 4.4 months, and manageable adverse events. These findings support the efficacy of C+CP as the standard first-line treatment regimen for sq-NSCLC.

3.3 PD-L1 inhibitor

3.3.1 PD-L1 ICIs monotherapy

Durvalumab is a humanized anti-PD-L1 protein monoclonal antibody that blocks the binding of PD-Ll to PD-1 and CD80. It recognizes and clears tumor cells and can be used as the first-line treatment for unresectable III NSCLC that has not progressed after

concurrent radiotherapy or chemotherapy and for progressing SCLC (40, 41). A phase III study [NCT02125461] showed a higher PFS in the durvalumab group than in the placebo group (PFS:16.8 months vs 5.6 months) (42). In addition, the ORR was higher in the durvalumab group than in the placebo group (28.4% vs 16.0%), with a DoR of 18 months. Similarly, the median time to death or distant metastasis was longer in the durvalumab group compared to the placebo group (23.2 months vs 14.6 months). In the phase III ARCTIC study (NCT02352948), 476 patients with advanced NSCLC received durvalumab as a consolidation therapy after chemoradiotherapy (43). The study found that patients treated with durvalumab had a longer median PFS benefit irrespective of PD-Ll expression levels. In light of these findings, in February 2018, the US FDA approved durvalumab for patients with NSCLC whose disease has not progressed after locally advanced chemoradiotherapy and who are inoperable.

Avelumab is an IgG1-type monoclonal antibody, which also has antibody-dependent cell-mediated cytotoxic effects compared to other PD-L1 inhibitors, causing direct lysis of tumor cells (44). Avelumab has a controlled safety profile and promising clinical activity in a population of patients with progressive, platinum-treated, metastatic, or recurrent NSCLC. Responses occurred in both squamous and non-squamous tumors, regardless of PD-L1 expression status. These findings support the therapeutic benefit of anti-PD-L1 antibodies in previously treated NSCLC patients. In addition, these results demonstrating the efficacy of avelumab provide a rationale for ongoing Phase 3 trials in the second-line NSCLC population and highlight the potential benefit of immunotherapy for patients with this difficult-to-treat disease (45).

3.3.2 PD-L1 ICIs combined with chemotherapy

Atezolizumab is an engineered humanized monoclonal anti-PD-L1 antibody that inhibits the binding of PD-L1 to PD-1 and B7.1 (also known as CD80), thereby restoring anti-cancer immunity (46). IMpower130 is the first to demonstrate the benefit of PD-L1 inhibitors in combination with chemotherapy for the first-line treatment of advanced NSCLC. The results of the study showed that atezolizumab+carboplatin+nab-paclitaxel(A+CnP) for first-line treatment of patients with EGFR/ALK wild-type nsq-NSCLC showed a better benefit in both OS (median OS: 18.6 months vs 13.9 months) and PFS (median PFS:7.0 months vs 5.5 months) compared to chemotherapy, with no new occurrence of AEs (47). In light of these findings, A+CnP was approved by the US FDA for the first-line treatment of metastatic nsq-NSCLC without EGFR/ALK mutations. In the phase III clinical trial IMpower131, which also compared the efficacy of immunotherapy plus chemotherapy with chemotherapy alone in advanced sq-NSCLC, there was an improvement in PFS in the A+CnP group compared with CnP group (PFS:6.3 months vs 5.6 months), with no difference in OS (48). The IMpower132 study focused on the efficacy of atezolizumab+ pemetrexed + platinum(A+PP) versus chemotherapy alone in patients with advanced nsqNSCLC and showed that atezolizumab in combination with chemotherapy improved PFS (PFS: 7.6 months vs 5.2 months), regardless of PD-L1 expression (49).

Sugemalimab (formerly CS1001) is an immunoglobulin G4 (IgG4, s228p) monoclonal antibody targeting PD-L1. Sugemalimab retains binding affinity to Fcγ receptor I and thus can effectively induce antibody-dependent cellular phagocytosis through cross-linking of PD-L1-positive tumor cells with macrophages prevalent in the tumor microenvironment and may further enhance tumor antigen presentation (50). In the GESTONE-302 trial, which investigated the PD-L1 inhibitor sugemalimab in combination with platinum-based chemotherapy (S+P) in NSCLC, sugemalimab combined with chemotherapy improved PFS compared with placebo combined with chemotherapy (median PFS:9.0 months vs 4.9 months), with a more prominent benefit, especially in the sq-NSCLC subgroup. Analysis of the subgroups indicated that these benefits remained unchanged, regardless of PD-L1 expression and NSCLC subtype. Their results confirmed that sugemalimab combined with platinum-based chemotherapy showed measurable improvements in PFS in NSCLC patients and could be a new first-line treatment option for NSCLC (51). An interim analysis of the phase 3 trial GESTONE-301 exhibited a significant and clinically meaningful improvement in PFS after concurrent or sequential chemoradiotherapy combined with sugemalimab (S+C)group compared with the placebo group. The results suggested that sugemalimab is an effective consolidation therapy for patients with locally advanced, unresectable stage III NSCLC without disease progression after chemoradiotherapy (52).

3.4 Combined treatment with dual ICIs

PD-1 and CTLA-4 are both immune checkpoint molecules but have very different mechanisms of action, negatively regulating the activation of T cells in the immune response at different stages. CTLA-4 prevents T cell activation and effector functions during the initial T cell activation phase, whereas PD-1 acts on activated T cells at a later stage of the immune response, inhibiting the degree of T cell activation and cytotoxicity (Figure 2A) (13). Clinical research has shown that the combination of PD-1/PD-L1 inhibitors and CTLA-4 inhibitors resulted in enhanced effector T-cell action and attenuated suppressor T-cell action, resulting in stronger anti-tumor effects than single-agent ICIs (Figure 2B) (53).

The CheckMate012 study was a trial of dual ICIs regimen, using nivolumab and ipilimumab as the first-line treatment for patients with NSCLC. The regimen showed excellent efficacy with a manageable safety profile. Patients with high PD-L expression benefitted more from combination therapy. This investigation was the first to support that dual immunotherapy can enhance the benefit of first-line treatment in NSCLC (54). The CheckMate 227 trial further confirmed that the dual ICIs arm (nivolumab plus ipilimumab, N+I) had a prolonged PFS (median PFS:7.2 months vs 5.4 months) and a more prominent improvement in ORR (45.3% vs 26.9%) compared to the chemotherapy alone arm, regardless of PD-L1 status. The results of this project were assessed by the Lung Cancer Symptom Scale and the European Five-Dimensional Health Scale, and patient-reported outcomes showed multiple symptoms

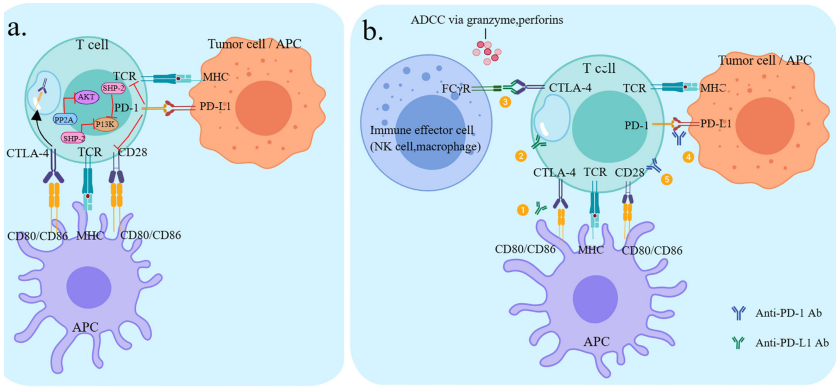

FIGURE 2
Checkpoint pathways of CTLA-4 and PD-1 and potential mechanisms of action of antibodies. **(A)** CTLA-4 and PD-1 pathways negatively regulate T-cell activation **(B)** ① Anti-CTLA-4 restores T-cell activation by inhibiting the interaction between CTLA-4 and CD80/CD86 on the APC.② Anti-CTLA-4 inhibits CD28 ligand CD80/86-mediated endocytosis *via* CTL A-4-mediated endocytosis.③Anti-CTLA-4 lgG1 antibody-ipilimumab, through its Fc segment, binds to FcγR° on immune effector cells (NK cells, monocytes/macrophages), resulting in ADCC effects and subsequent depletion of highly expressed CTLA-4 T cell subsets (e.g. Treg).④ Anti-PD-1 restores T-cell activation by inhibiting the interaction between PD-1 and PD-L1 on T cells (PD-L1 can be expressed by tumor cells and various immune cells.⑤ Anti-PD-1 restores Tcel activation through the interaction between PD-1 and CD28, which is the point of convergence between the two pathways.

and quality-of-life improvements compared to chemotherapy (55). The results of the CheckMate 9LA study were consistent with that of CheckMate 227. Compared to chemotherapy alone, regardless of tumor histology or PD-L1 expression, nivolumab plus ipilimumab in combination with two cycles of chemotherapy significantly improved survival. Efficacy and safety data support nivolumab plus ipilimumab combination chemotherapy has a favorable risk-benefit profile as first-line therapy for patients with advanced NSCLC (56). The main objective of the phase III clinical trial MYSTIC study was to explore the efficacy and safety of durvalumab plus tremelimumab versus conventional chemotherapy in the first-line treatment of advanced NSCLC and to explore its associated biomarkers. At tumor mutation burden (TMB) ≥16 mut/Mb, the OS of dual immune combination versus chemotherapy was 16.5 months and 10.5 months, with significant differences. In patients with low TMB, immunotherapy did not result in favorable OS, showing the importance of appropriate biomarkers (57). A phase 1b investigation demonstrated that, regardless of PD-l expression levels, the combination of durvalumab and tremelimumab showed good anti-tumor effects in NSCLC patients (58).

3.5 ICIs in combination with anti-angiogenic drugs

ICIs combined with anti-angiogenic drugs are based on the following theories. First, tumor angiogenesis inhibits the tumor microenvironment; vascular endothelial growth factor (VEGF) plays a key role in tumor angiogenesis and immunosuppression at different levels by binding to VEGF receptors 1-3 and neuropilin (59). Dendritic cells (DCs) play a central role in T cell initiation and activation. However, VEGF can inhibit the differentiation, maturation, and antigen presentation of DCs (60). In addition, VEGF can drive the suppressive effects on effector T cells by inhibiting the differentiation of progenitor cells to CD8+ T cells, reducing the proliferation and cytotoxic effects of CD8+ T cells, increasing the exhaustion of CD8+ T cells, promoting the polarization of tumor-associated macrophages (TAMs) to M2 type and recruiting immunosuppressive cells (such as Tregs, MDSCs and M2-like TAMs) to exert immunosuppressive effects (61). Target VEGF can diminish the expression of adhesion molecules on the endothelium of tumor vessels and decrease the ability of immune cells to adhere to and cross the vessel wall, thus preventing immune cells from entering the tumor (62). Second, the tumor immune microenvironment promotes tumor angiogenesis; neuropilin-1 can be transferred from DCs to T cells during the interaction between T cells and DCs, and the transferred neuropilin-1 can effectively bind VEGF secreted by DCs to boost tumor angiogenesis. Moreover, DCs and M2-like TAMs can promote angiogenesis by secreting the pro-angiogenic factor VEGF (63).

Mechanism of action of anti-angiogenic drugs: 1) Immune response is stimulated by increasing CD8+ T lymphocyte infiltration into the tumor (64); 2) immune signaling is suppressed by inhibiting T regulatory cell proliferation and DC maturation, and PD-1 expression in infiltrating tumor T lymphocytes exerts a regulatory effect; 3) TAMs are induced to polarize into an immune-supporting M1-like phenotype, and the expression of immune checkpoint molecules, such as PD-L1 and CTLA-4, on the surface of immunosuppressive cells and the secretion of immunosuppressive factors, such as VEGF, transforming growth factor β and interleukin 10, is reduced, thereby restoring the activation and function of immune cells (65); and 4) reducing vascular pressure, improving tissue hypoxia, inviting vascular normalization and relieving immunosuppression by depressing the permeability of tumor vessels (66). On the other hand, ICIs can activate CD8+ T lymphocytes and Th1 cells to secrete anti-tumor cytokines such as interferon γ and tumor necrosis factor, which can regulate the immune microenvironment while exerting anti-angiogenic and vascular

normalization effects (67, 68). During anti-angiogenic drug treatment, immunotherapy can be coupled with immunotherapy to enhance the transport of immunotherapeutic drugs and immune cells, strengthen the infiltration of immune cells into tumor tissues and activate the positive regulation of the body's immune function to achieve reinforcement of the anti-tumor effect. Therefore, the combination of anti-angiogenic and immunotherapy can theoretically produce a synergistic anti-tumor effect (Figure 3).

IMpower150, a representative study of ICIs-combined anti-angiogenesis, is the first randomized phase III clinical trial to demonstrate the benefit of ICIs in patients with EGFR mutations. The results showed that the atezolizumab+bevacizumab+carboplatin + paclitaxel (ABCP) arm prolonged PFS and OS in first-line nsq-NSCLC patients compared to the bevacizumab+carboplatin + paclitaxel (BCP) arm, including the EGFR mutation and ALK translocation populations. The exploratory analysis in the EGFR mutation population suggested that patients with EGFR-sensitive mutations or NSCLC treated with tyrosine kinase inhibitors can benefit from the ABCP regimen, adding a new treatment option for this patient population. Following this study, in December 2018, the US FDA approved, atezolizumab +bevacizumab+paclitaxel+carboplatin for the front-line treatment of EGFR/ALK-negative metastatic nsq-NSCLC, regardless of PD-L1 expression status (69). ONO-4538-52/TASUKI-52, a randomized, double-blind phase III clinical trial, evaluated nivolumab +bevacizumab+carboplatin+paclitaxel (NBCP) as a first-line treatment for nsq-NSCLC (70). The outcomes showed that the median PFS was significantly longer in the ABCP group than in the placebo group (median PFS:12.1 months vs 8.1 months), and prolonged PFS was observed in all patients with PD-L1 expression levels, with an ORR of 61.5% and 50.5%, respectively. In addition, the incidence of grade ≥3 treatment-related AEs was comparable in both groups. This regimen can be considered a new viable treatment strategy for patients with primary nsq-NSCLC. In the JVDF study (NCT02443324), 26 patients with advanced NSCLC were enrolled and were administrated ramucirumab +pembrolizumab (P+R) as first-line treatment. By the time of data cut-off, the overall ORR was 42.3%, the disease control rate (DCR) was 84.6%, the median PFS was 9.3 months, OS was not reached and the overall safety profile was excellent, with stratified analysis showing better efficacy in those with high PD-L1 expression than in those with low PD-L1 expression. This study revealed the clinical benefits of anti-angiogenic combination ICIs (71). A phase Ib/II clinical trial (NCT02501096), which included 21 patients with advanced NSCLC who received lenvatinib +pembrolizumab, showed an overall ORR of 33.3%, DCR of 80.9%, median PFS of 7.4 months and overall safety control. On account of these findings, a phase III clinical trial (NCT03829319) was initiated. Data from the first part of LEAP-006 suggested that the effectiveness of pembrolizumab+chemotherapy in combination with lenvatinib in patients with advanced NSCLC as the primary treatment is definite. Furthermore, data from 13 validated analyses showed that the ORR of this combination mode was xx. The second part of the randomized study is currently underway, and we look forward to the publication of the related data (72). Preliminary results from the phase I study (NCT03628521) using sintilimab

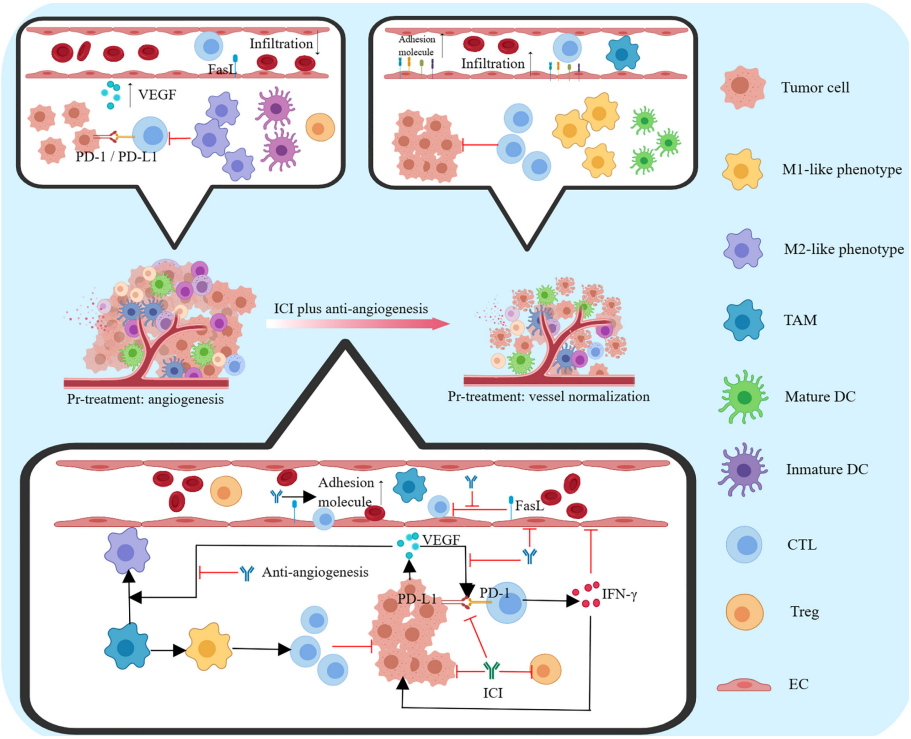

FIGURE 3
Mechanism of action of ICIs in combination with anti-angiogenic agents.

+anlotinib (S+A) in 22 patients with advanced NSCLC showed that the combination therapy was well tolerated by all patients, with an incidence of grade ≥3, treatment-related adverse events of 31.8%, an ORR of 77.3% and a DCR of 100%. A subgroup analysis according to patients' PD-L1 expression and TMB at baseline showed a consistent benefit of combination therapy in all subgroups. Although PFS was immature at the time of data cut-off, the regimen demonstrated good anti-tumor activity (73). A phase II study (NCT04239443) explored the efficacy and safety of apatinib +camrelizumab as a second-line and beyond-treatment option for advanced NSCLC. The results showed that among 91 evaluable subjects with nsq-NSCLC, ORR was 30.8%, DCR was 82.4%, median PFS was 5.9 months and OS was not achieved, with stratified analysis showing better clinical outcomes observed in patients with bTMB-high

4 Conclusion and prospect

With a large number of clinical trials and a growing body of data demonstrating the durable efficacy of ICIs in patients with advanced NSCLC, the clinical use of ICIs is changing the treatment paradigm and landscape for NSCLC. The study of ICIs for NSCLC has been extended to first-line treatment, and PD-1/PD-L1 ICI monotherapy has improved the prognosis of some patients with advanced NSCLC and become a more favorable treatment after molecular targeted therapy (Table 1). Combination therapy with ICIs avoids the intolerable AEs caused by chemotherapy. Combination therapy with ICIs and anti-angiogenic drugs has shown high anti-tumor activity and tolerable safety and is expected to become a new paradigm in the treatment of advanced NSCLC (Tables 2, 3). However, despite its excellent efficacy in NSCLC, ICIs have some limitations. First are the immune-related adverse events (irAEs). A growing body of research data suggests that although ICIs improve survival, a significant proportion of patients develop irAEs. Common target organs for irAEs include the skin, gastrointestinal tract, liver, lungs, and endocrine organs. Common irAEs for CTLA-4 ICIs are colitis, pituitary gland inflammation, and rash. Pneumonia, hypothyroidism, and arthralgia are often seen in PD-1/PD-L1 ICI irAEs. However, although these irAEs are elevated, they are generally within control with proper monitoring and management. Moreover, most irAEs are mild to moderate, although severe or life-threatening irAEs do occur, resulting in death in 1%–2% of patients. The current mainstay of treatment for irAEs is dose reduction or drug discontinuation, and for severe toxic reactions, immunotherapy should be permanently terminated. The second limitation is the lack of validated predictive biomarkers of efficacy. Some studies have shown that the expression level of PD-L1, mismatch repair gene expression status, and TMB, among others, have a certain correlation with the efficacy of PD-1/PD-L1 ICIs. Among them, PD-L1 is the most recommended immunotherapy-related oncology marker by the National Comprehensive Cancer Network guidelines. However, its application is limited by temporal dynamics, tumor heterogeneity, and different threshold detection methods. Therefore, PD-L1 expression may not be the best

TABLE 1 Summary of the efficacy and safety of ICIs monotherapy in NSCLC.

Study	Phase	Treatment			Efficacy				Safety		Most common Aes	Reference
		Experimental group			ORR	PFS	OS	DOR	Any AEs	≥G3AEs		
					(%)	(month)	(month)	(month)	(%)	(%)		
PD-1 ICIs monotherapy												
Keynote001	I	pembrolizumab			27	6.2	22.1	12.5	85.1	11.9	fatigue(27.7%),pruritus(14.9%),rash(13.9%),arthralgia(11.9%), hypothyroidism(13.9%), nausea(11.9%)	(19)
Keynote024	III	pembrolizumab			44.8	10.3	–	Not	73.4	26.6	fatigue(10.4%),pyrexia(10.4%), diarrhea(14.3%),	(20)
Keynote042	III	pembrolizumab	PT %	1	27	7.1	16.7	8.3	63	18	Hypothyroidism(11%) pneumonitis(3%)	(22)
				20	33	6.2	17.7	8.3				
				50	39	5.4	20	10.8				
CheckMate017	III	Nivolumab			20	3.5	9.2	Not	58	7	decreased appetite(11%), asthenia(10%), fatigue(16%),	(25)

(*Continued*)

TABLE 1 Continued

Study	Phase	Treatment		Efficacy			Safety		Most common Aes	Reference
		Experimental group	ORR	PFS	OS	DOR	Any AEs	≥G3AEs		
			(%)	(month)	(month)	(month)	(%)	(%)		
CheckMate063	II	Nivolumab	Not	–	8.2	Not	75	17	Fatigue(4%),pneumonitis(3%), diarrhea(3%)	(26)
CheckMate057	III	Nivolumab	19	2.3	12.2	17.2	69	10	Fatigue(16%),nausea(12%), decreased appetite(10%), anemia (10%)	(28)
CheckMate078	III	Nivolumab	16.6	2.8	12	Not	9	5	Rash(12%),fatigue(10%)	(27)
PD-L1 ICIs monotherapy										
CT02125461	III	Durvalumab	28.4	16.8	–	18	96.8	29.9	Cough(35.4%), Dyspnea(22.3%), Pneumonitis(33.9%)	(40)
ARCTIC	III	Durvalumab	22	3.8	11.7	9.5	56.5	22	–	(43)
Impower110	III	Atezolizumab	–	8.1	20.2	–	90.2	30.1	Anemia, neutropenia, thrombocytopenia	(74)
EMPOWER-Lung 1	III	Cemiplimab	39	8.2	Not	16.7	43	28	anemia (16%),neutropenia(10%),thrombocytopenia(8%)	(75)
CTLA-4 ICIs monotherapy										
NCT01772004	I	Avelumab	22	3	8.4	–	99	13	infusion-related reaction(21%),fatigue(25%),nausea(13%)	(45)

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TABLE 2 Summary of the efficacy and safety of ICIs combination therapy in NSCLC.

Study	Phase	Treatment	Efficacy				Safety		Most common Aes	Reference
		Experimental group	ORR	PFS	OS	DOR	Any AEs	≥G3AEs		
			(%)	(month)	(month)	(month)	(%)	(%)		
PD-1 ICIs Combined with Chemotherapy										
Rationale304	III	T+PP	57.4	9.7	Not	8.5	20	2	decreased neutrophil count (DeNE,44.6%), anemia (13.5%), thrombocytopenia(19.4%) leukopenia(21.6%)	(32)
Rationale307	III	A:T+CP	72.5	7.6	–	8.2	99.4	85.8	anemia,alopecia,DeNE	(33)
		B:T+CnP	74.8	7.6	–	8.6		83.9		
Orient11	III	S+PP	51.9	8.9	Not	Not	99.6	61.7	anemia (74.1%),DeNE(71.1%),decreased white blood count (DeWBC,67.7%)	(36)
Orient12	III	S+GP	44.7	6.7	Not	6.1	100	86.6	anemia (93.3%),DeNE(83.2%),DeWBC(88.8%), decreased platelet (72.6%)	(35)
Camel	III	C+PP	60.5	11.3	Not	17.6	99.5	69	DeNE,DeWBC, anemia	(38)
Camel SQ	III	C+CP	64.8	8.5	Not	13.1	–	–	DeNE(155%),DeWBC(30%), anemia (10%)	(39)
Keynote189	III	Pembrolizumab+PP	47.6	88	Not	11.2	99.8	67.2	Nausea, anemia, fatigue	(76)
Keynote407	III	pembrolizumab+CP	57.9	6.4	15.9	7.7	98.2	69.8	anemia,alopecia,neutropenia	(77)
PD-L1 ICIs Combined with Chemotherapy										
Impower130	III	A+CnP	49.2	7	18.6	8.4	99.6	81	neutropenia(32%), anemia (29%),DeNE(12%)	(47)
Impower131	III	A+CnP	49.7	6.3	14.2	7.3	97.9	68	Pneumonitis(3.0%),neutropenia(3.9%), anemia (2.1%)	(48)
Impower132	III	A+PP	47	7.7	17.1	10.1	98.6	54.6	Rash(25.8%),hypothyroidism(8.2%),pneumonitis (6.2%)	(49)
Gestone301	III	S+C	–	9	–	Not	76	9	pneumonia(2%) interstitial lung disease (2%)	(52)
Gestone302	III	S+P	–	9	–	–	99	54	DeNE (33%), anemia (13%), decreased platelet (10%) DeWBC(14%),	(51)
CTLA-4 ICIs Combined with Chemotherapy										
NCT01285609	III	I+CP	44	5.6	13.4	–	89	53	anemia (12%), diarrhea(7%), thrombocytopenia(7%) neutropenia(14%),	(15)

(Continued)

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TABLE 2 Continued

Study	Phase	Treatment			Efficacy			Safety		Most common Aes			Reference
		Experimental group			ORR	PFS	OS	DOR	Any AEs	≥G3AEs			
					(%)	(month)	(month)	(month)	(%)	(%)			
Combined treatment with dual ICIs													
CheckMate012	I	Nivolumab 1 mg/kg + Ipilimumab 1mg/kg q6w			33	5.6	Not	Not	73	40	Skin	Eastpointe	endocrine
											36%	23%	21%
		Nivolumab 3mg/kg + Ipilimumab 1 mg/kg q6w			38	3.9	–	Not	744	31		23%	21%
		Nivolumab3 mg/kg + Ipilimumab 1mg/kg q12			47	8.1	–	Not	84	42	39%	24%	11%
Checkmate227	III	PD-L1	≥1%	N+I	36.4	5.1	17.1	23.2	77.2	35.5	cutaneous (34.0% any grade, 4.2% grade≥3), endocrine (23.8% any grade, 4.2% grade≥3), gastrointestinal (18.2% any grade, 2.4% grade≥3), hepatic (15.8% any grade, 8.2% grade≥3)		
			<1%	N+I	27.3	5.1	17.2	18	75.7	35			
Checkmate9LA	III	Nivolumab 360mg q3w+ Ipilimumab 1mg/kgq6w +Chemotherapy			38	6.7	15.8	13	92	48	hepatic(14.4%), endocrine(25.7%), cutaneous(40.5%), gastrointestinal(23.3%)		
CheckMate568	II	Nivolumab 3mg/kg q2w+ Ipilimumab 1mg/kg q6w			30	4.2	–	Not	80	29	gastrointestinal toxicities (5%) Skin(30%)		
		Nivolumab 360mg q3w+ Ipilimumab 1 mg/kg q6w+ Chemotherapy			47	10.8	19.4	12.7	94	58			
ARCTIC	III	Durvalumab+ Tremelimumab			26	3.5	11.5	12.2	63.3	23.3	–		
ICIs Combined with anti-angiogenic drugs													
Impower150	III	ABCP			64	8.3	19.2	9	94.4	55	DeNE, neutropenia, hypertension		
TASUKI-52	III	NBCP			61.5	12.1	–	Not	64	56	DeNE, DeWBC, anemia		
JVDF	I	P+R			42.3	9.3	Not	–	84.6	42.3	Rash(26.9%),Fatigue(19.2%) Hypertension(19.2%), Pruritus (15.4%)		
NCT03628521	Ib/II	S+A			77.3	–	Not	–	–	31.8	Hematuria,hyperuricemia,hypertension		

Reference
(54)
(55)
(56)
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(70)
(71)
(73)

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TABLE 3 Summary of efficacy and safety of FAD-approved ICIs for NSCLC.

Study	Phase	Treatment			Efficacy			Safety		Most common Aes	Reference
		Experimental group			ORR (%)	PFS (month)	OS (month)	DOR (month)	Any AEs (%)	≥G3AEs (%)	
ICIs monotherapy											
Keynote001	I	pembrolizumab			27	6.2	22.1	12.5	85.1	11.9	fatigue(27.7%),pruritus(14.9%),rash(13.9%),arthralgia (11.9%), hypothyroidism(13.9%), nausea(11.9%)	(19)
Keynote024	III	pembrolizumab			44.8	10.3	–	Not	73.4	26.6	fatigue(10,4%),pyrexia(10.4%), diarrhea(14.3%),	(20)
Keynote042	III	pembrolizumab	PT%	1	27	7.1	16.7	8.3	63	18	Hypothyroidism(11%) pneumonitis(3%)	(22)
				20	33	6.2	17.7	8.3				
				50	39	5.4	20	10.8				
CheckMate017	III	Nivolumab			20	3.5	9.2	Not	58	7	decreased appetite(11%), asthenia(10%), fatigue(16%),	(25)
CheckMate063	II	Nivolumab			Not	–	8.2	Not	75	17	Fatigue(4%),pneumonitis(3%), diarrhea(3%)	(26)
CheckMate057	III	Nivolumab			19	2.3	12.2	17.2	69	10	Fatigue(16%),nausea(12%), decreased appetite(10%), anemia (10%)	(28)
CheckMate078	III	Nivolumab			16.6	2.8	12	Not	9	5	Rash(12%),fatigue(10%)	(27)
CT02125461	III	Durvalumab			28.4	16.8	–	18	96.8	29.9	Cough(35.4%), Dyspnea(22.3%), Pneumonitis(33.9%)	(40)
ARCTIC	III	Durvalumab			22	3.8	11.7	9.5	56.5	22	–	(43)
Impower110	III	Atezolizumab			–	8.1	20.2	–	90.2	30.1	Anemia, neutropenia, thrombocytopenia	(74)
ICIs combination therapy												
Keynote189	III	Pembrolizumab+PP			47.6	88	Not	11.2	99.8	67.2	Nausea, anemia, fatigue	(76)
Keynote407	III	pembrolizumab+CP			57.9	6.4	15.9	7.7	98.2	69.8	anemia,alopecia,neutropenia	(77)
Impower130	III	A+CnP			49.2	7	18.6	8.4	99.6	81	neutropenia(32%), anemia (29%),DeNE(12%)	(47)
Impower131	III	A+CnP			49.7	6.3	14.2	7.3	97.9	68	Pneumonitis(3.0%),neutropenia(3.9%), anemia (2.1%)	(48)
Impower132	III	A+PP			47	7.7	17.1	10.1	98.6	54.6	Rash(25.8%),hypothyroidism(8.2%),pneumonitis (6.2%)	(49)
Impower150	III	ABCP			64	8.3	19.2	9	94.4	55	DeNE, neutropenia, hypertension	(69)

(Continued)

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TABLE 3 Continued

Study	Phase	Treatment			Efficacy			Safety		Most common Aes			Reference
		Experimental group			ORR	PFS	OS	DOR	Any AEs	≥G3AEs			
					(%)	(month)	(month)	(month)	(%)	(%)			
CheckMate012	I	Nivolumab 1 mg/kg + Ipilimumab 1mg/kg q6w			33	5.6	Not	Not	73	40	Skin	Eastpointe	endocrine	(54)
											36%	23%	21%	
		Nivolumab 3mg/kg + Ipilimumab 1 mg/kg q6w			38	3.9	–	Not	744	31		23%	21%	
		Nivolumab3 mg/kg + Ipilimumab 1mg/kg q12			47	8.1	–	Not	84	42	39%	24%	11%	
Checkmate227	III	PD-L1	≥1%	N+I	36.4	5.1	17.1	23.2	77.2	35.5	cutaneous (34.0% any grade, 4.2% grade≥3), endocrine (23.8% any grade, 4.2% grade≥3), gastrointestinal (18.2% any grade, 2.4% grade≥3), hepatic (15.8% any grade, 8.2% grade≥3)			(55)
			<1%	N+I	27.3	5.1	17.2	18	75.7	35				
Checkmate9LA	III	Nivolumab 360mg q3w+ Ipilimumab 1mg/kgq6w +Chemotherapy			38	6.7	15.8	13	92	48	hepatic(14.4%), endocrine(25.7%), cutaneous(40.5%), gastrointestinal(23.3%)			(56)
CheckMate568	II	Nivolumab 3mg/kg q2w+ Ipilimumab 1mg/kg q6w			30	4.2	–	Not	80	29	gastrointestinal toxicities (5%) Skin(30%)			(78)
		Nivolumab 360mg q3w+ Ipilimumab 1 mg/kg q6w+ Chemotherapy			47	10.8	19.4	12.7	94	58				
TASUKI-52	III	NBCP			61.5	12.1	–	Not	64	56	DeNE, DeWBC, anemia			(70)

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predictive biomarker for efficacy. Therefore, future studies combining multiple other novel biomarkers to individualize the choice of ICIs treatment regimen are warranted. Third is acquired immune resistance. Although ICIs therapies have improved prognostic outcomes for many NSCLC patients, only a few patients have achieved durable responses after treatment with ICIs. We need to tap into novel immune checkpoint molecules as well as explore combination strategies of different ICIs to address drug resistance. The combination of ICIs with topical therapy (mainly radiofrequency ablation, cryoablation, and bronchial artery chemoembolization, etc.) lacks a large number of reliable clinical studies. Although it has been shown that these topical therapies combined with ICIs in the treatment of NSCLC, can improve the survival rate and prolong the survival of patients. However, there is still a lack of sufficient clinical data, and more evidence-based medical data is needed to validate the findings. This is a new direction for the treatment of NSCLC in the future, and it is worthwhile for us to follow the new research in this field. All of the above questions will be the direction of our future exploration and endeavors, guiding us to continue to improve and expand this area of research to ensure that more NSCLC patients can experience significant improvements in both survival time and quality of life.

Author contributions

Study concept and design: FW and XF. Analysis and interpretation of data: FW and TX. Drafting of the manuscript: FW and TX. Critical revision of the manuscript for important intellectual content: FW, TX, ZL, XF, and XG. Obtained funding: XF. Study supervision: XF and XG. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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VNLG-152R and its deuterated analogs potently inhibit/repress triple/quadruple negative breast cancer of diverse racial origins *in vitro* and *in vivo* by upregulating E3 Ligase Synoviolin 1 (SYVN1) and inducing proteasomal degradation of MNK1/2

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Triple-negative breast cancer (TNBC) and its recently identified subtype, quadruple negative breast cancer (QNBC), collectively account for approximately 13% of reported breast cancer cases in the United States. These aggressive forms of breast cancer are associated with poor prognoses, limited treatment options, and lower overall survival rates. In previous studies, our research demonstrated that VNLG-152R exhibits inhibitory effects on TNBC cells both *in vitro* and *in vivo* and the deuterated analogs were more potent inhibitors of TNBC cells *in vitro.* Building upon these findings, our current study delves into the molecular mechanisms underlying this inhibitory action. Through transcriptome and proteome analyses, we discovered that VNLG-152R upregulates the expression of E3 ligase Synoviolin 1 (SYVN1), also called 3-hydroxy-3-methylglutaryl reductase degradation (HRD1) in TNBC cells. Moreover, we provide genetic and pharmacological evidence to demonstrate that SYVN1 mediates the ubiquitination and subsequent proteasomal degradation of MNK1/2, the only known kinases responsible for phosphorylating eIF4E. Phosphorylation of eIF4E being a rate-limiting step in the formation of the eIF4F translation initiation complex, the degradation of MNK1/2 by VNLG-152R and its analogs impedes dysregulated translation in TNBC cells, resulting in the inhibition of tumor growth. Importantly, our findings were validated *in vivo* using TNBC xenograft models derived from MDA-MB-231, MDA-MB-468, and MDA-MB-453 cell lines, representing

different racial origins and genetic backgrounds. These xenograft models, which encompass TNBCs with varying androgen receptor (AR) expression levels, were effectively inhibited by oral administration of VNLG-152R and its deuterated analogs in NRG mice. Importantly, in direct comparison, our compounds are more effective than enzalutamide and docetaxel in achieving tumor growth inhibition/repression in the AR+ MDA-MD-453 xenograft model in mice. Collectively, our study sheds light on the involvement of SYVN1 E3 ligase in the VNLG-152R-induced degradation of MNK1/2 and the therapeutic potential of VNLG-152R and its more potent deuterated analogs as promising agents for the treatment of TNBC across diverse patient populations.

KEYWORDS

triple/quadruple negative breast cancer (TNBC/QNBC), MNK1 and MNK2 degrader, eIF4E signaling, Synoviolin 1 (SYVN1), VNLG-152R and deuterated analogs

1 Introduction

Breast cancer is a significant global health concern and a leading cause of cancer-related mortality among women worldwide. It is the second main cause of cancer-related death in the American women and the most detected cancer in women globally (1). Over the past three decades, the rate of incidence has been increasing by 0.3% every year though the death rate decreased significantly due to advanced medical intervention (1). Among all the subtypes, triple negative breast cancer (TNBC) and lately classified quadruple negative breast cancer (QNBC) are highly resilient and elude currently available treatment strategies (2). While TNBC lacks the expression of estrogen receptor (ER), progesterone receptor (PR) and expresses low levels of HER2, QNBC is characterized by low or no androgen receptor (AR) expression apart from the features of TNBC (3). More than 57% of TNBC diagnosed lack AR expression and may be sub-categorized as QNBC (2, 4). As TNBC and QNBC lack important pharmacological targets, both these subtypes are therapeutically challenging and highly metastatic in nature (5). Therefore, the development of novel therapeutic drugs that effectively inhibits TNBC/QNBC is an urgent ongoing medical need (6).

Interestingly, the TNBC subtype Luminal AR (LAR) that expresses AR is significantly driven by AR signaling and associated with decreased disease-free survival and poor overall survival (7). Meta-analyses of AR expression in TNBC reveals that 27.96% of the 4703 patients studied expressed AR (8). In the clinical trial to identify AR-positive TNBC patients, 80% of the 368 patients screened expressed AR and responded to AR inhibitor enzalutamide (7). Therefore, AR is considered a significant pharmacological target in combating TNBC. However, in the absence of AR in other sub-types such as QNBC, targeting other pathways such as MNK-eIF4E and mTORC1 is more rational (9). Phosphorylation of eIF4E by MNK1/2 is a critical step in mRNA 5'cap-dependent translation of many proteins that actively promote cell division and tumor growth (10).

Pharmacological targeting of oncogenic eIF4F translation initiation complex has been an attractive therapeutic strategy for the development of novel drugs to treat various cancers (11, 12). EIF4E, being the least abundant protein of the eIF4F complex is considered the rate-limiting factor in mRNA 5'-cap-dependent translation initiation (13). Phosphorylation of eIF4E is critical for the formation of eIF4F. MNK1 and MNK2 are the only kinases known to phosphorylate eIF4E when both are bound to the scaffolding protein eIF4G to form the translation initiation complex eIF4F (13–15). Further, MNK1/2 being at the center of eIF4E signaling and mTORC signaling (16), pharmacological inhibition of MNK1/2 is a potent strategy to combat various cancers including TNBC (17).

Previously, we reported the development of a novel MNK1/2 degrader VNLG-152R that promotes degradation of MNK1/2 in breast cancer cells (18) and inhibits TNBC *in vivo* (9). Additionally, we explored the potential of deuterated derivatives of VNLG-152R, which showed enhanced efficacy against TNBC cells *in vitro* and improved pharmacokinetic properties in mice models (19). The incorporation of deuterium, by replacing hydrogen atoms, has emerged as a promising strategy to enhance pharmacokinetic and therapeutic profiles of various drugs (20). The deuterated analogs were either better or equipotent to VNLG-152R in *in vitro* antiproliferative activities against MDA-MB-231 and MDA-MB-468 human TNBC cells. Importantly and as expected, the expression of Mnk1, peIF4E and their associated downstream targets, including cyclin D1 and Bcl2, were strongly decreased in VNLG-152R/deuterated analogs-treated TNBC cells signifying inhibition of Mnk1-eIF4E signaling (*i.e.*, target engagement). Among the seven deuterated analogs of VNLG-152R examined, three novel analogs (D6, D7 and H6) (Figure 1) exhibited enhanced pharmacokinetic parameters including prolonged residence time and extended elimination half-life in plasma in CD-1 female mice (19). These findings highlight the potential of deuterated analogs as promising candidates for further development in the treatment of TNBC.

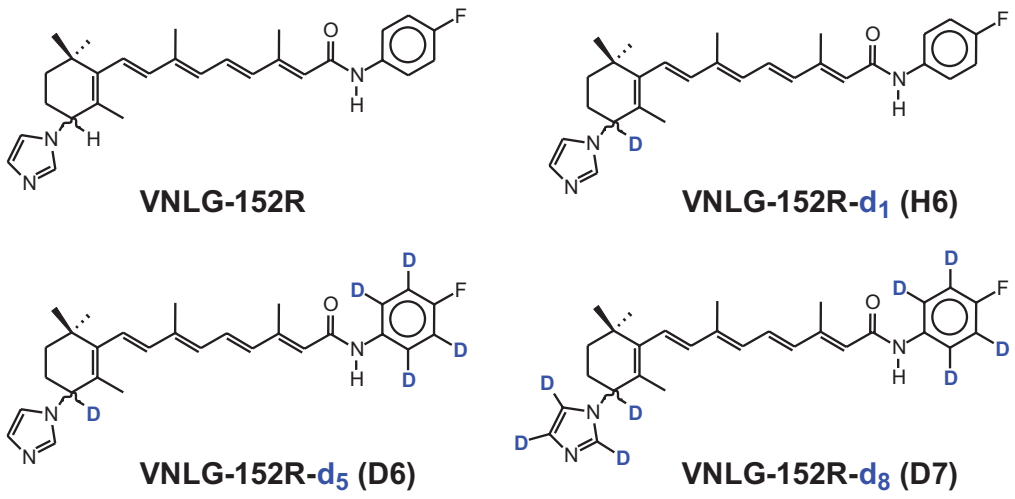

FIGURE 1
The chemical structures of VNLG-152R and its deuterated analogs D6, D7 and H6. The hydrogen atoms in the indicated positions were replaced by the heavy isotope deuterium to improve the pharmacokinetic properties and retention time in plasma for enhanced antitumor efficacy.

In this study, we unveil the key molecular mechanism behind degradation of MNK1/2 by VNLG-152R in breast cancer cells. Our findings highlight the upregulation of Synoviolin 1 (SYVN1), also called 3-hydroxy-3-methylglutaryl reductase degradation (HRD1), an E3 ligase by VNLG-152R and its significant role in ubiquitination and proteasomal degradation of MNK1/2. To broaden our investigation, we conducted a comprehensive evaluation comparing the *in vivo* efficacy of VNLG-152R's deuterated analogs, namely D6, D7 and H6, with the parent compound in three different tumor xenografts of MDA-MB-231 (derived from Caucasian female metastatic mammary adenocarcinoma, low AR/AR$^-$), MDA-MB-468 (derived from metastatic mammary adenocarcinoma of an African female patient and AR$^-$), and MDA-MB-453 of Caucasian female origin with high AR expression. Additionally, we compared the efficacies of VNLG-152R and the most potent deuterated analog, D7, to the efficacies of clinically relevant TNBC drugs, such as docetaxel (DTX) and enzalutamide (ENZ), in mice tumor xenografts MDA-MB-453 of Caucasian female origin with high AR expression (21, 22).

We must acknowledge the emerging racial disparities in TNBC occurrence and subsequent mortality rates, with women of African descent facing higher vulnerability (23). Hence, our investigation also encompassed the evaluation of VNLG-152R and its deuterated analogs in three distinct tumor xenografts representing diverse racial origins, including both Caucasian and African women, while considering their respective AR expression status. Through our comprehensive study, we aim to shed light on the intricate molecular mechanisms underlying TNBC and address the urgent need for effective therapeutic interventions tailored to specific racial populations. These findings hold promise in advancing personalized medicine approaches for the treatment of TNBC, ultimately contributing to the overall improvement of patient outcomes irrespective of their ethnicity.

In the current study, we employed *in vitro*, *in vivo*, molecular, and biochemical approaches to investigate the effects of VNLG-152R and its deuterated analogs on TNBC. Next-generation RNA-sequencing, differential gene expression analysis and HD Mass Spectrometry Proteome revealed significant upregulation of SYVN1, in response to VNLG-152R treatment. Furthermore, the modulation of multiple pathways by VNLG-152R contributed to the inhibition of TNBC. Biochemical analyses confirmed the presence of elevated levels of SYVN1 protein in both VNLG-152R-treated cells *in vitro* and tumor tissues from the treated mice. Notably, we show for the first time that VNLG-152R facilitated the ubiquitination of MNK1/2 by SYVN1, leading to its subsequent proteasomal degradation, which ultimately contributed to the inhibition of TNBC. Degradation of MNK1/2 further affected phosphorylation of eIF4E adversely, which in turn restricted mRNA 5'cap-mediated translation initiation thereby checking uncontrolled protein synthesis in tumor cells, effectively restricting tumor growth and proliferation.

2 Materials and methods

2.1 Cell culture, western blotting, and fine chemicals

The human breast cancer cell lines, MDA-MB-231, MDA-MB-468 and MDA-MB-453 representing triple negative breast cancer of Caucasian origin with no AR expression (QNBC), African origin with

low AR and Caucasian origin with high AR expression respectively obtained from ATCC (Manassas, VA) were cultured in the recommended media supplemented with 10% heat-inactivated standard fetal bovine serum (FBS, GIBCO) and 1% penicillin-streptomycin (10,000 U/ml, Life Technologies) at 37°C and 5% CO_2. Primary antibodies of MNK1, MNK2, eIF4E, p-eIF4E, SYVN1, Ubiquitin, β-actin, GAPDH and secondary HRP-conjugated anti-rabbit were obtained from Cell Signaling Technology, USA. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer supplemented with 1x protease inhibitors (Roche, Indianapolis, IN, USA), phosphatase inhibitors (Thermo Scientific, Waltham, MA, USA), 1 mmol/L EDTA and 1 mmol/L PMSF (Sigma) and immunoblotted as described earlier (24, 25). Immunoprecipitation of MNK1 was performed as reported previously using MNK1 primary antibody (26, 27). All fine chemicals were purchased from Sigma-Aldrich, St. Louis, MO. VNLG-152R and the deuterated analogs (D6, D7 and H6) were synthesized in house as described previously (19). The chemical structures of VNLG-152R and its deuterated analogs are presented in Figure 1.

2.2 RNA-sequencing and GSEA

MDA-MB-231 cells were treated with 10 μM VNLG-152R for 24 h in triplicates. Total RNA was isolated using RNAeasy Plus mini kit (Qiagen) following manufacturer's instructions. The RNA preparation was quantified and assessed its quality using Agilent 2100 Bioanalyzer. A RIN value of 8 or above was used for all samples. The sequencing libraries were prepared with the NEB Ultra II Directional RNA library prep kit. Further, the libraries were evaluated for quantity and size distribution using Qubit and Agilent 2100 Bioanalyzer. Sequencing was carried out on an Illumina NovaSeq S2 PE100 bp lane (Maryland Genomics, Institute for Genome Sciences, University of Maryland Baltimore). As a norm, Phred quality score (Q score; to measure the quality of sequencing) more than 90% of the sequencing reads reached Q30 (99.9% base call accuracy). Differential Gene Expression and Gene Set Enrichment analyses (GSEA) were performed to identify canonical cellular pathways modulated by VNLG-152R as reported previously (28).

2.3 siRNA-mediated knockdown of gene expression

Specific siRNA targeting SYVN1 and scramble siRNA (siControl) were purchased from Ambion (Foster City, CA, USA). MDA-MB-231 and MDA-MB-468 cells were grown in 6-well culture plates and transfected with siRNA using lipofectamine RNAiMax transfection reagent (Invitrogen, USA) in Opti-MEM reduced serum medium (Thermo Fisher Scientific, USA) for 48 h according to the manufacturer's protocol. Scrambled siRNA was transfected as control and SYVN1 knockdown was scored by immunoblot analyses.

2.4 Proteome profiling by high-definition mass spectrometry

MDA-MB-231 cells treated with VNLG-152R (10 μM, 24 h) or vehicle control were lysed in 4% deoxycholate and the lysates were washed, reduced and alkylated followed by trypsin-lysis as described (29). The tryptic fragment peptides were separated in a nanoACQUITY UPLC analytical column (BEH130 C18, 1.7 μm, 75 μm x 200 mm, Waters) over a 180 min linear acetonitrile gradient (3–43%) containing 0.1% formic acid in nano-ACQUITY UPLC system, Waters Corporation and analyzed in coupled Waters Synapt G2S HDMS mass spectrometry system. The spectra acquired using ion mobility linked parallel mass spectrometry (UDMSe) were analyzed as reported previously (30, 31).

Tandem mass spectra generated were aligned using UniProt human reference proteome. The resulting hits were further validated at a maximum false discovery rate of 0.01. The abundance ratio between the control and VNLG-152R treatments were calculated by comparing the MS1 peak volumes of peptide ions at the low collision energy cycle. The MS1 peptides were further validated by MS2 sequencing at higher collision energy cycle. Label-free quantifications were performed using aligned AMRT (Accurate Mass and Retention Time) cluster quantification as reported previously (32).

2.5 *In vivo* tumor xenograft studies

All animal studies in mice were performed in accordance with the humane use of experimental animals following review and approval by the Institutional Animal Care and Use Committee (IACUC), University of Maryland School of Medicine, Baltimore, MD, USA, per IACUC No. # 0221010 dated 03/09/2021. The human breast cancer cell lines, MDA-MB-231, MDA-MB-468 and MDA-MB-453 representing triple negative breast cancer of diverse ethnic origin and AR expression status were used to induce tumor xenografts in immunodeficient female NRG mice (age 5–7 weeks) procured from the Veterinary Resources, University of Maryland School of Medicine, Baltimore, MD, USA. The animals were housed under sterile conditions and fed with sterile pellets and water *ad libitum*. After a week of acclimatization, 3-5x10^6 cells in 100 μl were subcutaneously injected into the left flank of mice. After 21-25 days of inoculation and upon reaching the tumor volume ~100 mm^3, the animals were randomly grouped into five animals per group. The control animals were orally administered with vehicle (20% β-cyclodextrin in saline, PO) and other compounds administered (PO or IP as indicated) with indicated doses of test compounds and duration. The animals were carefully observed daily for general health and body weight recorded three times a week. The tumor size was measured three times a week using digital calipers and tumor volume calculated using the formula length (mm) x $width^2$ (mm) x 0.5 (mm^3). Upon reaching a tumor length of approximately 20 mm or a tumor volume of 2000 mm^3, whichever was achieved first in the control groups (approximately 6 weeks after breast cancer cell inoculation), the study was promptly

concluded. Subsequently, the mice were humanely euthanized, and the tumors were surgically removed for further analysis.

2.6 Statistical analysis

Statistical comparisons were made by one-way ANOVA followed by Multiple comparisons test using GraphPad Prism 9.0 software (GraphPad Software, Inc.). A probability value with *p < 0.05, **p<0.001 and ***p<0.0001 were considered statistically significant. As specified in the figures, values in data are expressed as the mean ± SEM of three or more independent experiments.

3 Results

3.1 MNK1/2 and eIF4E are upregulated in breast cancer: TCGA and CPTAC database

Notably, transcriptome and proteome analyses using data from The Cancer Genome Atlas (TCGA) and Clinical Proteomic Tumor Analysis Consortium (CPTAC) have revealed consistent upregulation of MNK1/2 and eIF4E in most breast cancer cases at mRNA and protein level except MNK1 mRNA (Figures 2A–F). Though MNK1 mRNA is marginally upregulated in breast cancer, MNK1 protein is significantly abundant in the cancer tissue (Figure 2B). Upregulation is evident at the protein level in all three genes, with breast tumor tissues from cancer patients exhibiting significantly higher levels of MNK1/2 and eIF4E (Figures 2B, D, F). Remarkably, elevated levels of eIF4E have been associated with poor overall survival in breast cancer patients (Figure 2G). Further, the analysis of patient data based on racial backgrounds indicated relatively higher levels of MNK1 protein in patients of African descent (Figure 2H). Among the major races represented in the database, Caucasian patients exhibited relatively higher levels of eIF4E expression in tumor tissues, followed by the African race (Figure 2I). These findings underscore the consistent dysregulation of MNK1/2 and eIF4E in breast cancer and provide insights into potential racial disparities in their expression patterns. It also emphasizes the importance of further investigations to unravel the underlying molecular mechanisms and implications in breast cancer disparities.

3.2 SYVN1 is constitutively upregulated in VNLG-152R-treated TNBC cells and associated with MNK1/2 degradation

We first carried out the total proteome profiling of TNBC cells MDA-MB-231 using High-Definition Mass Spectrometry (HDMS) to visualize the differently expressed proteins upon treating with VNLG-152R (Figure 1). Among the differentially expressed proteins, SYVN1, an E3 ligase was found to be upregulated three-fold in the treated cells (Figure 3A). Based on our previous studies

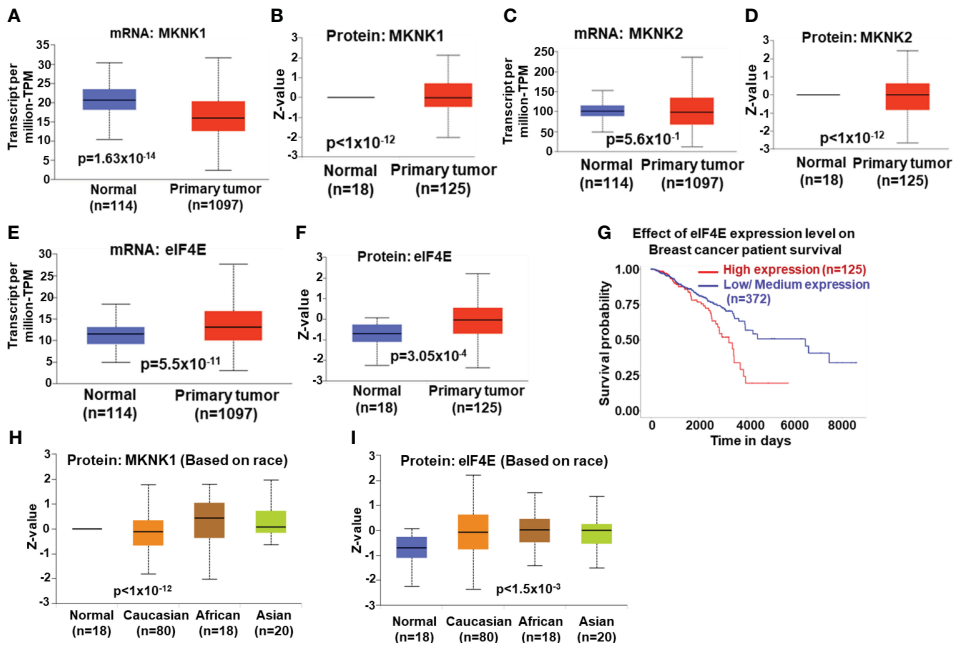

FIGURE 2
Expression (mRNA) and protein levels of MNK1/2 and eIF4E are significantly high in tumor tissues of breast cancer patients: TCGA (The Cancer Genome Atlas) and CPTAC (Clinical Proteomic Tumor Analysis Consortium). **(A-F)** The mRNA levels in tumor tissues of 1097 breast cancer patients were compared to the mRNA levels in the adjacent normal tissues of 114 individuals. The protein levels of MNK1/2 and eIF4E were analyzed from tumor tissues from 125 patients against that of normal tissue from 18 individuals. Both mRNA and protein levels of MNK1/2 and eIF4E are significantly higher in tumor tissue from breast cancer patients compared to the adjacent normal tissue. **(G)** Increased level of eIF4E is correlated to poor overall survival rate in breast cancer patients. **(H, I)** Protein level of MNK1 and eIF4E is significantly high in clinical tumor specimens of African and Caucasian races respectively and are likely to benefit from therapies targeting MNK1/2-eIF4E signaling.

demonstrating the ubiquitin-proteasomal degradation of MNK1/2 induced by VNLG-152R in breast (9, 18) and prostate (33, 34) cancer cell lines and the role of SYVN1 as an E3 ligase involved in ubiquitination and proteasomal degradation of several proteins (35–42), we hypothesized that SYVN1 might play a key role in the ubiquitination and subsequent degradation of MNK1/2. Immunoblotting for SYVN1 confirmed its increased expression in VNLG-152R-treated cells compared to the control with concomitant decrease in MNK1/2 and its product p-eIF4E (Figure 3B). To further validate the involvement of SYVN1 in MNK1/2 degradation, we performed immunoblotting in MDA-MB-231 and MDA-MB-468 cells treated with VNLG-152R in the presence or absence of the SYVN1 inhibitor LS102 (43, 44) or SYVN1 siRNA. As predicted, VNLG-152R did not significantly affect the levels of MNK1/2 when SYVN1 inhibitor or siRNA was present, but it significantly decreased the levels of MNK1/2 in the absence of SYVN1 inhibitor or siRNA (Figure 3B). To address a concern raised by an astute reviewer, we note that LS102 is an inhibitor of the enzymatic activity of SYVN1 and hence we do not see (or expected) decreased levels of SYVN1. As MNK1/2 are the only known kinases known to phosphorylate eIF4E (13–15), the degradation of MNK1/2 by VNLG-152R was accompanied by decrease in eIF4E phosphorylation, indicating the active role of SYVN1 in the degradation of MNK1/2 mediated by VNLG-152R. Furthermore, we observed a dose-dependent increase in SYVN1 expression and a corresponding decrease in MNK1/2 and p-eIF4E levels upon treatment with increasing concentrations of VNLG-152R (Figures 3C, D). As expected, we also observed a dose-dependent decrease in other downstream oncoproteins involved in breast cancer cell migration, invasion, and cell cycle progression such as WNK1 (kinase with no lysine (K) 1) (45, 46), and Cyclin-D1 (47, 48), respectively (Figure 3D).

3.3 SYVN1 induces proteasomal degradation of MNK1/2 through ubiquitination

After ascertaining the involvement of SYVN1 in the degradation of MNK1/2, we proceeded to investigate the ubiquitination of MNK1/2 through a proteasomal degradation inhibition assay. MDA-MB-231 cells were treated with MG-132, a known proteasomal inhibitor (49) prior to treating the cells with VNLG-152R briefly (2 h) to recover ubiquitinated MNK1/2, immunoblotted and probed with ubiquitin antibody. As expected, ubiquitinated MNK1/2 was accumulated in cells treated with MG-132 and VNLG-152R, while reduced ubiquitination was observed in cells treated with SYVN1 siRNA (Figure 4A). Thus, treating MDA-MB-231 and MDA-MB-468 cells with proteasome inhibitor MG132 in presence of VNLG-152R did not significantly alter the MNK1/2 levels, suggesting the proteasomal pathway of degradation of MNK1/2. The level of MNK1/2 was comparable to that of control

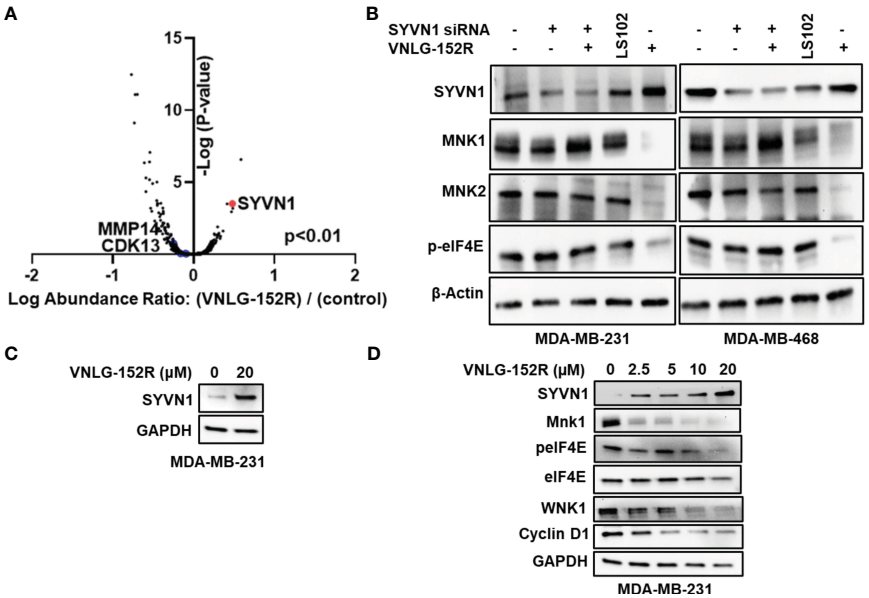

FIGURE 3
SYVN1 is upregulated in VNLG-152R-treated TNBC cells and correlated to MNK1/2 degradation. **(A)** Whole Proteome profiling by high-definition mass spectrometry (HD-MS) showed that SYVN1 is upregulated three-fold compared to the vehicle control ($p<0.01$). MDA-MB-231 cells were treated with 10 μM VNLG-152R for 24 h and processed as detailed in the methods section. **(B)** Degradation of MNK1/2 by VNLG-152R in QNBC is mediated by its ubiquitination by SYVN1. Immunoblots show upregulation of SYVN1 protein in VNLG-152-treated TNBC cells with concurrent degradation of MNK1/2. Knockdown of SYVN1 using siRNA or its inhibition by known inhibitor LS102 abrogated VNLG-152R-mediated degradation of MNK1/2 suggesting active role of SYVN1 in VNLG-152R-mediated MNK1/2 degradation in TNBC cells. As MNK1/2 are the only kinases known to phosphorylate eIF4E, a decrease in MNK1/2 levels further affects the level of p-eIF4E, thus limiting mRNA 5′cap-dependent translation initiation in TNBC cells. β-actin served as protein loading control. **(C)** Immunoblot showing upregulation of SYVN1 when MDA-MB-231 cells were treated with 20 μM VNLG-152R. **(D)** Dose-dependent effect of VNLG-152R on the expression of SYVN1, MNK1, eIF4E, peIF4E, WNK1 and Cyclin D1. MDA-MB-231 cells were treated with VNLG-152R (0-20 μM) for 24h. Cells were lysed with RIPA buffer and 40 μg of protein used in analyzing protein expression of, SYVN1, MNK1, eIF4E, peIF4E, WNK1 and Cyclin D1 respectively by immunoblotting. GAPDH was used as the loading control.

when treated with MG-132 and VNLG-152R whereas we observed significant decrease of MNK1/2 in VNLG-152R treatment alone, further reenforcing the proteasomal degradation of MNK1/2 (Figure 4B).

However, there are two major pathways involved in degradation of cellular proteins *viz*. ubiquitin-proteasome system (UPS), which is specific in nature and associated with targeted protein degradation and more generic autophagy-lysosomal degradation that degrades protein aggregates and organelles, which is less specific but tightly regulated (50, 51). We ruled out the potential involvement of the autophagy-lysosomal pathway in the degradation of MNK1/2 by using Bafilomycin-A1 (Baf-A1), a standard inhibitor of lysosomal autophagy (52). Addition of Baf-A1 to cultured MDA-MB-231 cells did not inhibit VNLG-152R-mediated degradation of MNK1/2, indicating that the lysosomal pathway is not involved in the degradation of MNK1/2 mediated by VNLG-152R (Figure 4C). As expected, the levels of SYVN1 were elevated in the VNLG-152R treated cells (Figure 4C).

3.4 RNA-sequencing, GSEA and HD mass spectrometry-proteome profiling demonstrates inhibition of mTORC1 signaling and reveal pathways perturbed by VNLG-152R

After establishing the role of SYVN1 in the degradation of MNK1/2 induced by VNLG-152R, we proceeded to assess the impact of VNLG-152R on canonical pathways relevant to breast cancer. To investigate the effect of 10 μM VNLG-152R on the cellular transcriptome of MDA-MB-231 cells, we conducted RNA sequencing and GSEA studies. Notably, VNLG-152R induced differential expression (DE) of 337 genes (Figure 5A). Gene Set Enrichment Analysis (GSEA) of these differentially expressed genes revealed the inhibition of key cancer pathways such as mTORC1 signaling and NUP153, while p53 was upregulated (Figure 5B). The inhibition of mTORC1 signaling is apparently due to MNK1/2 degradation by VNLG-152R, corroborating the biochemical data presented in the study. NUP153 (Nucleoporin 153) contributes to cell migration and proliferation and regulates the nuclear translocation of endothelial nitric oxide synthase (eNOS) by forming a multimeric complex (53). It is reported that eNOS is critical for maintaining tumorigenicity of cancer cells (54).

Further, the HDMS Proteome profiling and subsequent pathway analysis revealed a shift in total proteome of VNLG-152R-treated cells to reflect decreased levels of several pathway proteins involved in the biological processes such as cell adhesion to the matrix and cell-to-cell adhesion that are critical for breast cancer progression and cell migration were decreased 4-5-fold upon treating TNBC cells with VNLG-152R (Figure 5C). Particularly noteworthy, protein translation in the cells were decreased by 12-fold (Figure 5C), apparently due to the decreasing levels of MNK1/2 that is necessary for phosphorylating eIF4E, a pre-requisite for the formation of mRNA 5' cap-dependent translation initiation complex eIF4F.

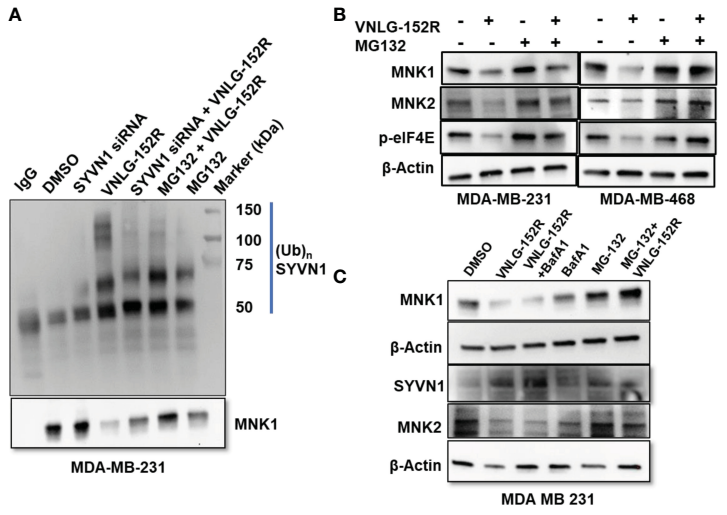

FIGURE 4
Inhibition of proteasomal degradation but not lysosomal degradation accumulates ubiquitinated MNK1. **(A)** MDA-MB-231 cells were treated with MG-132 prior to treating cells with VNLG-152R for short duration (2h). The cells were lysed and immunoprecipitated MNK1 using anti-MNK1 and probed with anti-ubiquitin. The cells treated with VNLG-152R resulted in accumulation of higher amount of ubiquitinated MNK1 compared to the controls. Short duration of treatment with VNLG-152R minimizes MNK1 degradation and facilitates maximum recovery of ubiquitinated MNK1. **(B)** Treatment of TNBC cells with proteasome inhibitor MG-132 in presence of VNLG-152R did not significantly alter the MNK1/2 levels, suggesting the proteasomal pathway of degradation of MNK1/2. Decrease in MNK1/2 is reflected by decreased levels of phosphorylated eIF4E. **(C)** MDA-MB-231 cells were treated with VNLG-152R in presence or absence of lysosome inhibitor Bafilomycin-A1 (BafA1) or proteasome inhibitor MG-132. Inhibition of lysosome by BafA1 did not abrogate VNLG-152R-mediated degradation of MNK1/2 but inhibition of proteasome by MG-132 abolished MNK1/2 degradation. This further suggests that the VNLG-152R-mediated degradation of MNK1/2 is through proteasomal pathway and not by lysosomal degradation. β-actin served as protein loading control.

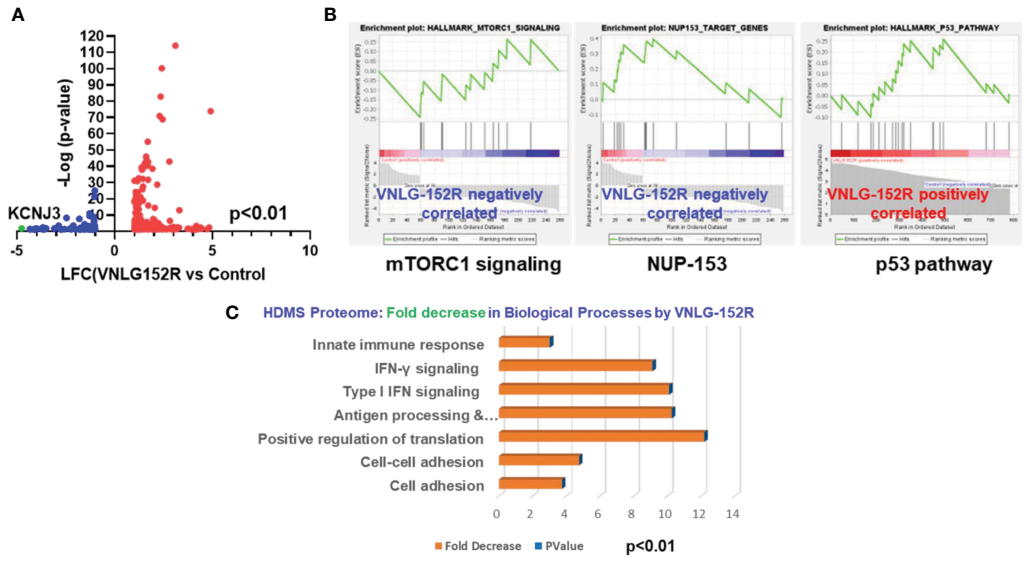

FIGURE 5
VNLG-152R modulates transcriptome and proteome of TNBC cells in favor of cancer inhibition. **(A)** RNA-sequencing and differential gene expression analysis show that 337 genes were differentially expressed when MDA-MB-231 cells were treated with 10 μM VNLG-152R for 24h; 259 genes were upregulated (red dots) and 78 downregulated (blue dots). **(B)** GSEA of differentially expressed genes demonstrate inhibition of MTORC1 and NUP-153 pathways but activation of p53 pathway by VNLG-152R. **(C)** Whole proteome profiling by high-definition mass spectrometry (HD-MS) of VNLG-152R-treated (10 μM for 24 h) MDA-MB-231 cells demonstrate modulation of several pathways. Notably, protein translation is inhibited 12-fold apparently due to MNK1/2 degradation besides inhibiting other biological processes such as cell adhesion to the matrix, cell to cell adhesion and IFN signaling critical to breast cancer progression. Statistical significance was computed at $P < 0.01$.

3.5 VNLG-152R and its deuterated analogs demonstrate potent inhibition of TNBC growth and inhibit tumor growth *in vivo* in diverse racial tumor xenograft models

3.5.1 MDA-MB-231 tumor xenograft in mice: caucasian female patient with low/no AR

Tumor xenografts in mice originated from the widely used TNBC model, MDA-MB-231 cell line, derived from pleural effusion of a 51-year-old Caucasian female with metastatic mammary adenocarcinoma is highly aggressive, metastatic, and fast-growing (55). Interestingly, many reports suggest that it lacks expression of AR protein though presence of AR mRNA is detected (22). When the mice bearing MDA-MB-231 tumor xenograft were orally administered with 20 mg/kg VNLG-152R, five days a week, it resulted in 87% tumor growth inhibition (TGI) as measured by tumor volume (Figures 6A–C). Remarkably, the deuterated analogs D6, D7, and H6 demonstrated even higher tumor growth inhibition (94% each for D6 and H6, respectively), with D7 causing *67% tumor regression* compared to the initial tumor volume. The percentage change in tumor volume was plotted for the groups (Figure 6B) and for the individual animals in the groups (Figure 6C) show significant tumor regression in D7-treated group and 1-3 animals in the D6- and H6-treated groups. The weight of excised tumors was plotted and corresponded to the tumor volume (Figure 6D). Figure 6E shows the photograph of all the excised tumors after termination of the study which corroborates the tumor volumes shown in Figures 6A-C. Immunoblotting analysis of excised tumor tissue revealed significantly decreased levels of MNK1, accompanied by increased expression of SYVN1, confirming the expected molecular response to treatment (Figure 6F). Moreover, downregulation of the antiapoptotic protein BCL2, upregulation of the pro-apoptotic protein BAX, and decreased expression of Cyclin D1, crucial for cell cycle progression, were observed in the treated tumor tissues of the Caucasian model of TNBC/QNBC *in vivo*. We did not assess the levels of AR in the MDA-MD-231 tumors because we (*data not shown*) and others have shown that MDA-MB-231 cells have undetectable level on AR protein (22). Importantly, the body weight of the control and treated animals did not show significant differences, indicating the absence of treatment-induced toxicity of the test molecules at the given dose (Figure 6G).

3.5.2 MDA-MB-468 tumor xenograft in mice: female patient of African descent with low/no AR

MDA-MB-468 cell line is derived from metastatic adenocarcinoma of the breast from a female patient of African ancestry, expresses no AR and characterized by aggressive lymphatic metastasis (56). In mice tumor xenograft model of MDA-MB-468, oral administration of 20 mg/kg VNLG-152R and its deuterated analogs (D6, D7, and H6) resulted in significant inhibition of tumor growth. VNLG-152R inhibited tumor growth by 80.5%, while D6 exhibited a higher inhibition rate of 92.8%. Remarkably, both D7 and H6 completely inhibited tumor growth and induced *tumor regression* by 37.1% and 6.6%, respectively (Figures 7A–C). Importantly, the percentage change in tumor volume demonstrated significant tumor regression in at least two mice in the groups treated with the deuterated analogs (Figures 7A-C). The weights of the excised tumors from all animals were consistent with the tumor volume, further confirming the

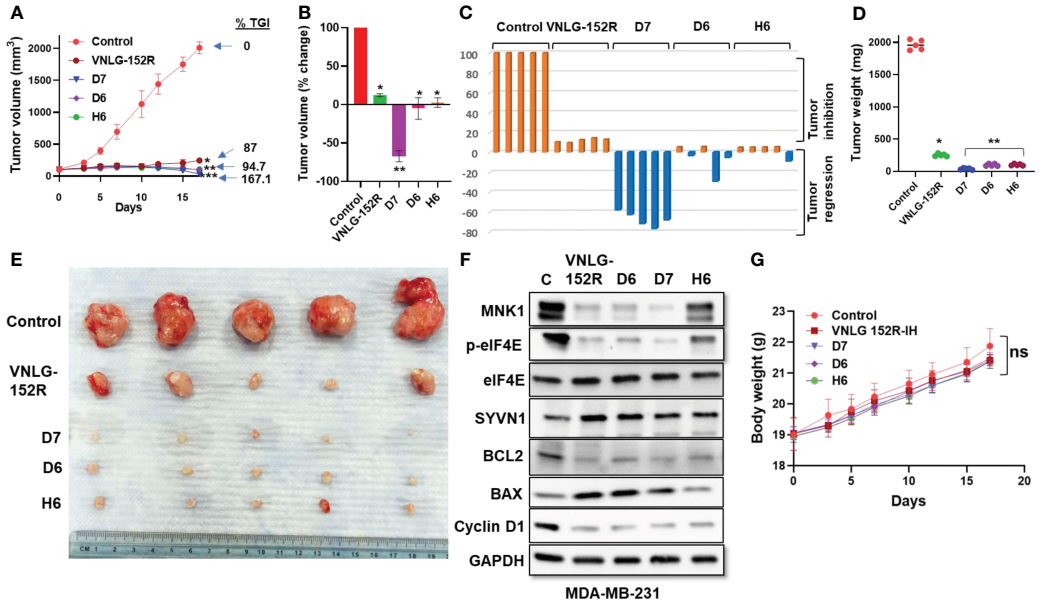

FIGURE 6
VNLG-152R and its deuterated analogs inhibit QNBC of Caucasian origin with no AR in NRG mice tumor xenograft model. MDA-MB-231 tumor xenografts were transplanted to NRG mice by subcutaneous injection of 5 x 10^6 cells to the left flank. Oral administration of VNLG-152R and its deuterated analogs (20 mg/kg body weight, PO) significantly inhibited tumor growth and resulted in tumor regression without apparent host toxicity. **(A)** Tumor volume was measured periodically as indicated and plotted against time (days), all *p* values are compared to vehicle control: *$P < 0.001$, **$P < 0.0001$; **(B, C)** Comparison of percentage change in tumor volume of animals in the group and the individual mice in the group (waterfall plots); **(D)** All excised tumors were weighed and plotted for comparison of tumor mass. **(E)** Tumors excised from all the test animals at the end of the experiment and photographed; **(F)** Immunoblots of key proteins in the excised tumor tissue show significant reduction in MNK1/2 protein and decreased level of phosphorylated eIF4E. The level of SYVN1 is higher in the treated animals compared to the control. **(G)**. The body weights of mice periodically taken and plotted show no apparent host toxicity of the test compounds. Statistics: All *P* values are compared to vehicle control: *$P < 0.001$, **$P < 0.0001$.

reduction in tumor mass following treatment with VNLG-152R or its analogs (Figure 7D). Furthermore, the oncogenic proteins BCL2 and Cyclin D1 were downregulated, while the proapoptotic protein BAX was upregulated in the excised tumors treated with VNLG-152R and the deuterated analogs (Figure 7F). Immunoblotting analysis of key proteins in the excised tumor tissue revealed significant downregulation of MNK1, accompanied by a decrease in p-eIF4E, which can be attributed to elevated levels of SYVN1 compared to the control (Figure 7C). Furthermore, the oncogenic proteins BCL2 and Cyclin D1 were downregulated, while the pro-apoptotic protein BAX was upregulated in the excised tumors treated with VNLG-152R and the deuterated analogs. As with the MDA-MB-231 tumors, we did not assess the impact of treatments on AR as the MDA-MB-468 cells do not express detectable levels of AR (22). Throughout the study period, the body weight of the animals did not show any significant changes in the treatment groups compared to the control, indicating that the administered compounds were not associated with significant toxicity at the given dose (Figure 7G).

3.5.3 MDA-MB-453 tumor xenograft in mice: caucasian female patient with high AR

Finally, we tested the antitumor efficacy of VNLG-152R and its most potent deuterated analog, D7 in MDA-MB-453 xenograft tumor model in female NRG mice in head-to-head comparison with Enzalutamide (ENZ) and Docetaxel (DTX). It is noteworthy that unlike a recent report which found that MDA-MB-453 tumors grew very slowly in either female or male SCID mice (22), our study clearly established that MDA-MB-453 xenograft tumors grew exceptionally well in female NRG mice (Figure 8A). MDA-MB-453 cell line represents a type of aggressive TNBC and was originally developed from metastatic breast cancer of a Caucasian female patient with metastatic sites involving the nodes, brain and both pleural and pericardial cavities (57). Unlike the other TNBC models investigated in this study, MDA-MB-453 expresses high levels of AR (22). Despite being less proliferative in nature, the LAR (luminal androgen receptor) subtype of TNBC is less responsive to chemotherapy than the basal type (58–60). When the mice transplanted with MDA-MB-453 tumor xenografts were treated with VNLG-152R and its deuterated analog D7, tumor growth was significantly inhibited as shown by the tumor volume (Figures 8A-C). VNLG-152R exhibited 84.2% inhibition of tumor growth, while D7, the most promising analog in other models, completely inhibited tumor growth and led to a remarkable 52.4% *tumor regression*. As anti-androgen therapy is a preferred clinical treatment option in AR-positive TNBC (7, 61–64), we compared the test compounds with clinically relevant anti-androgen, ENZ and chemotherapeutic, DTX, which inhibited tumor growth by 78.6% and 74.9%, respectively (Figures 8A–C). *It is important to state here that ENZ* (65) *and DTX* (66) *were administered at their optimal preclinical dosing regimens, and, it should be noted that higher doses of DTX have been shown to be toxic to mice.* The percentage change

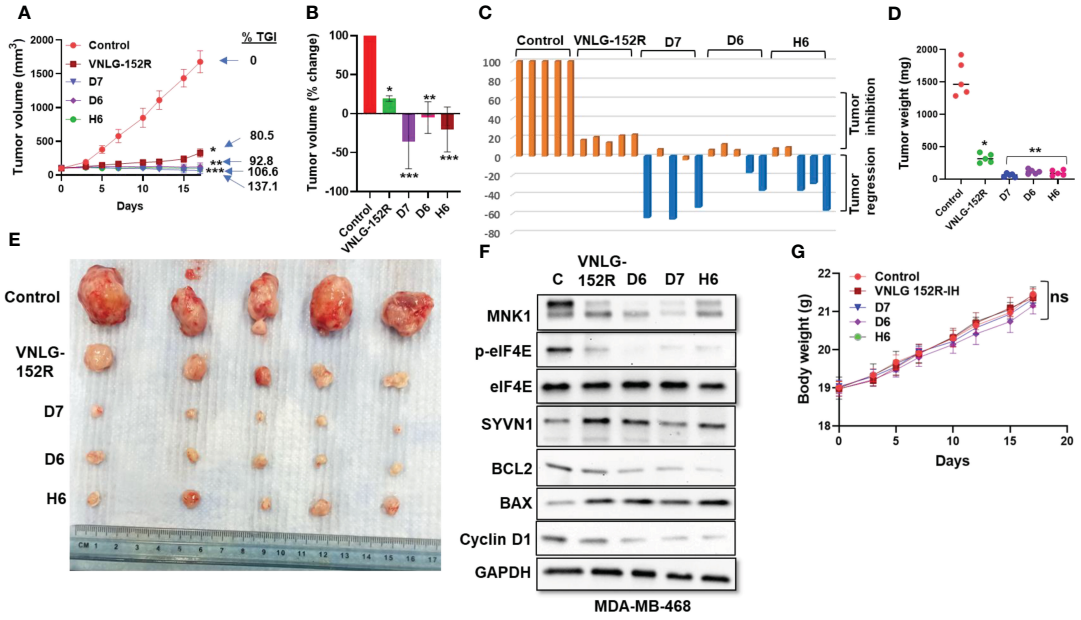

FIGURE 7
VNLG-152R and its deuterated analogs effectively inhibit TNBC of African origin with low or no AR expression *in vivo* in NRG mice. NRG mice were subcutaneously injected with 5x10^6 MDA-MB-468 cells in the left flank to establish tumor xenografts. Oral administration of VNLG-152R and its deuterated analogs (20 mg/kg body weight, PO) effectively suppressed tumor growth and induced tumor regression. **(A)** Tumor volume was periodically measured and plotted over time to assess the growth of tumors in response to the treatments; **(B, C)** Percentage change in volume of tumor from all animals in the group and that of individual mice (waterfall plots). **(D)** All excised tumors were weighed and plotted for comparison of tumor mass. **(E)** Tumors excised from all the test animals at the end of the experiment and photographed; **(F)** Immunoblots of proteins of interest in the excised tumor show reduction in level of MNK1/2. Further, the level of phosphorylated eIF4E is decreased and SYVN1 is higher in the tissue of treated animals. **(G)** The body weights of mice periodically taken and plotted show no signs of host toxicity of the test compounds. *Statistics:* All *P* values are compared to vehicle control: $*P < 0.01$, $**P < 0.001$, $**P < 0.0001$.

in tumor volume for individual animals and the treatment group is presented in Figures 8B, C, highlighting significant *tumor regressions* in all animals of the D7-treated group with a mean value of 52.4%. The weights of the excised tumors from all animals corresponded to the tumor volumes, providing further confirmation of the decrease in tumor mass after treatment with VNLG-152R or its analog (Figure 8D). Figure 8E shows the photograph of all the excised tumors after termination of the study which corroborates the tumor volumes shown in Figures 6A–C. Immunoblotting analysis of key proteins in the excised tumor tissue revealed upregulation of SYVN1 and a concomitant decrease in MNK1, resulting in reduced levels of p-eIF4E, modulation of apoptosis (BAX/BCL-2 ratios), depletion of cyclin D1 like the observations in other TNBC models (Figure 8F). In this model, and as expected (33, 34, 67), we also observe significant depletion of AR in tumors treated with VNLG-152R and D7 (Figure 8F). Consistent with the other studies, the tested compounds did not exhibit any toxic effects on the animals at the studied dose, as evidenced by stable body weight throughout the study period (Figure 8G).

4 Discussion

The pharmacological intervention of TNBC is an intricate challenge due to its diverse sub-types and unique molecular signatures, each presenting its own complexities. Additionally, patients of different racial backgrounds respond differently to available drugs. the pharmacological outcome is largely dependent on molecular signatures and ethnicity, with the African women registering the least overall survival (68). Due to this racial disparity in overall survival and response to drugs, it is imperative to study the efficacy of novel putative drugs in *in vivo* models of TNBC representing different racial origin.

The standard treatment regimens with hormone or HER2-targeted therapies are not an option in treating TNBC/QNBC patients (69). One of the alternative strategies is to pharmacologically target dysregulated translation machinery in the tumor cell as demonstrated by inhibition of MNK1/2 by eFT508 and other agents (70, 71). Notably, MNK1/2 are the only kinases known to phosphorylate eIF4E critical for the formation of translation initiation complex eIF4F (13–15). Since MNK1/2 are at the crossroads of other signaling pathways vital for the cancer development and progression such as mTORC1-4E-BP1 signaling and eIF4E signaling axes (16, 72), restraining MNK1/2 significantly inhibits cancer cells proliferation, cell migration, invasion, and metastasis (71).

The present study further extends our current understanding of the benefits of pharmacologically targeting MNK1/2 in TNBC/QNBC and unravels the molecular mechanism of VNLG-152R-mediated degradation of MNK1/2. Transcriptome and proteome-guided study further suggested the role of E3 ligase SYVN1 in

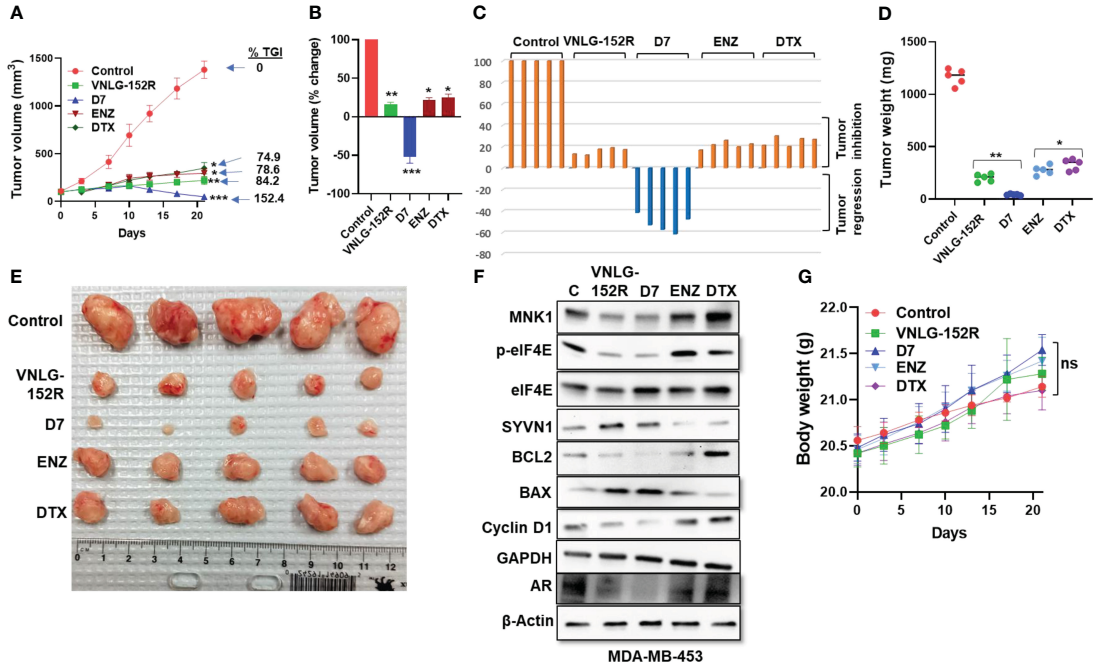

FIGURE 8
VNLG-152R and its deuterated analogs inhibit TNBC of Caucasian origin with high AR in NRG mice tumor xenograft model. MDA-MB-453 tumor xenografts were transplanted to NRG mice by subcutaneous injection of $3x10^6$ cells to the left flank. Oral administration of VNLG-152R and its deuterated analog D7 (20 mg/kg body weight, PO) significantly inhibited tumor growth and resulted in tumor regression without apparent host toxicity. DTX was administered by IP injection (5 mg/kg body weight). **(A)** Tumor volume was measured periodically as indicated and plotted against time (days) and shows significant inhibition of tumor growth in treated animals. Notably, D7 caused 52.4% tumor regression. **(B, C)** Percentage change in tumor volume of mice in different groups and that of the individual animals (waterfall plots). **(D)** All excised tumors were weighed and plotted for comparison of the tumor mass. **(E)** Tumors excised from all the test animals at the end of the experiment and photographed. **(F)** Immunoblots of putative proteins in the excised tumor tissue show a decrease in MNK1/2 and consecutive reduction of p-eIF4E level in the tumor of treated animals. **(G)** The body weights of mice periodically taken and plotted show no apparent host toxicity of the test compounds as there is no significant difference in body weights. *Statistics:* All *P* values are compared to vehicle control: $*P < 0.01$, $**P < 0.001$, $**P < 0.0001$.

MNK1/2 degradation. Interestingly, biochemical, and molecular studies emphasized the involvement of SYVN1 in ubiquitination of MNK1/2 as the presence of SYVN1 inhibitor LS102 or siRNA-knockdown of SYVN1 abolished the MNK1/2 degradation by VNLG-152R. Furthermore, immunoblots showed the presence of elevated levels of ubiquitinated MNK1/2 upon VNLG-152R treatment when proteasome was inhibited using the known proteasome inhibitor MG-132, suggesting the proteasomal degradation of ubiquitinated MNK1/2. VNLG-152R and the analogs might act as a molecular glue that brings together SYVN1 and MNK1/2 facilitating proximity-induced ubiquitination and subsequent proteasomal degradation as depicted in Figure 9. This study, including our previous studies (9, 18, 19, 33, 34), clearly establishes VNLG-152R and its analogs as monomeric molecular glues that induce MNK1 and MNK2 ubiquitin-proteasomal degradation to inhibit oncogenic eIF4F complex.

It is well established that E3 ligases, including SYVN1 can have opposite effects as either tumor suppressors (TS) or oncogenes depending on the context or type of cancer (73–75). With regards to SYVN1, previous studies have shown that it functions as a tumor suppressor in breast (37, 39, 41, 76, 77) and ovarian (40) cancers. On the contrary, the tumor-promoting (oncogenic) effects of SYVN1 have been revealed in colon cancer (78), lung cancer (79, 80), and hepatocellular carcinoma (81, 82).

Another significant finding of this study is that VNLG-152R caused dose-dependent depletion of WNK1 (Figure 3D) which is implicated in cell migration, invasion, and metastasis in multiple cancer types including glioblastoma (83), prostate cancer (84), non-small cell lung cancer (85), and breast cancer (45, 46, 86, 87). Because metastasis is the major cause of mortality in patients with breast cancer (88), we posit that our compounds can be developed as small molecules therapeutics with the characteristics of inhibiting both cell proliferation and metastasis, which would undoubtedly have a major impact on mortality in patients with breast cancer.

Transcriptome and proteome analyses further demonstrate inhibition of oncogenic pathways such as mTORC1 and NUP152 signaling in TNBC cells treated with VNLG-152R. Our study reveals a remarkable finding that VNLG-152R and the deuterated analogs are capable of inhibiting TNBC in patients of different ethnicity and molecular signatures. This includes patients with higher levels of MNK1 and eIF4E expression in tumors irrespective of AR expression status. Besides delineating the molecular mechanism of action of VNLG-152-induced degradation of MNK1 and MNK2, we clearly demonstrate that VNLG-152R and its deuterated analogs effectively inhibit TNBC tumor xenografts of both Caucasian and African origins, including those with low or no AR expression as well as the Caucasian race with high AR expression.

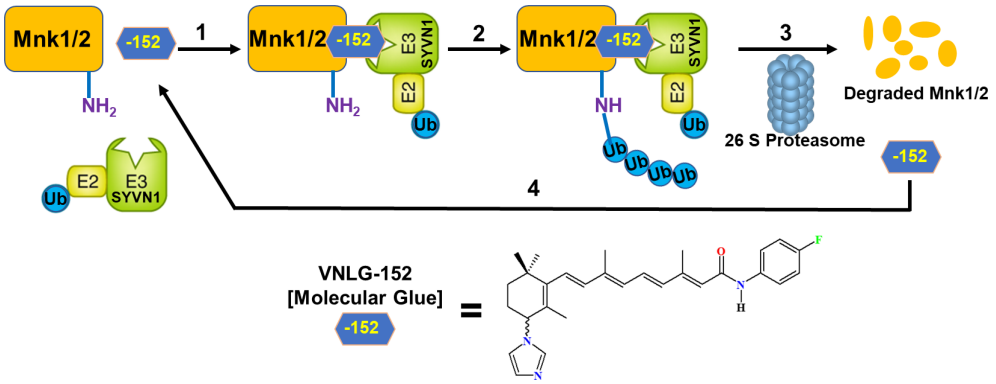

FIGURE 9
Schematic representation of mechanism of action of VNLG-152R in inhibiting TNBC by degrading MNK1/2. A ternary complex formed upon binding of VNLG-152R (-152; Molecular Glue), target proteins (MNK1/2) and SYVN1 E3 ligase complex (step 1), to promote MNK1/2 protein ubiquitination (Ub) (step 2), followed by MNK1/2 degradation by the proteasome and release of VNLG-152R (step 3). Free VNLG-152R can then bind another molecule of MNK1/2 (step 4) to repeat the degradation cycle.

5 Conclusion

In conclusion, we have clearly established that SYVN1 is the prime E3 ligase implicated in the VNLG-152/deuterated analogs-induced ubiquitin-proteasomal degradation of MNK1 and MNK2 degradation *in vitro* and *in vivo*. Because SYVN1 has been shown to act as a tumor suppressor in TNBC models, *in vitro* and *in vivo*, we propose that this phenomenon may also contribute to the anti-tumor efficacy of our compounds. Indeed, the inhibition of MNK1/2-mediated eIF4E phosphorylation reduces the formation of the translation initiation complex eIF4F, effectively restraining dysregulated protein synthesis central to tumor growth, progression and metastasis. Our findings highlight the significant potential of VNLG-152R and its deuterated analogs in effectively combating TNBC/QNBC across patients of diverse racial backgrounds, regardless of their genetic background and AR expression status. These results emphasize the broad applicability and efficacy of these compounds in addressing the challenges associated with TNBC/QNBC treatment in a racially diverse population. The data presented here clearly justify the on-going Investigational New Drug (IND) studies with VNLG-152R under the auspices of Isoprene Pharmaceuticals, Inc., in view Phase I clinical trials in women with TNBC and solid tumors.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: GEO repository, accession number GSE242514.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Institutional Animal Care and Use Committee (IACUC), University of Maryland School of Medicine, Baltimore, MD, USA, per IACUC No. # 0221010 dated 03/09/2021. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

The study was conceptualized and designed by RT, ET and VN. RT, ET and PP performed the experiments and acquired data. The data was analyzed by RT, ET, DW, VR, WH, MK and VN. RT, ET and VN wrote the manuscript. VN supervised the entire study. All authors contributed to the article and approved the submitted version.

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Conflict of interest

VN is the lead inventor of VNLG-152R and its deuterated analogs, the patents and technologies thereof are owned by the University of Maryland, Baltimore. PP, RT, ET are co-inventors of the deuterated analogs of VNLG-152R. Authors RT, VR and VN were employed by the company Isoprene Pharmaceuticals, Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Publisher's note

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Progress of research on PD-1/PD-L1 in leukemia

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Leukemia cells prevent immune system from clearing tumor cells by inducing the immunosuppression of the bone marrow (BM) microenvironment. In recent years, further understanding of the BM microenvironment and immune landscape of leukemia has resulted in the introduction of several immunotherapies, including checkpoint inhibitors, T-cell engager, antibody drug conjugates, and cellular therapies in clinical trials. Among them, the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) axis is a significant checkpoint for controlling immune responses, the PD-1 receptor on tumor-infiltrating T cells is bound by PD-L1 on leukemia cells. Consequently, the activation of tumor reactive T cells is inhibited and their apoptosis is promoted, preventing the rejection of the tumor by immune system and thus resulting in the occurrence of immune tolerance. The PD-1/PD-L1 axis serves as a significant mechanism by which tumor cells evade immune surveillance, and PD-1/PD-L1 checkpoint inhibitors have been approved for the treatment of lymphomas and varieties of solid tumors. However, the development of drugs targeting PD-1/PD-L1 in leukemia remains in the clinical-trial stage. In this review, we tally up the basic research and clinical trials on PD-1/PD-L1 inhibitors in leukemia, as well as discuss the relevant toxicity and impacts of PD-1/PD-L1 on other immunotherapies such as hematopoietic stem cell transplantation, bi-specific T-cell engager, chimeric antigen receptor T-cell immunotherapy.

KEYWORDS

leukemia, programmed cell death protein 1, programmed death-ligand 1, immunotherapy, PD-1/PD-L1 mAbs

1 Introduction

The current standard clinical treatment for leukemia, as a non-solid malignant tumor, mainly includes chemotherapy and hematopoietic stem cell transplantation (HSCT). However, the treatment process faces a series of problems such as chemotherapy insensitivity, chemoresistance, post-transplant relapse, and intolerance in elderly patients (1–4), thereby greatly limiting the progress of treatment for patients with leukemia.

Therefore, developing effective methods with low adverse reactions is currently imperative to ameliorate the prognoses of leukemia patients. The immune milieu of bone marrow (BM) is dramatically altered in patients with leukemia, where tumor cells prevent themselves from being cleared by immune system by affecting suppressive immune responses (5–8). Moreover, tumor cells in the blood, BM, and lymphoid tissue are also more accessible to immune cells than solid tumors. Furthermore, the efficacy of allogeneic HSCT (allo-HSCT) demonstrates that leukemia is a typical immune-responsive tumor type (9). Thus, immunotherapy is an obvious choice for treating hematological malignant tumors. In hematologic tumors, currently used immunotherapies include allo-HSCT, bi-specific T-cell engager (BiTE), chimeric antigen receptor (CAR) T-cell immunotherapy (CAR-T), immune-checkpoint inhibitors (ICIs), and other monoclonal antibodies (mAbs) targeting tumor-cell surface antigens (10–13). In recent years, the role of immune escape in leukemia progression and development of immunotherapy have been elucidated, employing ICIs to block suppressor molecules on the surface of T cells, thereby reversing the "exhausted" state of T cells to an "activated" one to kill tumor cells, has proved to be a promising option.

Immune checkpoint (IC) is a signal regulating T-cell receptor (TCR) antigen recognition during immune response. Programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) as an important IC modulating immune response. PD-1 (CD279), a type I transmembrane protein inhibitory checkpoint molecule is expressed on various immune cells, such as naive and activated B cells, effector T cells, regulatory T cells (Tregs), dendritic cells (DCs), activated monocytes, macrophages, natural killer (NK), and immature Langerhans cells (14). PD-1 receptors bind two ligands of the B7 family, PD-L1 and programmed death-ligand 2 (PD-L2). PD-L1 (CD274) is expressed on the surface of hematopoietic cells, such as DCs, macrophages, T cells, and B cells (15, 16). PD-L2 (PDCD1LG2) is expressed on monocytes, myeloid DCs, and activated CD4+ or CD8+ T-cell subsets (17). PD-L1 and PD-L2 differ in expression patterns but have the same effect, and binding of PD-1 to either ligand leads to T-cell dysfunction or exhaustion, resulting in diminished intensity of antigen-specific T-cell response in tumor tissues (18–21). In hematological malignant tumors, the expression rate of PD-L1 in malignant cells is 37%–58% (22). Leukemia cells highly express checkpoint-inhibitor receptors for sharing an immune-cell lineage (9, 23), making them potential targets for this therapy. This review centers around PD-1 signaling, summarizes its molecular functions in hematological malignant tumors and the achievements of ICIs in preclinical development and clinical settings.

2 Mechanisms involved in tumor immune escape through PD-1/PD-L1

As a pair of co-stimulatory signals, PD-1 and PD-L1 jointly constitute PD-1/PD-L1 signaling pathway. Under physiological conditions, the binding of PD-L1 on cell surface to PD-1 on lymphocyte surface inhibits lymphocyte function and induces the apoptosis of activated lymphocytes. The activation of the PD-1/PD-L1 pathway reduces the damage of immunoreactions to surrounding tissues and prevents the progression of autoimmune diseases (24). However, the activation of this pathway causes the binding of PD-L1 expressed on tumor cells to PD-1 on tumor infiltrating lymphocytes, decreasing the immune effect of T cells in the local tumor microenvironment (TME), thereby mediating tumor immune escape and promoting cancer progression (25–27). Researches have shown that PD-L1 expression is upregulated in tumor cells, which activates PD-1/PD-L1 downstream pathways by specifically binding to PD-1 on the surface of cytotoxic T lymphocytes (CTLs) to deliver negative regulatory signals. In turn, it induces the exhaustion of activated T cells and the loss of immunoreactivity, leading to a diminished intensity of antigen-specific CTL responses in tumor tissues (18–21). Besides, Tregs as important suppressive immune cells in TME contribute to cancer initiation and progression. The PD-1/PD-L1 pathway promotes Tregs transformation and enhances their immunosuppressive capacity (28–30). In addition to T cells, other immune cells are implicated in the regulation of immune tolerance induced by the PD-1/PD-L1 pathway. Tumor-associated macrophages (TAMs) upregulate PD-L1 expression of tumor cells (31), whereas tumor cell-secreted versican and derived exosomes induce upregulation of PD-L1 expression in TAMs, which is associated with M2 polarization of TAMs. TAMs with high expression of PD-L1 more significantly inhibit effector T cells and promote tumor growth and metastasis (32–34). Tumor cells increase PD-1 expression on B cells (35, 36), and PD-1+ B cells significantly suppress the proliferation and reduce the viability of CD4+ and CD8+ T cells via the PD-1/PD-L1-dependent pathway (37). NK cells can obtain PD-1 from leukemia cells by endocytosis in tumor cells, and PD-L1 in tumor cells interacts with PD-1 of NK cells to reduce NK cell responses and produce more aggressive tumors (38–40) (Figure 1).

PD-1 signaling is a pivotal molecule mediating immune escape in TME. Blocking PD-1 signaling attenuates tumor cell suppression of immune cells and improves immune system recognition and cytotoxicity of tumor cells. The increasing understanding of immune function and immune escape mechanisms has led to exploitation of therapeutic mAbs targeting PD-1 signaling (25). Up to now, FDA has successively approved four mAbs (pembrolizumab, nivolumab, cemiplimab, and dostarlimba) targeting PD-1 and three mAbs (atezolizumab, avelumab, and durvalumab) targeting PD-L1 for the treatment of solid and hematological malignancies (15, 16, 41–46), as presented in Table 1.

3 Role of PD-1/PD-L1 in the development of leukemia

Leukemia can be divided four major clinical categories: acute myeloid leukemia (AML), chronic myeloid leukemia (CML), acute lymphoblastic leukemia (ALL), and chronic lymphocytic leukemia (CLL). This review provides a theoretical basis for drug discovery and clinical application of PD-1/PD-L1 pathway by summarizing

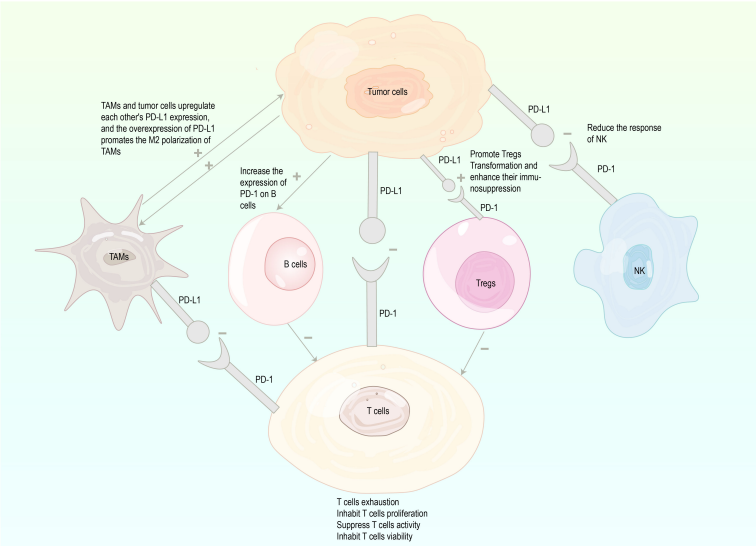

FIGURE 1
Mechanisms involved in tumor immune escape through the PD-1/PD-L1 (inhibition marked with -, enhancement marked with+), PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; TAMs, tumor associated macrophages; Tregs, regulatory T cells; NK, natural killer.

and analyzing the role of PD-1 signaling in various types of leukemia.

3.1 AML

AML is a heterogeneous disease with various genetic and epigenetic alterations. Its pathogenesis is the accumulation and expansion of immature myeloid cells in the peripheral blood (PB) and BM, resulting in hematopoietic dysfunction. Historically, AML has been regarded as an immunoreactive malignancy and remains the most common indication to receive allo-HSCT (7). PD-1 expression is generally high on T cells in AML patients with *de novo* and relapsed/refractory (R/R) after chemotherapy, and partial recovery is achieved in patients with complete remission (47–49). Moreover, the level of PD-1 on NK cells and PD-L1 on regulatory B cells (Bregs) increases in AML patients (47, 48, 50, 51). High expression of PD-1 coincides with the T-cell exhaustion (52–54). The overexpression of PD-1 signaling is relevant to poor overall survival of AML patients (55). Above studies suggest PD-1 signaling may influence the development and poor prognosis of AML by increasing T-cell exhaustion. Contrary to this conclusion, Schnorfeil et al. (56) found the level of PD-1 expression on PB CD4+ and CD8 + T cells of AML patients at diagnosis was similar to that of healthy controls, but significantly increased in relapse after stem cell transplantation. T-cell function is not impaired during this

TABLE 1 FDA approves mAbs for PD-1/PD-L1.

Drugs	Target	Timeline and cancer type
Pembrolizumab	PD-1	2014: Melanoma; 2015: NSCLC; 2016: HNSCC; 2017: Hodgkins Lymphoma, MSI-H or dMMR cancer, Gastric cancer, Bladder Cancer; 2018 Merkel cell carcinoma, Hepatocellular carcinoma, Cervical cancer, PMBCL; 2019: RCC, SCLC, Esophagus cancer; 2020: Colorectal cancer, Cutaneous squamous-cell carcinoma, TMB-high cancers; 2021: Breast cancer, Endometrial Carcinoma
Nivolumab	PD-1	2014: Melanoma; 2015: NSCLC, RCC; 2016: Hodgkins Lymphoma, HNSCC; 2017: Colorectal cancer; Hepatocellular carcinoma, Bladder Cancer; 2018: SCLC; 2020: Esophagus cancer, Malignant Pleural Mesothelioma; 2021: Gastric cancer
Cemiplimab	PD-1	2018: Cutaneous squamous-cell carcinoma;2021: NSCLC, Basal Cell Carcinoma
Dostarlimab	PD-1	2021: dMMR solid cancers, Endometrial Carcinoma
Atezolizumab	PD-L1	2016: NSCLC, Bladder Cancer; 2019: SCLC, Breast cancer; 2020: Melanoma, Hepatocellular carcinoma; 2022: ASPS
Durvalumab	PD-L1	2017: Bladder Cancer; 2018: NSCLC; 2020: SCLC; 2022: Hepatocellular carcinoma, Billiary track
Avelumab	PD-L1	2017: Merkel cell carcinoma, Bladder Cancer; 2019 RCC

FDA, Food and Drug Administration; mAbs, monoclonal antibodies; PD-1, programmed cell death protein 1; PD-L1, programmed cell death-ligand 1; NSCLC, non-small cell lung cancer; HNSCC, head and neck squamous cell carcinoma; MSI-H, high microsatellite instability; dMMR, deficient mismatch repair; PMBCL, Primary mediastinal large B-cell lymphoma; RCC, renal cell carcinoma; SCLC, small cell lung cancer; TMB, tumor mutational burden; ASPS, Alveolar soft part sarcoma.

process. They thought that this pattern is associated with a shift toward effector memory cells in patients with recurrent AML and T-cell exhaustion does not play a major role in AML. Besides, AML cells induce generation and expansion of Tregs by PD-1 signaling, and Tregs promote the proliferation of AML cells by secreting IL-10 and IL-35 (57, 58). In addition to regulating immune cells, PD-1/PD-L1 drives AML progression by regulating tumor-associated proteins, for example, the expression of PI3K and p-AKT decreases after PD-L1 knockdown, which induces G2/M cell cycle arrest and apoptosis, and the upregulation of PD-L1 increases the expression of PI3K/AKT and enhances the proliferation of tumor cells (59). PD-L1 is overexpressed in AML leukemia-initiating cells, where it increases cyclin D2 expression by enhancing JNK phosphorylation, ultimately promoting the entry of leukemia-initiating cells into cell cycle and proliferation (60). Epigenetic therapy (EGT), particularly with hypomethylating agents (HMAs) either alone or in combination, continues to be successfully used in treating elderly AML, although resistance is a frequent and ultimately near universal outcome (61). Liu et al. (62) found that EGT treatment induces the expression of PD-L1 mRNA and PD-L1 induces the occurrence of EGT resistance. To sum up, PD-1 signaling can promote AML progression by regulating immune cells, oncoproteins, and the occurrence of drug resistance, inhibition of PD-1 signaling can be a breakthrough for successful treatment of AML.

3.2 CML

CML is a myeloproliferative disorder characterized by BCR-ABL oncoprotein with high tyrosine kinase activity, which promotes the proliferation and inhibits the apoptosis of cancer cells (63). PD-1 signaling on specific T cells leads to T-cell exhaustion, and leukemia cells inhibit effector T-cell proliferation through PD-1/PD-L1 interactions, blocking PD-1 signaling contributes to improved CML control in pre-clinical mouse models by restoring the function of CML-specific CTLs (64). The quantity of bcr-abl fusion gene, as the initiation and core factor of CML pathogenesis, is positively correlated with the PD-1 expression level on CD8+ T cells. When CML is treated with tyrosine kinase inhibitors (TKIs), a target drug for bcr-abl, the PD-1 expression level of CD8+ T cells in the complete hematological response group is significantly lower than that in the control group, chronic phase, and blast phase (22). However, leukemia stem cells (LSCs) are resistant to specific TKIs and cause disease relapse after drug discontinuation in CML, besides, CTL transfer therapy leads to upregulation of PD-L1 on LSCs, which protects LSCs from CTL-mediated elimination. In contrast, PD-1 blockade during CTL transfer results in long-term survival of CML mice, suggesting that LSCs were either eliminated or effectively controlled by PD-1 blockade (65, 66). The Tregs are also increased in CML patients at diagnosis and in patients refractory to TKI treatment, and these Tregs have higher levels of PD-1 expression (67, 68). Which suggests PD-1-blocking antibodies given directly prior to and temporarily after TKI discontinuation may block the immune inhibitory effects of Tregs on CD4+/CD8+T-cells, blocking aberrant PD-1 signaling may result in greater success in TKI cessation studies.

3.3 ALL

ALL results from a clonal expansion of abnormal lymphoid progenitors of B cell (BCP-ALL) or T cell (T-ALL) origin that invades BM, PB, and extramedullary sites (69). Similar to other types of leukemia, PD-1 expression increases on T-cell subsets in B-ALL patients and is more prominent at relapse, PD-L1/L2 expression increases on LSCs (70). PD-1+ LSCs are used for T-ALL initiation and relapse, they can upregulate genes related to the MYC pathway, leukemic stemness, and early T-cell progenitor development, and downregulate genes related to apoptosis, cell cycle, and PI3K/AKT signal pathway (71). To determine whether PD-L1 expression on ALL cells inhibits T-cell responses, Blaeschke et al. (72) co-cultured second-generation anti-CD19 CAR-T cells with CD19+ and CD19+/PD-L1+ target cells. Result shows that CAR-T cells co-cultured with PD-L1+ target cells decrease the levels of Th1 cytokine secretion. Which indicates that PD-1 signaling mediates T-cell inhibition after/during T cells against BCP-ALL. In summary, above studies suggest that enhancing T-cell response by inhibiting PD-L1/L2 is a promising therapeutic option.

3.4 CLL

CLL is characterized by the accumulation and clonal proliferation of mature and typically CD5+CD23+ B-cells within PB, BM, lymph nodes, and spleen (73). Several studies have shown that PD-1 signaling is significantly upregulated in CLL patients, and the high level of PD-1/PD-L1 is closely related to disease grade and poor prognosis (74–78). Epstein–Barr virus (EBV) is one of the human tumor viruses, it can transform B-cells into tumor cells. In CLL patients, EBV load is positively correlated with the expression of PD-1 signaling on CD4+ and CD8+ T cells. In EBV (+) patients, the higher the level of PD-1 signaling on T cells, the higher the risk of lymphocyte doubling and treatment initiation (79). Gassner et al. (80) found that inhibiting the interaction of PD-1/PD-L1 can reactivate the cytotoxic effect of exhausted T cells in CLL mouse model. To study the mechanism of PD-1 signaling in CLL, Qorraj et al. (81) collected PB mononuclear cells from CLL patients. They found that triggering PD-1 on monocytes hampers phagocytosis, glycolysis, and Bruton's tyrosine kinase (BTK)-signaling. Conversely, the immune metabolic dysfunctions and antitumor activity of monocytes can be reversed by disrupting PD-1 signaling. In conclusion, PD-1 signaling inhibits immune cell activity and interferes with immune metabolic processes. The blockade of PD-1 signaling may improve the prognosis of CLL.

4 Regulation of the PD-1/PD-L1 pathway in leukemia

In addition to PD-1 and PD-L1 antibodies directly acting on PD-1 signaling, other proteins, genes, and drugs affect the level of PD-1/PD-L1. When mAbs are insensitive or patients are intolerant to adverse reactions, we may consider indirectly inhibiting immune escape of tumor cells by regulating related proteins and genes or applying relevant drugs (Figure 2).

4.1 AML

In AML patients, B lymphocyte-induced maturation protein 1 (Blimp-1) directly binds to the promoter of PD-1 and impairs T-cell activity by upregulating PD-1. The knockdown of Blimp-1 can reverse the T-cell functional defect (82). IFN-γ induces PD-L1 expression in myeloid precursor cells and primary cells (57, 83). Stattic, a small molecule inhibitor of STAT3, interferes with IFN-γ-induced PD-L1 expression in AML (84). PD-1 level decreases after the initial HMA/ventoclax (Bcl-2 inhibitor) treatment on all CD4+ T-cell subpopulations except naïve in AML patients (48). In an immunocompetent murine leukemia model, guadecitabine (a second-generation HMA) negatively regulates inhibitory accessory cells in TME by reducing PD-1+ T cells and the AML-mediated expansion of myeloid-derived suppressor cells. Consequently, functionally active leukemia specific T cells increase (85). NA-AML ($Nras^{G12D/-}$; $Asxl^{-/-}$AML) cells overexpress PD-L1/PD-L2, and the level of PD-L1 is associated with the upregulation of AP-1 transcription factor (TF). AP-1 inhibitor or short-hairpin RNAs against AP-1 TF Jun decreases PD-L1 expression (86). The overexpression of miR-200c and miR-34a causes the significant downregulation of PD-L1 level. MUC1 attenuates the interference of miR-34a and miR-200c on PD-L1 translation by negatively regulating the expression of miR-34a and miR-200c, and silencing of MUC1 leads to increased miR-34a and miR-200c. In turn, PD-L1 expression is reduced (87).

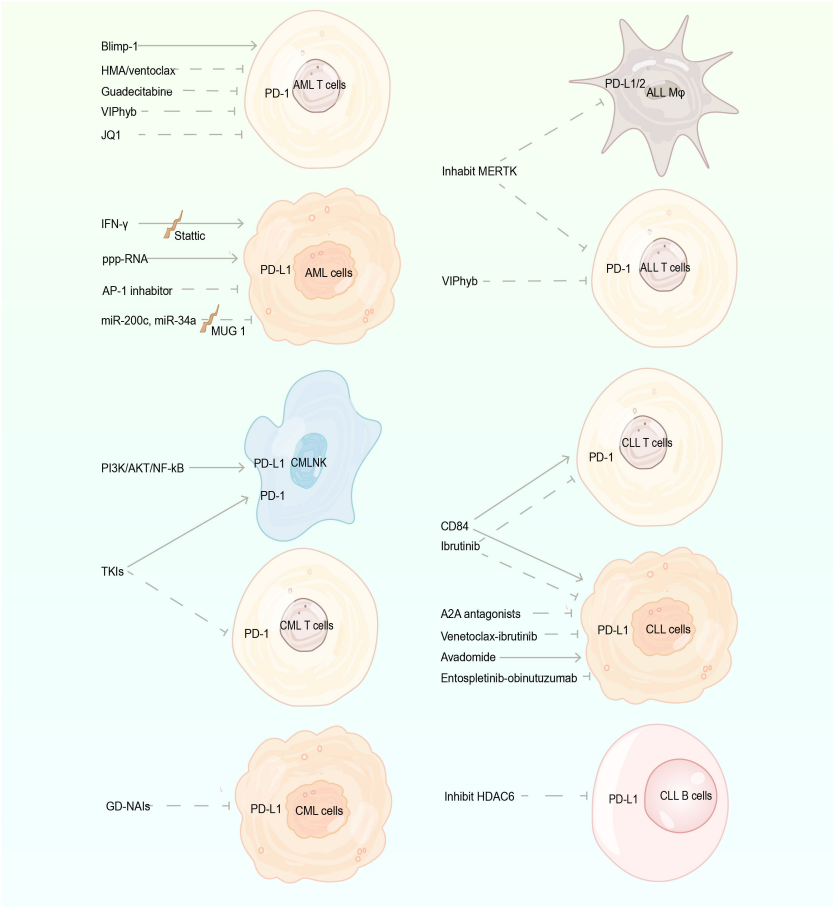

FIGURE 2
The regulation of the PD-1/PD-L1 pathway. PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myeloid leukemia; CLL, chronic lymphocytic leukemia; Blimp-1, B lymphocyte-induced maturation protein 1; HMA, hypomethylating agents; TKIs, tyrosine kinase inhibitors; GD-NAIs, *Gynura divaricate*-non-alkaline ingredients; Mφ, monocytes/macrophages; A2A, Adenosine A2A receptor.

4.2 CML

Myeloid leukemia cells induce PD-L1 expression on NK cells via PI3K/AKT/NF-kB pathway (88), thus, inhibiting this pathway may block PD-L1 expression. The level of PD-1 on CD8+ T cells is reduced in CML patients treated with TKIs dasatinib and imatinib (22). *Gynura divaricata* (L.) DC. is a widely used herbal medicine, whose non-alkaline ingredients regulate PD-1 signaling, significantly inducing apoptosis and inhibiting proliferation of CML cells (89).

4.3 ALL

A leukemic microenvironment supports the survival of ALL cells and their immune evasion through multiple interactions (69). In an ALL mouse model, inhibition of MERTK significantly decreases the expression PD-L1/L2 on CD11b+ monocytes/macrophages and PD-1 on CD4+ and CD8+ T cells in the leukemic microenvironment, reducing the incidence of splenic FOXP3+ Tregs at sites of leukemic infiltration. Consequently, T-cell activation increases, and immune-mediated ALL clearance is promoted (90). Murine models of AML and T-ALL reveal that VIPhyb, a peptide antagonist of VIP signaling, enhances IFN-γ secretion and suppresses PD-1 expression in CD4+ and CD8+ T cells (91).

4.4 CLL

CD84-mediated intercellular interactions upregulate the level of PD-1 on T cells and PD-L1 on CLL-cells via the Akt-mTOR pathway, resulting in T-cell exhaustion. Conversely, the downregulation of CD84 expression reverses these phenomena and reduces the expression level of other exhaustion markers (92). The activation of Adenosine A2A receptor (A2A) induces immune tolerance and is closely associated with immune escape of tumor cells (93). In CLL cells, hypoxia causes the emergence of a population of PD-1+ and IL-10–secreting T cells, and adding A2A antagonists attenuates Tregs generation, TGF-β induction, PD-1 expression, and IL-10 synthesis and secretion. Thus, leukemia cells become more susceptible to pharmacological agents while restoring immune competence and T-cell proliferation (94). Ibrutinib, a covalent inhibitor of BTK, is approved for treatment of patients with R/R or treatment-naive CLL (95). Cubillos et al. (96) found that ibrutinib can decrease PD-1 and PD-L1 expression by driving Th1-selective pressure in T cells. Kondo et al. (95) suggested that ibrutinib enhances antitumor immune responses by inhibiting STAT3-induced selective and persistent downregulation of PD-L1 on CLL cells and PD-1 in CD4+ and CD8+ T cells. In a venetoclax (VEN)–ibrutinib combination treatment, the number of PD-1 +CD8+ T cells, Tregs, and follicular helper T cells decreases more than fivefold, thereby reducing the immunosuppressive characteristics of CLL (97). The SYK inhibitor entospletinib in combination with obinutuzumab downregulates the expression of PD-1 in CD4+ and CD8+ T-cell subsets of CLL patients, partially reversing the T-cell exhausted phenotype (98).

5 PD-1/PD-L1 and allo-HSCT

Allo-HSCT is a potentially curative therapy for various hematologic malignancies. It relies on the graft-versus-leukemia (GVL) effect mediated by donor-derived alloreactive T cells. However, graft-versus-host disease (GVHD) is also mediated by the same T cells and remains a major clinical problem related to considerable morbidity and mortality (99, 100). The occurrence of GVHD and T-cell suppression is positively correlated with the expression level of PD-L1 (101). The loss of GVL effect is relevant to PD-1 overexpression in allograft recipients, and blocking PD-L1 largely restores GVL efficacy without triggering GVHD (102). Besides, HSCT leads to differential upregulation of PD-1 ligands in tissues, which compartmentalizes CTL activity and thus creates niches for tumor escape. PD-1 blockage can restore CTL sensitivity to antigens and homogenize the effect of graft against tumor (103). These suggest that improving GVL and reducing GVHD by blocking PD-1 signaling can yield considerable results. Ni et al. (100) found that the exhaustion of CD4+ T cells leads to PD-L1 upregulation in donor CD8+ T cells and recipient tissues. which increased PD-L1/PD-1 interplay between donor CD8+ T cells and recipient tissues contributes to preventing GVHD by promoting the apoptosis and exhaustion of T-cell in GVHD target tissues, and enhanced PD-L1/CD80 interplay between CD8+ T cells contributes to retaining GVL responses by improving T-cell expansion and survival. Accordingly, the influence of the PD-L1-mediated effect on HSCT depends on the tissue microenvironment, the existence of CD4+ T cells, and the natural interacting partner expressed by CD8 + T cells. This suggests that we can enhance the PD-1 signaling-mediated GVL effect and reduce the PD-1 signaling-mediated GVHD by changing the above conditions. Besides, VIPhyb also increases the anti-leukemic effect after allogeneic BM transplantation by downregulating PD-1 and PD-L1 expression on donor immune cells (104). In clinical trial, Tschernia et al. (105) found that the use of pembrolizumab before allo-SCT reduced 100-day mortality in AML patients (17% vs 0%) and did not increase grade III-IV acute GVHD. The chronic GVHD is not found in patients who have received pembrolizumab before allo-SCT and cyclophosphamide after transplantation. This suggests that ICI treatment prior to allo-SCT is effective and safe, and post-transplant cyclophosphamide can eliminate the GVHD risk and severity. The above studies provide an empirical and theoretical basis for ICIs combined with HSCT in the treatment of leukemia.

In addition to utilizing the GVL effect of hematopoietic stem cells (HSCs), Hu et al. (106) enhanced the delivery of checkpoint inhibitors by using the *in situ* activation of platelets and the homing ability of HSCs. They constructed HSC-platelet-aPD-1 conjugates and then injected them into mice bearing AML cells, the therapeutic effect of checkpoint blocking is significantly enhanced. With regard to the drug-delivery mode of PD-1/PD-L1, Chen et al. (107) introduced a transdermal cold atmospheric plasma (CAP)-

mediated IC blockade (ICB) therapy. The ICB delivered via microneedles enhanced the immune response mediated by T cells. Han et al. (108) used HEK293T-derived vesicles with PD-1 receptors on their surface to destroy PD-1 signaling, while the internal space of the vesicle allows for the packaging of an indoleamine 2,3-dioxygenase inhibitor, which further enhanced the antitumor effect. This suggests that in addition to drug development for ICIs, new technologies for applying ICIs are also of interest worthy of attention.

6 Efficacy of PD-1/PD-L1 mAbs treating leukemia alone or in combination

Studies on leukemia treatment with PD-1/PD-L1 mAbs are rapidly increasing in number. They are primarily divided into basic research and clinical stages. Herein, we provide guidance and rationale for subsequent clinical applications by analyzing their pooled data.

6.1 PD-1/PD-L1 mAbs for AML

Current clinical treatments for AML are primarily chemotherapy and allo-HSCT. However, due to the emergence of resistance to chemotherapy and GVHD, more effective and safer drugs to treat AML need to be developed (109, 110). Nivolumab, a PD-1 mAbs, is applied in an index case of recurrent myeloproliferative neoplasms after HSCT. Before infusion of nivolumab, AML blasts show high expression of chemokines, whereas T cells are characterized by the expression of interferon-responsive genes. This baseline inflammatory signature disappears after infusion of nivolumab, and the clinical responses are characterized by the temporary expansion of polyclonal CD4+ T-cell populations, the contraction of AML subsets exhibiting megakaryocytic characteristics, and elevated PD-L1 expression (111). Several studies show that the combination of PD-1/PD-L1 mAbs is promising research, for instance, the combined blockade of PD-1 signaling and Tim-3 have an additive effect on inhibiting tumor growth in advanced AML mouse models (112). The combination of IL-15 and PD-1 blockers activates AML-NK cells and enhances the killing ability of NK by increasing the release of perforin, granzyme, and IFN-γ (113). In addition, inhibiting the effects of other therapies on PD-1 expression can also yield considerable results, for instance, exogenous short 5′-triphosphate-modified RNA (ppp-RNA) can direct the immune response toward tumor cells. However, ppp-RNA treatment induces PD-L1 expression on AML cells and establishes therapeutic sensitivity to anti-PD-1 *in vivo*, the combination of anti-PD-1 and ppp-RNA is superior to either regimen alone in the survival rate of a mouse model (114). The DAC/VEN therapy (HMA decitabine combined with BCL-2 inhibitor venetoclax) effectively targets leukemia cells while upregulating PD-1 expression in AML patients. Nivolumab combined with DAC/VEN can enhance antitumor effect and eliminate circulating blasts and LSCs/progenitor cells (115). The above studies indicate that considering PD-1/PD-L1 antibodies as an adjuvant treatment scheme for AML can effectively enhance the sensitivity of cell therapy and chemotherapeutic agents, which is a promising combination-chemotherapy option. A number of clinical studies have been conducted on PD-1 mAbs combined with other chemotherapeutic drugs in the treatment of AML, such as cytarabine (116), azacytidine (117), decitabine (118), and these treatment regimens are clinically feasible and have shown encouraging results.

Tumor progression leads to increased Tregs and elevated PD-1 expression on CD8+ CTLs in AML mouse model, which reduces the recognition and activation of tumor-specific CTLs (58). PD-L1 siRNA-mediated silencing augments the expression of T cell activation markers (CD69 and CD137) and improves CTL degranulation (CD107a) (119). CTL infusion combined with PD-1 blockade suppresses Tregs (120). PD-1 blockade in combination with Tregs exhaustion or CTL infusion induces significantly more AML tumor reduction than either treatment alone (58, 120). Additionally, combining DC-based immunotherapy with PD-1 blockade might be a promising approach to eliminating LSCs (121). In sum, PD-1/PD-L1 blockade combined with cell therapy represents a significant new approach that can be easily translated into clinical applications to enhance T cell-mediated cytotoxic responses.

6.2 PD-1/PD-L1 mAbs for CML

TKIs and HSCT are the mainstay of treatment for CML (122, 123), and immune mechanisms may help maintain treatment-free remission. The direct interplay between NK cells and K562 myeloid leukemia cells induces the PD-L1 expression of NK cells. Compared with PD-L1-NK cells, PD-L1+ NK cells are activated effector cells with strong killing activity against tumor cells *in vitro*. The binding of the PD-L1 mAbs atezolizumab to PD-L1 upregulates PD-L1 expression on the surface of NK cells and provides more binding sites for PD-L1 mAbs, resulting in continuous activation of p38. This phenomenon further propagates strong activation signals toward NK cells to maintain their cytotoxic and cytokine-secretion features. *In vivo*, the combination of PD-L1 mAbs and NK cell-activating cytokines significantly enhances the antitumor activity of NK cells against myeloid leukemia lacking PD-L1 expression (88). This finding suggests that PD-L1 mAbs have a unique therapeutic effect on PD-L1- tumors, this is independent of PD-1. Dasatinib, a second-generation TKI, upregulates PD-1 expression on $CD56^{dim}$NK cells and increases dysfunctional $CD56^{neg}$NK cells that highly express PD-1. Nivolumab enhances the cytotoxic activity of both subsets but more efficiently in the $CD56^{dim}$ subset compared with the $CD56^{neg}$ subset (39). Which suggests the combination of TKIs and PD-1/PD-L1 mAbs may be an approach for the successful treatment of CML patients. Recent evidence shows PD-1 expression on CD4+ and CD8+ T-cells, including on CML-reactive PR1-CTL in TKI-naive but also TKI-treated remission CML patients (124–126), which suggests T-cell

exhaustion also in deep molecular remission, this provides a rationale for the treatment with checkpoint blocking antibodies to PD-1/PD-L1. However, a clinical trial of the combination of dasatinib and nivolumab for the treatment of CML showed that this approach did not show meaningful clinical activity in patients with CML in chronic phase or accelerated phase who received ≥2 prior TKIs with progression, resistance, or suboptimal response to most recent therapy (127). A phase II trial of the effectiveness of pembrolizumab and dasatinib, imatinib mesylate, or nilotinib in treating patients with CML and consistently detecting minimal residual disease (defined as the level of a gene product called bcr-abl in the blood) is currently underway (www.clinicaltrials.gov as # NCT03516279).

6.3 PD-1/PD-L1 mAbs for ALL

ALL has genetic heterogeneity, and the incidence is much higher in children. The current therapies for ALL are primarily multidrug chemotherapy, which has a high response rate but also has a high recurrence rate, leaving much room for improvement (128, 129). The phenotypic exhaustion of CD4+ T-cells predicts recurrence and poor overall survival in B-ALL. In a Ph+ B-ALL mouse model, the application of PD-L1 antibody clonally expands leukemia-specific CD4+ T-cells with helper/cytotoxic phenotype and reduces the expression of exhaustion markers. The combination of PD-L1 mAbs and TKI nilotinib also significantly improves the efficacy of nilotinib against BCR-ABL+ B-ALL (130). Axl ablation in macrophages can elicit the susceptibility of PD-1 refractory treatment naive B-ALL to PD-1 checkpoint blockade and promote antileukemia immunity (131). A new peptide, nABPD1, is designed to specifically bind PD-1. It enhances cytokine-induced killer (ICIK) cell-mediated antitumor activity by protecting ICIK cells through blockade of PD-1 signaling (44). There is a lack of clinical studies on the use of PD-1/PD-L1 mAbs in ALL, especially in young people, and as far as the current studies are concerned, they show unsatisfactory results for MRD in adults(median age is 52.5y) with ALL (132). Two studies of PD-1 mAbs for the treatment of ALL in children, adolescents, and young adults are currently underway (www.clinicaltrials.gov as # NCT05310591, NCT04546399).

6.4 PD-1/PD-L1 mAbs for CLL

Chemotherapy and anti-CD20 mAbs therapy are the standard of care for patients with CLL (133–135). Currently, it is prominent to improve the complete remission rate and reduce chemotherapy-induced immunosuppression. Studies suggest that early blockade of PD-L1 effectively prevents the immune dysfunction induced by tumor cells and thus avoids CLL development in mice. This includes the prevention of exhaustion-like and aberrant T-cell phenotypes, and the restoration of MHC class II-expressing dendritic cells and mature macrophages (108, 136). Ioannou et al. (137) concluded that although PD-L1 mAbs are superior to PD-1 mAbs in inducing anti-CLL T-cell activity, PD-1 mAbs and PD-L1 mAbs monotherapies are largely ineffective in overcoming T-cell tolerance in CLL. Avadomide is a cereblon E3 ligase modulator drug that stimulates T-cell immune synapse while increasing PD-L1 expression, it triggers IFN-driven T-cell responses and converts noninflamed CLL tumors into CD8+ T cell-inflamed ones, making CLL sensitive to PD-1/PD-L1 immunotherapy. The combination of avadomide and PD-1/PD-L1 blockade effectively reinvigorates previously exhausted patient T cells and contributes to more T cell killing in CLL. HDAC6 gene silencing or inhibition decreases PD-L1 expression on B cells of Eμ-TCL1 mice model, and the combination of HDAC6 inhibitor ACY738 and anti-PD-1/anti-PD-L1 further enhances the cytotoxicity of T cells (138). Rivas et al. (139) found that the treatment of CLL with anti-PD-L1 in combination with IL-10 produces more IFN-γ+, memory CD8+ T-cells, and cytotoxic effector KLRG1+, and fewer exhausted T-cells than anti-PD-L1 alone. CLL animal experiments show that PD-1/PD-L1 antibody as a combination chemotherapy regimen definitely affects tumor inhibition. However, according to the results of the current clinical study, PD-1 mAbs have limited efficacy in CLL patients, but reassuringly they show a promising therapeutic option in patients with Richter's transformation (140–142).

6.5 PD-1/PD-L1 mAbs and CAR-T

CAR-T cell therapy has contributed to a revolution in the therapy of patients with hematological malignancies (143). However, the activation of CAR-T cells can lead to persistently high levels of PD−1 and eventually cause the exhaustion of T cells (144). Several studies have shown that the integration of PD-1-mediated inhibitory signaling into CAR-T significantly improves the function of conventional CAR-T, and it even may have an almost equivalent or better anti-tumor effect and a lower side effect compared with the CAR-T plus PD-1 antibody (72, 145, 146). More studies on PD-1 signaling with CAR-T are shown in Table 2. These studies suggest that PD-1 signaling blockade combined with CAR-T can enhance the efficacy of CAR-T. To date, a variety of CAR-T with PD-1 inhibition have been designed, and they have achieved gratifying results in preclinical studies. PD-1 signal blocking combined with CAR-T may produce greater benefits compared with chemotherapeutic drugs. However, there is a lack of clinical studies in this area, and the clinical effects and adverse effects are unclear.

6.6 PD-1/PD-L1 mAbs and BiTE

Blinatumomab (BiTE antibody) is a novel immunotherapy that recruits the forces of T cells and guides them against lymphoblastic cells by binding CD3 expressed on the surface of T cells and CD19 expressed on the surface of B cell lines (151, 152). It was approved by FDA in 2014 for the treatment of Ph-negative R/R precursor B-ALL. However, approximately 50% of R/R B-ALL patients do not respond to blinatumomab. Non-responders consistently express higher levels of PD-1 during blinatumomab treatment, and the levels of PD-L1 and PD-L2 increase on residual tumor cells in BM after treatment. The T-cell responses of blinatumomab against

TABLE 2 CAR-T combined with PD-1/PD-L1 for leukemia treatment.

Condition	CAR-T product	Design	Phase	Outcome
(72) ALL	CD19 CAR-T, CD22 CAR-T	Anti-CD19 and anti-CD22 CAR T cells combined with PD-1-CD28 fusion protein	Preclinical trial	Increase function of CAR-T cells against leukemia and protect CAR-T cells from leukemia-induced suppression
(147) CLL	CD19 CAR-T	-	Clinical trial	The percentage of CAR-T cells with CD8+PD-1+ phenotype is significantly lower in complete-remission patients compared with partially responding and nonresponding patients.
(146) CML	CD19/ △PD-1 CAR-T	Integrate PD-1 shRNA into a third-generation CAR plasmid	Preclinical trial	Suppress the immunosuppression of TME and prolong the activation time of CAR-T cells
(145) CML	aPDL1-CART	Integrate a PD-L1-targeted scFv fusion protein into a CAR	Preclinical trial	Successfully prevent the development of PD-L1-expressing leukemia xenografts in immunocompromised mice
(144) AML	CLL-1 CAR-T	Silence the expression of PD-1 in CLL-1 CAR-T	Preclinical trial	The killing ability of CLL-1 CAR-T is further enhanced
(148) AML	CD19-CAR-T, CD123-CAR-T	CAR-T treated with JQ1 [JQ1(BET inhibitors) can suppress PD-1 expression in T cell]	Preclinical trial	The antileukemia potency and anti-exhaustion ability of CAR-T cells are enhanced
(149) R/R AML	CLL-1 CAR-T	CLL-1 CAR-T cells with PD-1 knockdown in 2 patients	Clinical trial	Both patients achieved molecular complete remission with incomplete hematologic recovery at 28 days
(150) ALL	PD-1-CD28 IFP CAR-T	Fuse different variants of the extracellular domain of PD-1 to the intracellular domain of CD28 to create multiple variants in the protein length of the PD-1-CD28 IFP	Preclinical trial	IFP variants with physiological PD-1 length ameliorate CAR T cell effector function and proliferation in response to PD-L1+ tumor cells *in vitro* and prolonged survival *in vivo*

CAR-T, chimeric antigen receptor T-cell immunotherapy; ALL, acute lymphoblastic leukemia; PD-1, programmed cell death protein 1; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; shRNA, short hair RNA; TME, tumor microenvironment; PD-L1, programmed death-ligand 1; CLL-1, C-type lectin-like molecule-1; BET, bromodomain and extra terminal domain BET; IFP, immunostimulatory fusion protein.

leukemia are potentiated by blocking CTLA-4 and PD-L1 signaling pathways (153, 154). This finding illustrates that the response of blinatumomab is correlated with the molecular level of IC. Wunderlich et al. (155) reported that pembrolizumab combined with blinatumomab increases the clearance of B-ALL in mice and reverses T-cell lymphopenia induced by blinatumomab. PD-1 inhibition also enhances the efficacy of blinatumomab in a UCB/PDX model of recurrent pediatric B-ALL. Krupka et al. (156) constructed the CD33/CD3 BiTE antibody AMG 330. They found that PD-L1 on primary AML cells is strongly upregulated after adding AMG 330 in the ex vivo culture, and blocking PD-1/PD-L1 axis enhances the AMG 330-induced lysis of AML cells by reversing T-cell-induced immune escape. Herrmann et al. (157) fused the extracellular domain of PD-1 (PD-1ex), which naturally holds a low affinity to PD-L1, with an αCD3.αCD33 BiTE®-like scaffold to form a bifunctional checkpoint inhibitory T-cell-binding (CiTE) antibody. The CiTE antibody is more potent in binding to AML cells and T cells, thereby increasing the function of T-cell effectors, and minimizing iRAEs associated with the systemic application of ICB. From the above several ex vivo studies and animal experiments, we can conclude that BiTE and PD-1 signaling blockade have good synergy in leukemia treatment. Nevertheless, there are few completed clinical studies on the combination therapy of blinatumomab and PD-1/PD-L1 mAbs for leukemia. A large sample phase II trial comparing blinatumomab alone to blinatumomab with nivolumab in patients with relapsed B-ALL is currently underway (www.clinicaltrials. as # gov NCT04546399).

6.7 Clinical trials of PD-1/PD-L1 mAbs in leukemia

A number of clinical trials on leukemia treatment with PD-1/PD-L1 mAbs have been performed nowadays. The overall response rate (ORR) of pembrolizumab alone is 0% in eight patients with AML (158). When combined with cytarabine, the ORR is 46% (116); the former grade 3–4 iRAEs are 25% (158), and the latter grade ≥3 iRAEs are 14% and self-limiting (116). The median recurrence-free survival (RFS) of AML patients treated with nivolumab alone is 8.48 months (159). When combined with cytarabine–idarubicin, the RFS is 18.54 months (160); the former grade 3–4 iRAEs are 27% (159), and the latter are 13.6% (160). From the above data, we assume that the efficacy of pembrolizumab and nivolumab alone is significantly lower than that of the combination, and the incidence of iRAEs is also higher with the single agent than with the combination. The median overall survival (mOS) is 11.1 months for AML with 200 mg of pembrolizumab in combination with 1.5–2 g/m^2 cytarabine (116), 21 months when combined with 1.5–2 mg/m^2 cytarabine (105), and 10.8 months when combined with decitabine (118). Regarding the data from the

current sample, the efficacy of pembrolizumab combined with low-dose cytarabine is superior to that of the high-dose one, and the efficacy of pembrolizumab combined with decitabine or high-dose cytarabine is similar. The iRAEs are 42% in AML patients treated with pembrolizumab alone, the grade 3–4 iRAEs are 25% (158), the iRAEs are 40% when treated with nivolumab alone, the grade 3–4 iRAEs are 27% (159), and the incidence of adverse events is similar for both drugs. The mOS for AML treated with avelumab–azacitidine combination is 4.8 months (161), and that with durvalumab–azacitidine combination is 13.0 months (162). The ORR for AML treated with avelumab–azacitidine combination is 10.5% (161), that with nivolumab–azacitidine combination is 33% (117), and that with durvalumab–azacitidine combination is 31.3% (162). However, the grade 3–4 iRAEs in the avelumab combination are less than 7.7% (163). Based on the current sample alone, avelumab is less effective than pembrolizumab, nivolumab, and durvalumab, but its incidence of iRAEs is much lower. Due to differences in sample size and patient disease status among studies, comparisons of efficacy and adverse effect assessments of PD-1/PD-L1 mAbs among studies are subject to large errors. Regarding the current sample, PD-1/PD-L1 mAbs are effective in the treatment of leukemia, but the effect of single drug therapy is weak, and the effect of combination is more considerable. The occurrence of iRAEs is also not negligible, and large sample data are required to clarify the curative effects and adverse effects of PD-1/PD-L1 mAbs. Studies on PD-1/PD-L1 mAbs in CML, ALL, and CLL are few, and more details about clinical trials on PD-1/PD-L1 mAbs in the treatment of leukemia are shown in Table 3.

6.8 Future clinical scenarios of PD-L1/PD-1 inhibitors in AML

In sum, setting of either consolidation or maintenance where, in the presence of PD-L1/PD-1 inhibitors at least partially restored immune system, they could promote measurable residual disease negativity. A very interesting therapeutic application, albeit of limited use, of checkpoint inhibitors in AML, could be in the post allo-HSCT setting, where, in the presence of AML relapse/progression, these agents might be useful in augmenting the immune reactivity of the graft, boosting the GVL effect, at the expense of also enhancing iRAEs, in combination with other chemotherapeutic drugs might improve drug sensitivity in patients with R/R AML, and in combination with other T-cell based immunotherapies such as CAR-T, BiTE, and Treg exhaustion might enhance cytotoxic responses.

7 Limitations of ICB in the treatment of leukemia

7.1 Limited efficacy of ICB treatment

Different from other preclinical studies, co-blockade of PD-1 with Tim-3 or PD-1 with TIGIT fails to restore the proliferation and degranulation of CD8+ T-cells from CLL patients (173, 174). There are many other suppressive checkpoint molecules in T cells such as A2A receptor, CD276, B7-H4, CD272, CTLA-4, LAG-3, etc. (16), and they may also play an important role in exhaustion of CD8+ T-cells. Besides, Radpour et al. (175) suggest that CD8+ T cells in AML are dysfunctional mainly due to epigenetic silencing of activating IC receptors rather than signaling by immune inhibitory IC receptors. Kalinin et al. (176) block PD-1/PD-L1 signaling in CD19 CAR-T cells by co-expression of CD19-CAR and PD-1-specific VHH domain of anti-PD-1 nanobody. Results show that although the activation of CAR-T cells with low PD-1 level increases, the survival and cytotoxicity of these cells are diminished. Functional impairment caused by disrupted PD-1 signaling is accompanied by faster maturation and upregulation of exhaustion marker TIGIT in CAR-T cells. This result proves that for prolonged CAR-T activity and successful target cell killing, the strength of activation signal provided by CAR should be balanced by negative signal from IC. It suggests simply eliminating/knocking out PD-1 is not enough if one wants to optimize CAR-T cells by disposing of negative co-stimulation. Moreover, AML is an aggressive, rapid progressive disease, which does not allow the immune system to develop a proper antileukemic response. A study shows robust antigen-specific T cell responses are generated against AML cells after localized implantation (subcutaneous), but not a systemic (intravenous) route, the latter generates a tolerant state towards the malignant cells. Which suggests the ideal scenario for promoting a leukemia-specific T cell response will likely be in the minimal residual disease setting (177). Furthermore, AML has a low mutational burden and the newly formed antigens are expressed in different other tissues of the host (16). In conclusion, there are some experiments that have not found the exact effect of PD-1 signal blocking, and the reasons for poor PD-1 efficacy are complex. This may explain why PD-1 mAbs have suboptimal clinical efficacy.

7.2 Adverse reactions of ICB treatment

Additionally, the application of PD-1/PD-L1 mAbs is greatly limited by adverse drug reactions during the clinical treatment of leukemia patients. Godfrey et al. (158) concluded from a prospective study that treatment with pembrolizumab after allo-SCT is feasible, but it may be associated with serious iRAEs. A case study has reported the combined use of azacitidine and tislelizumab (an PD-1 mAbs) to treat relapsed AML posttransplantation. AML patients achieve complete remission, but the patients successively develop serious iRAEs and GVHD, eventually dying from GVHD complications (178). Significant ICI-related toxicity can occur in multiple tissues and organs, such as pneumonia, glomerulonephritis, hepatitis, gastroenteritis, dermatitis, neurotoxicity, and others. Fortunately, these symptoms are usually alleviated with the prompt use of steroids (179). However, among the 75 R/R AML patients treated with nivolumab, 85% develop infections during the study period, and they are mostly severe. R/R AML patients treated with nivolumab are more likely to develop infections when treated with corticosteroids than those who are not (164). More adverse events during leukemia

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TABLE 3 Clinical trials of PD-1/PD-L1 mAbs in leukemia.

Study population	Number (n)	ICIs	Target	Study design	Therapy regimen	Clinical benefits	iRAE
(116)R/R AML	37	Pembrolizumab	PD-1	Phase II, open-label, single-arm,	Pembrolizumab 200 mg after 1.5–2 g/m^2 cytarabine	ORR 46% CRc rate 38% mOS 11.1 months	Grade ≥3 iRAEs are 14% and self-limiting
(118)R/R AML	10	Pembrolizumab	PD-1	Open-label, single-arm, single-institution	Pembrolizumab 200 mg on day 1 of every 3-week cycle, with decitabine 20 mg/m^2 on days 8–12 and 15–19 of alternative cycles starting with cycle 1.	mOS 10.8 months	iRAEs are 30%
(105)R/R AML	9	Pembrolizumab	PD-1	Phase II, retrospective matched cohort	Cytarabine 1.5–2 mg/m^2 every 12 hours days 1–5 followed by pembrolizumab 200 mg on day 14, every 3 weeks for up to 2 years	mOS 21 months 1-year RFS 44% 1-year OS 67%	NR
(158)AML, MRD, and lymphoma relapsed after SCT	12	Pembrolizumab	PD-1	Prospective study	Pembrolizumab 200 mg every 3 weeks for up to 2 years	AML ORR 0%	iRAEs are 42% Grade 3–4 iRAEs are 25%
(159)High-risk AML	15	Nivolumab	PD-1	Phase II, open-label, single-arm,	Nivolumab 3 mg/kg every 2 weeks for cycle 6, then nivolumab every 4 weeks for cycle 12, finally, nivolumab every 3 months until disease relapse	6-month RFS 57.1% median RFS 8.48 months	iRAEs are 40%; Grade 3–4 iRAEs are 27%
(164)R/R AML	75	Nivolumab	PD-1	Single-center retrospective cohort study	Azacitidine with nivolumab or azacitidine with nivolumab plus ipilimumab	All but 2 patients are withdrawn from the CPI trial before completion	53% experience one or more iRAEs and grade 2–3 iRAEs are the most common
(117)R/R AML	70	Nivolumab	PD-1	Phase, open-label, non-randomized	Azacitidine 75mg/m^2 days 1–7 with nivolumab 3mg/kg on day 1 and 14, every 4–6 weeks	ORR 33% CR/CRi 22%	Grade 3–4 iRAEs are 11%
(160)AML or High-risk MDS	44	Nivolumab	PD-1	Phase, single-arm	Cytarabine 1.5 g/m^2 on days 1–4 and idarubicin 12 mg/m^2 on days 1–3. Nivolumab 3 mg/kg is started on day 24 and continues every 2 weeks for up to a year in responders	Median RFS 18.54 months mOS 18.54 months.	Grade 3–4 iRAEs are 13.6%
(161)R/R AML	19	Avelumab	PD-L1	Phase Ib/II, open-label, single-center, non-randomized	Azacitidine 75 mg/m^2 on days 1–7 and avelumab 3 mg/kg or 10 mg/kg on days 1 and 14, every 28-day	ORR 10.5% mOS 4.8 months	Two patients experience iRAEs of grade 2 and grade 3 pneumonitis
(163)R/R AML	7	Avelumab	PD-L1	Phase Ib/II, open-label, parallel cohort	Azacitidine 75 mg/m^2 on days 1–7, Avelumab 10 mg/kg (max dose: 2000 mg) on day 1 and day 14, gemtuzumab ozogamicin 3 mg/m^2 (max dose: 4.5 mg) on day 8	CR 14%	Grade ≥3 iRAEs are 0%
(163)R/R AML	13	Avelumab	PD-L1	Phase Ib/II, open-label, parallel cohort	Azacitidine 75 mg/m^2 on days 1–7, venetoclax 400 mg on days 1–28 (cycle 1)/days 1–21 (cycles 2+), avelumab 10 mg/kg (max dose: 2000 mg) on day 1 and day 14	CRi 15% mOS 4.8 months	One patient experience grade 3 iRAE
(165)AML	7	Avelumab	PD-L1	Phase I, open-label, single-arm	Decitabine 20mg/m^2 days 1–5, every 28-day, avelumab 10mg/kg day 1, every 14-day	CR 20% mOS 3.2 months	NR
(162) Elderly AML	64	Durvalumab	PD-L1	Phase, open-label, randomized	Azacitidine 75 mg/m^2 on days 1–7 with durvalumab 1500 mg on day 1 every 4 weeks	ORR 31.3% OS 13.0 months DOR 24.6 weeks	iRAEs. are 28.1%

(Continued)

TABLE 3 Continued

Study population	Number (n)	ICIs	Target	Study design	Therapy regimen	Clinical benefits	iRAE
(166)AML	16	Atezolizumab	PD-L1	Phase Ib, open-label, non-randomized, multicenter	Guadecitabine 60 mg/m^2 on days 1–5 and atezolizumab 840 mg on day 8 and day 22 in 28-day cycles	87.5% patients die during the trial period due to disease progression or AEs	Grade 3–4 TRAEs of Atezolizumab are 18.8%
(167)R/R AML	11	Atezolizumab	PD-L1	Phase Ib. open-label, multicenter, non-randomized	Atezolizumab (840 mg) on day 22 of cycle 1, in subsequent 28-day cycles, atezolizumab on days 8 and 22. Magrolimab two priming doses of 1 mg/kg on days 1 and 4 of cycle 1, then 15 mg/kg on day 8, and 30 mg/kg on day 11 of cycle 1, starting on day 15, magrolimab maintenance 30 mg/kg/week.	18.2% patients withdraw from the study, and 81.8% patients die	AEs related to atezolizumab are 36.4%
(168)R/R AML	27	Tislelizumab	PD-1	Phase II, open-label, single-arm, nonrandomized	Azacitidine 75 mg/m^2 daily, day 1–7 or decitabine 20 mg/m^2 daily, day 1–5 plus CAG regimen (cytarabine 100 mg every 12h, day 1–5; aclarubicin 20 mg daily, day 1–5 or idarubicin 10 mg day 1, 3 and 5; and G-CSF 5 μg/kg/day, from day 0 to end) with tislelizumab 200 mg day 6 or day 8	ORR 63% CR 44% CRi 7% mOS 9.7 months EFS 9.2 months	Grade 2–3 iRAEs are 14.8%
(127)CML	31	Nivolumab	PD-1	Phase Ib	Dasatinib 100 mg (CP) or 140 mg (AP) once daily and nivolumab 0.3 mg/kg, 1 mg/kg, or 3 mg/kg every 2 weeks for ≤2 years followed by ≤1 year of dasatinib only	26% patient achieve MMR at months 12 29% patient achieve MMR at months 24	Only 2 serious AEs (both grade 2) are considered drug-related
(169)MDS and CMML	33	Atezolizumab	PD-L1	Phase I/II, multicenter	Guadecitabine 30 mg/m^2 and escalating to 60 mg/m^2 days 1–5, atezolizumab 840mg days 8 and 22 of a 28-day cycle.	ORR 33% mOS 15.1 months Median PFS 7.2 months	iRAEs are 36% (4 grade 3, 3 grade 2, 5 grade1)
(132) ALL and MRD	12	Pembrolizumab	PD-1	Phase	Pembrolizumab 200 mg every 3 weeks	mOS 12.7 months 8% experience a complete MRD response, which last 3 weeks	iRAEs are 8% (grade 3 Stevens-Johnson syndrome)
(140) R/R CLL	17	Pembrolizumab	PD-1	Phase Ib	Pembrolizumab 200 mg every 3 weeks plus dinaciclib 7 mg/m^2 on day 1 and 10 mg/m^2 on day 8 of cycle 1 and 14 mg/m^2 on days 1 and 8 of cycles 2 and later	ORRs 29.4% median PFS 5.2 month median DOR 10.3 months mOS 21.7 months	TRAEs, any grades are 76.5%, grade 3–4 are 52.9%
(141)CLL and SLL	36	Nivolumab	PD-1	Phase I/IIa, open-label; two-part	Ibrutinib (420 mg or 560 mg) in combination with nivolumab (3 mg/kg every 2 weeks)	ORRs 61% median DOR 19.2 months The median duration of stable disease or better is 19.7 months	The most common grade 3–4 iRAEs are rash (8%) and increased ALT (2%)
(142)CLL and SLL	10	Nivolumab	PD-1	Phase II	Nivolumab 3 mg/kg every 2 weeks each 4-week cycle, starting cycle 1 day 1 for a total of 24 cycles, ibrutinib 420 mg once daily starting cycle 2 day 1	CR/CRi 30%	One patient experiences a grade 2 immunological toxicity

(Continued)

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TABLE 3 Continued

Study population	Number (n)	ICIs	Target	Study design	Therapy regimen	Clinical benefits	iRAE
(170)AML relapsed after SCT	1	Tislelizumab	PD-1	Case report	Tislelizumab 100 mg on day 1 and azacitidine 100 mg on days 1–7	Achieve CR	Patient experience moderate GVHD and iRAEs
(171)AML relapsed after SCT	1	Pembrolizumab	PD-1	Case report	Pembrolizumab 100 mg	CR lasting 10 months or more	NR
(171)AML relapsed after SCT	1	Nivolumab	PD-1	Case report	Nivolumab 0.3–1 mg/kg, 5 times a week	Achieve molecular disease stabilization	NR
(171)AML relapsed after SCT	1	Nivolumab	PD-1	Case report	Nivolumab 100 mg	No objective response	NR
(172)ALL relapsed after SCT	1	Nivolumab	PD-1	Case report	Nivolumab 40 mg every 2 weeks	PET-CT show near complete resolution of pre-existing lesions, with residual low-grade metabolic uptake in the renal lesion	Owing to hepatic derangement, nivolumab is suspended
(172)ALL relapsed after SCT	1	Nivolumab	PD-1	Case report	Nivolumab 40 mg every 2 weeks for two cycles, then 80 mg every 2 weeks	Blast counts remain static for 9 weeks, but increase after the fifth dose of nivolumab	LDH and serum phosphate increase, and generalized bone pain

ICIs, immune checkpoint inhibitors; iRAE, immune-related adverse events; R/R, relapsed/refractory; AML, acute myeloid leukemia; PD-1, programmed cell death protein 1; ORR, overall response rate; CR, complete remission; CRc, composite complete remission; OS, overall survival; mOS, median overall survival; RFS, recurrence-free survival; NR, not report; MRD, measurable residual disease; SCT, stem cell transplantation; CPI, checkpoint inhibitor; CRi CR with incomplete recovery; MDS, myelodysplastic syndrome; PD-L1, programmed death-ligand 1; DOR, duration of response; AE, adverse events; TRAE, treatment-related adverse event; G-CSF, granulocyte-colony-stimulating factor; EFS, event-free survival; CML, chronic myeloid leukemia; CP, chronic Phase; AP, accelerated Phase; MMR, major molecular response; CMML, chronic myelomonocytic leukemia; PFS, progression-free survival; ALL, acute lymphoblastic leukemia; MRD, measurable residual disease; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; ALT, alanine aminotransferase; LDH, lactate dehydrogenase.

TABLE 4 Adverse events after PD-1/PD-L1 blockade.

Study population	Antibody	Participants (n)	grade ≥3 hematological adverse events	grade ≥3 Nonhematological adverse events	solutions
(116)R/R AML	Pembrolizumab	37	Febrile neutropenia 62%; Hemolytic anemia 3%	Hypokalemia 3%; ALT increase 5%; AST increase 5%; Alkaline phosphatase increase 5%; Lymphocytic infiltration of liver 3%; Catheter-related infection 8%; Clostridium difficile colitis 3%; Hepatic infection 3%; Lung infection 26%; Typhlitis 3%; Pulmonary edema 3%; Maculopapular rash 5%	Median time to administration of systemic steroids after pembrolizumab and total duration of steroids is 15 (range, 5–23) and 14 (range, 1–35) days, respectively. iRAEs are self-limiting and fully resolve after administration of systemic steroids.
(158)AML, MRD and Lymphoma relapsed after SCT	Pembrolizumab	12	Hemolytic anemia 8%; Idiopathic thrombocytopenic purpura 8%	Fatigue 8%; Fever 17%; Pneumonitis 17%; Hyperthyroidism 8%; Secondary malignancy 8%	Steroid therapy/discontinue pembrolizumab therapy

(Continued)

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TABLE 4 Continued

Study population	Antibody	Participants (n)	grade ≥3 hematological adverse events	grade ≥3 Nonhematological adverse events	solutions
(171)AML relapsed after SCT	Pembrolizumab	1	NR	Skin GVHD	Complete remission after 30 days with topical corticosteroids
(159)High-risk AML	Nivolumab	15	Febrile neutropenia 7%; Hemolysis 7%	ALT increase 13%; Pneumonitis 13%; Hypotension 7%; Abdominal pain 7%; Vomiting 7%; Sepsis 7%; AST increase 7%	Steroid therapy/discontinue nivolumab therapy
(160)AML or High-risk MDS	Nivolumab	44	Febrile neutropenia 32%	Nausea 2%; Diarrhoea 16%; Muscle weakness 2%; Syncope 2%; Elevated transaminases 2%; Elevated bilirubin 2%; Rash 5%; Colitis 4%; Pancreatitis 2%; Cholecystitis 2%; Small bowel obstruction 2%	All patients are treated with steroids and nivolumab interruption and are successfully re-challenged with nivolumab
(164)R/R AML	Nivolumab	75	Neutropenia 84%; Lymphopenia 79%; Combined cytopenia 71%	85% patients develop an infection during the study period, with bacterial (72%), fungal (16%), viral (11%), and parasitic (< 1%)	Infliximab/steroid therapy/ antimicrobials/antibacterial
(161)R/R AML	Avelumab	19	Anemia 10.5%; Neutropenia 10.5%; Lymphopenia 5.3%	Diarrhea 5.3%; Fatigue 5.3%; Nausea 5.3%; Anorexia 5.3%; Pneumonitis 5.3%	Self-resolved/steroid therapy/anti-infective therapy/antiviral
(163)R/R AML	Avelumab	13	Febrile neutropenia 23%	Fatigue 8%; Gastrointestinal hemorrhage 8%; ALT/AST increase 8%; Increased bilirubin 8%; Infection 46%; Pericarditis 8%; Syncope 8%	NR
(165)AML	Avelumab	7	Febrile neutropenia 86%	Fatigue 14%; Weight 14%; Hypertension 57%; Edema 14%; Hypoxia 57%; Acute kidney injury 14%; Hypokalemia 29%; Oral mucositis14%; Pneumonitis 29%; Heart failure 29%	NR
(166)AML	Atezolizumab	16	Febrile neutropenia 56.3%; Anemia 18.8%; Thrombocytopenia 18.8%; Neutrophil count decrease 12.5%	Pneumonia 31.3%; Sepsis 18.8%; Hypokalemia 18.8%; Hypophosphatemia 18.8%; Failure to thrive 12.5%; Pneumonia aspiration 12.5%	NR
(167)R/R AML	Atezolizumab	11	Anemia 36.4%	Pneumonia 36.4%; Fatigue 18.2%; Hypokalemia 36.4%; Hypertension 18.2%	NR
(162)Elderly AML	Durvalumab	64	TEAEs: Thrombocytopenia 42.2%; Anemia 30%; Neutropenia 36%	TEAEs: Constipation 57.8%; imAEs: Pneumonitis 6.25%; Dermatitis 1.5%; Enteritis 1.5%; Arthritis 1.5%; Myocarditis 1.5%; Hepatitis 1.5%; Thyroiditis 1.5%; Bullous pemphigoid 1.5%; Colitis 1.5%; Progressive multifocal leukoencephalopathy 1.5%	NR
(127)CML	Nivolumab	31	Anemia 13%; Thrombocytopenia 16%; Neutropenia 16%; Febrile neutropenia 6%	Diarrhea 13%; Rash 6%; Nausea 3%; Vomiting 3%; Pyrexia 3%; Asthenia 3%	NR
(132)ALL and MRD	Pembrolizumab	12	Neutrophil count decrease 8%	Hypertension 25%; Stevens-Johnson syndrome 8%	After initiation of prednisone, all lesions resolve within days for Stevens-Johnson syndrome.
(141)CLL and SLL	Nivolumab	36	Neutropenia 53%; Anemia 25%; Thrombocytopenia 14%; Febrile neutropenia 11%	Rash 6%; Pneumonia 14%; Increased lipase 14%; Hypokalemia 8%; Increased amylase 8%; ALT increase 3%; Hypertension 6%	NR

(Continued)

TABLE 4 Continued

Study population	Antibody	Participants (n)	grade ≥3 hematological adverse events	grade ≥3 Nonhematological adverse events	solutions
(180)AML	Nivolumab	1	NR	PD-1 inhibitor-associated vitiligo-like depigmentation	Routine skin surveillance and no additional treatment
(181)AML relapsed after allo-SCT.	Pembrolizumab	2	NR	Pembrolizumab induce acute corneal toxicity after allo-SCT	Topical steroids, artificial tears and therapeutic soft contact lens/Topical steroids, topical serum eye drops, therapeutic soft contact lens and punctal plugs, bilateral temporary tarsorrhaphy
(182)CLL	Pembrolizumab	1	Autoimmune hemolytic anemia	NR	Prednisone/Rituximab/Ibrutinib

R/R, relapsed/refractory; AML, acute myeloid leukemia; ALT, alanine aminotransferase; AST, aspartate aminotransferase; iRAEs, immune-related adverse events; MRD, measurable residual disease; SCT, stem cell transplantation; NR, not reported; GVHD, graft versus host disease; MDS myelodysplastic syndromes; TEAEs, treatment-emergent adverse events; imAEs, immune-mediated adverse events; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; CLL, chronic lymphocytic leukemia; SLL, small lymphocytic lymphoma; allo-PBSCT, allogeneic peripheral blood stem cell transplantation.

treatment with ICIs are shown in Table 4. Chemotherapy intolerance is an important cause of treatment discontinuation in leukemia patients, and reducing adverse effects during ICI therapy while aiming to improve their efficacy is equally important. Accordingly, the development of well-tolerated ICIs and the exploration of clinical protocols with few adverse effects of ICIs are the keys to solving the problem. However, given the insufficient data on the clinical application of ICIs for leukemia, further exploration is required to optimize ICI therapy.

8 Conclusion

Blocking PD-1/PD-L1 achieves encouraging outcomes as shown by ex vivo studies and animal models, but clinical trials on PD-1/PD-L1 mAbs as single-agent in leukemia treatment show suboptimal results and varying degrees of adverse drug reactions. Fortunately, combinations of PD-1/PD-L1mAbs with other immunotherapies have shown quite promising, including the enhancement of GVL effect and reduction of GVHD in HSCT, the improvement of T-cell response in BiTE or CAR-T, and the application to multidrug chemotherapy to enhance drug sensitivity. In conclusion, ICB therapy opens new horizons for tumor immunotherapy, and future research will focus on refining combination regimens of ICIs to modulate the immune environment so that leukemia patients can maximize the benefits of ICB therapy.

Author contributions

HC: Data curation, Investigation, Writing – original draft, Conceptualization. TW: Conceptualization, Investigation, Writing – original draft. XZ: Investigation, Writing – original draft, Data curation, Formal Analysis. SX: Conceptualization, Supervision, Writing – review & editing. HS: Supervision, Writing – review & editing, Investigation. YS: Conceptualization, Supervision, Writing – review & editing, Funding acquisition, Project administration, Resources. YL: Supervision, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Resources.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The novel high-affinity humanized antibody IMM40H targets CD70, eliminates tumors via Fc-mediated effector functions, and interrupts CD70/CD27 signaling

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Background: A significant level of CD70 can be detected in various types of tumor tissues and CD27 is expressed on Treg cells, but CD70 expression is low in normal tissues. The interaction between CD70 and CD27 can stimulate the proliferation and survival of cancer cells and increase the level of soluble CD27, which is associated with poor prognosis in patients with lymphoma and certain solid tumors. Thus, it is a promising therapeutic target for the treatment of many major CD70+ cancer indications, including CD70+ lymphoma, RCC, NSCLC, HNSCC and OC.

Methods: IMM40H was obtained through hybridoma screening and antibody humanization techniques. IMM40H was evaluated for its binding, blocking, Fc-dependent effector functions and antitumor activity characteristics in various *in vitro* and *in vivo* systems. The safety and tolerability profile of IMM40H were evaluated through single and repeated administration in cynomolgus monkeys.

Results: *In vitro* cell-based assays demonstrated that IMM40H had considerably stronger CD70-binding affinity than competitor anti-CD70 antibodies, including cusatuzumab, which enabled it to block the interaction of between CD70 and CD27 more effectively. IMM40H also exhibited potent Fc-dependent effector functions (ADCC/CDC/ADCP), and could make a strong immune attack on tumor cells and enhance therapeutic efficacy. Preclinical findings showed that IMM40H had potent antitumor activity in multiple myeloma U266B1 xenograft model, and could eradicate subcutaneously established tumors at a low dose of 0.3 mg/kg. IMM40H (0.3 mg/kg) showed therapeutic effects faster than cusatuzumab (1 mg/kg). A strong synergistic effect between IMM01 (SIRPα-Fc fusion protein) and IMM40H was recorded in Burkitt's lymphoma Raji and renal carcinoma cell A498 tumor models. In cynomolgus monkeys, the highest non-severely toxic dose (HNSTD) for repeat-dose toxicity was up to 30 mg/kg, while

the maximum tolerated dose (MTD) for single-dose toxicity was up to 100 mg/kg, confirming that IMM40H had a good safety and tolerability profile.

Conclusion: IMM40H is a high-affinity humanized IgG1 specifically targeting the CD70 monoclonal antibody with enhanced Fc-dependent activities. IMM40H has a dual mechanism of action: inducing cytotoxicity against CD70+ tumor cells via various effector functions (ADCC, ADCP and CDC) and obstructs the proliferation and activation of Tregs by inhibiting CD70/CD27 signaling.

KEYWORDS

IMM40H, CD70/CD27 signaling, CD70, targeting CD70 antibody, ADCC

1 Introduction

Cluster of differentiation 70 (CD70) is a tumor necrosis factor family cell surface antigen. It is involved in lymphocyte maturation and proliferation and is intermittently produced by mature dendritic cells and a small proportion of activated B and T lymphocytes (1, 2). However, solid and hematological cancers express CD70 constitutively, and this expression is associated with a poor prognosis (3–6). Trimer type II transmembrane protein CD70 is the most common version of CD70, while CD27 is its receptor. The secretion of soluble CD27 (sCD27) and proteolytic shedding of the ectodomain of CD27 occur after CD70 binds to CD27 (CD70-CD27), which activates the nuclear factor-κB (NF-κB) and c-Jun kinase pathways and promotes the proliferation and survival of malignant cells (7). The CD70-CD27 signaling pathway can promote regulatory T cells (Tregs) mobilization or survival, leading to immune monitoring in the tumor microenvironment (8).

Monoclonal antibodies are the main focus of CD70-targeted immunotherapy, and they have either been tested as monotherapeutic agents or in conjunction with other medications. CD70 antibodies are not available commercially. For inhibiting CD70/CD27 signaling, cusatuzumab (ARGX-110) is the most rapidly progressing anti-CD70 monoclonal antibody (9). Due to its effector actions, such as antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytotoxicity (CDC), and increased antibody-dependent cell-mediated cytotoxicity (ADCC), cusatuzumab can destroy tumor cells directly (9). Combination treatment with azacitidine and cusatuzumab is safe and effective for individuals with untreated AML who are not candidates for intense chemotherapy, as determined by the results of a Phase I/II trial. Cusatuzumab may cause long-lasting remissions by eliminating CD70+ leukemia stem cells (LSCs) (10–12).

In this study, we reported the novel targeting CD70 monoclonal antibody IMM40H, which has higher affinity and stronger blocking activity than competitor anti-CD70 antibodies. Besides enhancing the immune defense against tumors by disrupting the communication between CD70-CD27 and Tregs, IMM40H induces cytotoxicity over CD70+ tumor cells through several effector functions (ADCC, ADCP, and CDC). Preclinical data have shown that IMM40H has potent antitumor activity in various CDX models, including Raji, U266B1, and A498 tumor cells; IMM40H was also safe and well-tolerated in cynomolgus monkeys.

2 Materials and methods

2.1 Cell culture

The SP2/0, Raji, Daudi, Jurkat, and Jeko-1 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences, and the U266B1 and A498 cell lines were purchased from the American Type Culture Collection (ATCC). Jurkat-CAR-CD27 and FcgRIIIA (158V) target-activated NK (FcR-TANK^{TM}) cells were self-modified in our laboratory. The logarithmic growth phase was reached in all cell lines before harvesting. The SP2/0, Raji, U266B1, Daudi, Jurkat, Jurkat-CAR-CD27, and Jeko-1 cell lines were maintained in an incubator at 37°C and 5% CO_2 with the RPMI-1640 medium (Gibco, Cat#11875093) containing 10% fetal bovine serum (Gibco, Cat#10091148) and 1% penicillin-streptomycin (Gibco, Cat#15140122). MEM medium (Gibco, Cat#11095080) with 1% penicillin-streptomycin and 10% FBS was used for the cultivation of A498 cells. TANK serum-free medium (Immuneonco, Cat#CT001-1) was used for the cultivation of FcR-TANK cells.

2.2 Humanization and development of antibodies

After immunizing with the human CD70 full-length extracellular domain (39–193) fused to mIgG1-Fc, we used the conventional hybridoma technique to screen for anti-human CD70 antibodies. Positive fusions were evaluated for specific CD70 binding to U266B1 by fusing splenocytes from vaccinated animals with the Sp2/0 myeloma cell line. We cloned and sequenced one of

the hybridoma clones, designated 26A3. By grafting CDRs onto human germline frameworks, 26A3 was humanized. For detailed information on the preparation of IMM40H, can refer to the approved patent (US11613584B1).

2.3 Antibody expression, purification, and characterization

CHO-S cells were cultured in TransFx-C CHO Transient transfection medium (Hyclone, Cat#SH30942.02). Co-transfection of expression vectors expressing the antibody heavy chain and light chain was achieved by transient transfection using a polyethylenimine transfection reagent (Polysciences, Cat#24765). After 8–10 days, the cells were collected for their supernatants and loaded to Protein A Sepharose columns (Bestchrom, Cat#AA0273). Wash buffer (20 mM phosphate buffer (PB) +140 mM NaCl, pH7.4 ± 0.1) was added to the columns, and the antibodies were then eluted with the elution buffer (25 mM NaAc+ 100 mM NaCl, pH3.5 ± 0.1). Using 2 M Tris, the pH of the collected fractions was adjusted to 5.2 ± 0.2. Size-exclusion high-performance liquid chromatography (SEC-HPLC) was performed to examine the purity of the eluted antibodies.

2.4 Determining the binding of IMM40H to the trimer CD70 protein

The binding of IMM40H to the trimer CD70 protein (ACROBiosystems, Cat# CDL-H52Da) was analyzed by ELISA. Trimer CD70 protein (100 ng/well) in PBS was overnight incubated at 4°C in flat-bottom 96-well plates (Thermo Fisher Scientific, Cat#442404). The plates were blocked in blocking buffer (PBS + 3% skim milk) at room temperature for 2 h, then washed thrice with wash buffer (PBS + 0.05% Tween-20). The plates were incubated with two-fold serially diluted IMM40H at the starting concentration of 10 μg/mL. Peroxidase-conjugated anti-human IgG secondary antibody (Jackson Immuno, Cat#109–006–008) was added after washing thrice and incubated for 1 h. After incubating the plates with the substrate solution TMB (KPL, Cat#51200050) for 10 min and adding 2 M sulfuric acid to terminate the reaction, the plates were analyzed using a microplate reader (BioRad, iMARK).

2.5 Determining the binding of IMM40H to CD70+ tumor cells

The binding of IMM40H to CD70+ tumor cells (including Raji, U266B1, SP53, and A498) was analyzed via flow cytometry assays. We used hIgG1-Fc (In house) as an isotype control. The tumor cells were incubated with IMM40H, Cusatuzumab, and hIgG1-Fc at different serially diluted concentrations for 45 min at 4°C. Then, PBS containing 0.5% BSA (Sangon Biotech, Cat#A500023–0100) was added to remove free antibodies. The 500-fold diluted FITC-conjugated anti-human IgG Fc 2nd antibody (Sigma, Cat#F9512) was added to the samples and incubated at 4°C for 45 min in the dark. After washing, the FITC fluorescence signal of the cells was analyzed by performing flow cytometry assays (Luminex, Guava® easyCyte™ 8HT Base System).

2.6 Antibody affinity assay

Surface plasmon resonance, SPR device (Biacore T200, GE Healthcare) was used for evaluating the affinity of IMM40H to recombinant human CD70 trimer. Following the amine coupling methodology described by the manufacturer, the anti-human IgG (Fc) antibody (GE Healthcare, Cat# BR-1008–39) was diluted to 25 μg/mL in 10 mM sodium acetate (pH 5.0) and then attached to a CM5 biosensor (GE Healthcare, Cat#BR100530). The SPR experiment was performed at 25°C in 1xHEPES running buffer (pH 7.4, 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% Tween-20). The samples, which were diluted to 10 μg/mL in 1xHEPES (pH 7.4), were captured on the anti-hIgG(Fc) antibody surface. A concentration series of 3.125–0.049 nM trivalent human CD70 was injected over the captured antibodies at 30 μL/min to measure association and dissociation. The anti-hIgG(Fc) antibody capture surface was regenerated between test cycles with 30 s injections of 3 M magnesium chloride. Biacore T200 Evaluation Software v.3.1 was used to fit the rate constants ka (kon, association rate) and kd (koff, dissociation rate) from the reference flow cell and 0 nM blank-subtracted sensorgrams to a 1:1 binding model.

2.7 Blocking the CD70/CD27 signaling assay

We engineered a recombinant Jurkat cell line that expressed a chimeric CD27 receptor (Jurkat-CAR-CD27) to further define the target-blocking action in a biological setting. The chimeric antigen receptor (CAR)-CD27 consisted of an extracellular CD27 domain that was linked to CD8α-hinge, CD28-TMD/ICD, and CD3ζ signal domains in a specific order. When the Jurkat-CAR-CD27 cells were incubated with recombinant Raji cells (CD70+) for 24 h, the former cells underwent activation-induced cell death (AICD), which was coupled with an increase in the expression of CD69. However, when the interaction between CD27 and CD70 was blocked by CD70 mAb, CD70-induced cell death was inhibited and CD69 expression remained stable. The experimental steps are briefly described as follows. The Jurkat-CAR-CD27 cells and Raji cells were mixed in a 5:1 ratio and incubated with serially diluted concentrations of antibodies in a humidified incubator at 5% CO_2 and 37°C for 24 hours. After incubation, CD69 (Biolegend, Cat#310906) and CD3 (Biolegend, Cat#300412) antibodies were added to each well, and then, the expression of CD69 on Jurkat-CAR-CD27 cells was determined via flow cytometry assays.

2.8 *In vitro* Fc-mediated effector function (ADCC/ADCP/CDC) assay

2.8.1 ADCC assay

The target cells were labeled with 200 nM carboxyfluorescein succinimidyl ester (CSFE; Sigma, Cat#21888) and incubated with different concentrations of antibodies for 30 min. Then, in an E/T ratio of 1:2, FcgRIIIA (158V) target-activated NK (FcR-TANK^{TM}) cells (In house) were added to wells for 4 h at 5% CO_2 and 37°C. Propidium Iodide (PI) solution (Sigma, Cat#P4170) was used for staining the cells. Then, the flow cytometry assay was performed to collect the cells, and the PI-positive stained cells were calculated. The intensity of ADCC was calculated using the formula: Lysis% = [(E+T+Ab) % PI positive cell – (E+T) % PI positive cell]/(100 – T % PI positive cell) × 100%.

2.8.2 ADCP assay

We collected THP-1 cells and washed them in RPMI-1640 medium with 10% fetal bovine serum (Gibco, Cat# 10091148) and 1% penicillin-streptomycin (Gibco, Cat# 15140122). In a flat 96-well plate, 100 µL of 4×10^5/mL THP-1 cells and 200 ng/mL PMA (Sigma, Cat#P-050) were incubated for 48 h at 37°C and 5% CO_2. After counting and harvesting the target cells, 200 nM CSFE was added to the cells and incubated at 37°C in the dark for 30 min to label them. After washing twice with full culture medium, the target cells were seeded at a density of 1×10^5 cells/well (50 µL, 2×10^6/mL). Antibodies were serially diluted and added to the plate at 50 µL/well and incubated for 2 h (Effector : Target = 2:5) at 37°C and 5% CO_2. After incubation, the plates were washed with PBS to remove free target cells. Adherent macrophages were digested with 10 µL/well 0.25% Trypsin-EDTA and resuspended in 150 µL/well PBS buffer. The phagocytic index was determined by flow cytometer and defined as the percentage of macrophages that have phagocytosed the target cells. The phagocytic index was calculated using the formula: phagocytic index % = (E+T+Ab) % CFSE positive cell – (E+T) % CFSE positive cell.

2.8.3 CDC assay

Different concentrations of antibodies were used for incubating target cells with normal human serum complement (Quidel, Cat#A113) at 37°C and 5% CO_2 for 4 h before being stained with a PI solution. A flow cytometry assay was performed to collect the cells and the percentage of PI-positive cells was calculated. The CDC intensity was calculated using the formula: Lysis % = Experimental Sample Lysis %- No Antibody Lysis %.

2.9 *In vivo* xenograft mouse model

2.9.1 U266B1/A498 xenograft model in CB17-SCID mice

We resuspended 200 µL of cells in cold PBS and injected them into the right side of the back near the axilla of female CB17- severe combined immunodeficient (SCID) mice aged 6-8 weeks (5×10^6 A498 or U266B1 cells). After the tumors in both models grew to an average of 126 and 195 mm^3, respectively, the animals were randomly divided into treatment groups (eight mice per group) based on the size of the tumor and the weight of the mice. In both experiments, IMM40H was administered intraperitoneally. In the U266B1 study, 0.3, 1, and 3 mg/kg QW of IMM40H was administered for four weeks. Bortezomib (Xian Janssen) was IV administered at 0.5 mg/kg BIW for four weeks and Cusatuzumab was administered via IV at 1 mg/kg QW for four weeks. In the A498 study, 3, 10, 30 mg/kg IMM40H, 10 mg/kg IMM01, and 10 mg/kg IMM40H combo with 5 mg/kg SIRPα-Fc fusion protein IMM01 were administered BIW for four weeks. The tumor volume and body weight were measured twice a week. When the tumor size met the euthanasia threshold (3,000 mm^3), the animals were euthanized. When a sufficient number of mice in any group met the euthanasia threshold, the study was terminated.

2.9.2 Raji orthotopic xenograft model in CB17-SCID mice

To establish disseminated disease, female CB-17 SCID mice aged 6-8 weeks were injected with 100 µL, 5×10^6 Raji cells resuspending cold PBS per animal into the lateral tail vein. The animals were randomized to various treatment groups (eight mice per group) based on body weight (BW) after three days of tumor cell inoculation. Treatment began with twice-weekly tail vein injections for three consecutive weeks. The following dosages were used: IMM40H (1, 3, and 10 mg/kg), IMM01 (0.3 mg/kg), and IMM40H (3 mg/kg) with IMM01 (0.3 mg/kg). The mice were euthanized when they displayed symptoms of an excessive tumor burden, such as weight loss > 20%, stooped posture, paralysis, lethargy, cranial edema, or dehydration. The BW of the mice was monitored at least twice a week.

2.10 Pharmacokinetics study in non-human primates

Acute toxicity, Pharmacokinetics (PK), and ToxicoKinetics (TK) profiles of IMM40H were evaluated following intravenous (IV) administration in cynomolgus monkeys. The appropriate standard operating procedures (SOPs) provided by WuXi AppTec (Suzhou) Co., Ltd. were followed for all experiments. The Protocol complied with the requirements of the Animal Welfare Act Regulations (9 CFR 3).

Acute toxicity study was evaluated after IMM40H was administered as a single-dose IV infusion to cynomolgus monkeys. Eight cynomolgus monkeys (4 animals/sex) were randomly assigned to four groups (1/sex/group). Different concentrations of IMM40H (0, 20, 50, and 100 mg/kg) were administered to the monkeys in different groups. The animals were monitored for 14 days and various parameters were assessed, including viability (morbidity/mortality), autopsy, clinical pathology and observations, food intake, and body weight.

The IMM40H PK study was conducted after a single IV infusion was administered to male and female cynomolgus monkeys to determine the serum PK properties and evaluating

the presence of anti-drug antibodies (ADA) in serum. In total, 18 cynomolgus monkeys (9 animals/sex) were assigned to three groups (3 animals/sex/group), which were IV-administered with different concentrations of IMM40H (0.5, 1.5, or 3 mg/kg). Blood samples were collected from the peripheral vessel of the animals following administration after 0, 0.5, 1, 2, 8, 24, 48, 72, 96, 120, 168, 336, 504, and 672 h. ADA samples were collected following administration after 0, 168, 336, 504, and 672 h. The concentration of IMM40H in serum was measured by an ELISA method, and ADA was analyzed using an ECL method.

In the TK study, male and female cynomolgus monkeys were characterized after they were administered IMM40H via IV infusion for five doses (on days 1, 8, 15, 22, and 29). In total, 40 monkeys (5/sex/group) were randomly assigned into four groups (0, 3,10, and 30 mg/kg) in the TK study. On days 1 and 22, blood samples were collected for the TK analysis at 0, 0.5, 2, 6, 24, 48, 72, 96, and 168 h after the end of the infusion.

2.11 Statistical analysis

We used GraphPad Prism 8.0 (GraphPad Software, Inc.) for statistical analysis. For comparisons involving three or more groups, a one-way repeated ANOVA was performed with Holm-Sidak correction. T-tests were performed for comparisons involving two groups. All differences were considered to be statistically significant at $P \leq 0.05$. In figures, asterisks denote statistical significance (*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).

3 Results

3.1 Humanization and generation of anti-CD70 monoclonal antibodies

The binding activity of the selected hybridoma clones to U266B1 cells was evaluated via FACS. One of the most promising positive clones, 26A3, could inhibit the interaction between human CD70 and CD27 in addition to binding human CD70 (Figures 1A, B).

To improve its Fc-dependent effector capabilities, 26A3 was humanized by grafting its CDRs onto human germline frameworks, and then, it was built as human IgG1 with S298A, E333A, and K334A alterations. FACS, ELISA, and SPR were then used to evaluate the humanized 26A3 (IMM40H) for its antigen-binding affinity. The FACS and ELISA results showed that the humanized 26A3 antibody (IMM40H) had equivalent CD70-binding ability to the chimeric antibody (Supplementary Figures 1A, B). The results of the affinity analysis showed that IMM40H also had a similar CD70 binding affinity to that of the chimeric version (kD = 3.22E-11M for humanized Ab, kD = 3.15E-11M for chimeric Ab) (Figure 2). Also, 26A3 could cross-react with CD70 from cynomolgus monkey but not from mouse (Supplementary Figure 2).

3.2 IMM40H exhibited the strongest specific binding activity to CD70+ tumor cells

Only stromal cells from the thymic medulla and mature dendritic cells were found to contain CD70 protein, indicating that its distribution outside the lymphoid organs is quite restricted (13, 14). CD70 is extensively expressed in Hodgkin and non-Hodgkin lymphomas, chronic lymphocytic leukemia, and multiple myeloma. It is also substantially expressed in renal cells (15, 16). A therapeutic anti-CD70 antibody may have broader applications since aberrant CD70 expression is associated with a poor prognosis in solid and hematologic cancers. FACS analysis was used to determine whether IMM40H bound to CD70 on various solid and hematologic tumor cells. Subsequently, IMM40H showed the highest binding activity to Raji and U266B1 cells among the competitor anti-CD70 antibodies (Figures 3A, B). In the low nanomolar range, IMM40H bound to both A498 and SP53 cells with high affinity (Supplementary Figures 3A-E). Upon transfection with CD70, IMM40H bound to the CD70-negative cell line CHO (Chinese Hamster Ovary) and Jurkat (T cell lymphoma cell line), proving its specificity for CD70 (Supplementary Figures 4A, B).

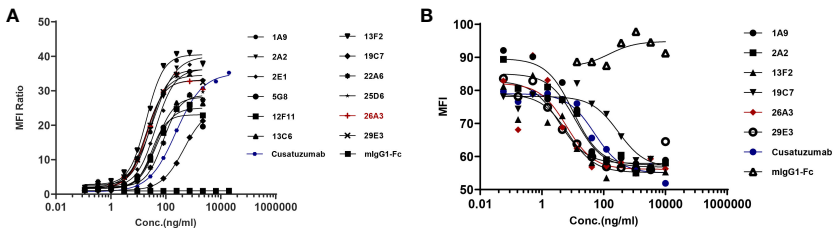

FIGURE 1
Identification of the lead anti-CD70 antibody. **(A)** Binding of the candidates to U266B1 cells, as determined by flow cytometry assays. **(B)** Blocking the interaction of CD27 with U266B1 cells, as determined by flow cytometry assays. Candidates with U266B1 cells were pre-incubated. Then, biotinylated-CD27 protein (Acro Biosystems, Cat#TN7-H82F6) and PE-conjugated Streptavidin (Biolegend, Cat# 405204) were added to detect the binding signal of CD27 and U266B1 cells. The results showed that 26A3 lead had the optimal binding and blocking activities, and thus, it was selected as the final candidate for generation.

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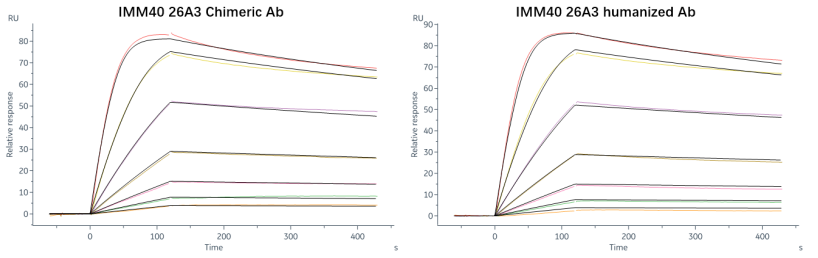


Method	Ligand	Capture Level (RU)	Analyte	Analyte Conc.	1:1 binding				
					ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi² (RU²)
Human IgG (Fc) Capture	IMM40 26A3 Chimeric Ab	239	Human CD70 Protein, His, Flag Tag (active trimer)	3.125-0.049 nM	2.39E+07	7.71E-04	**3.22E-11**	82.0	0.57
Human IgG (Fc) Capture	IMM40 26A3 humanized Ab	247	Human CD70 Protein, His, Flag Tag (active trimer)	3.125-0.049 nM	2.26E+07	7.12E-04	**3.15E-11**	86.9	0.83

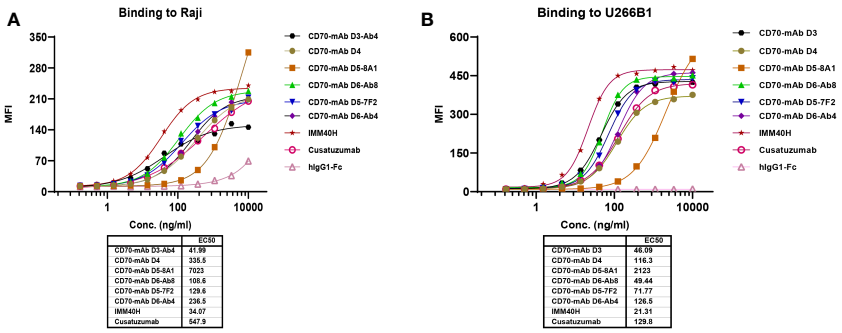
FIGURE 2
CD70 target affinity measured by SPR. The results of the affinity analysis showed that the humanized 26A3 (IMM40H) has equivalent CD70 binding affinity to the chimeric 26A3 (kD = 3.22E-11M for humanized Ab, kD = 3.15E-11M for chimeric Ab).

3.3 IMM40H exhibited the strongest blocking activity of interrupting CD70/CD27 signaling

Inhibition of the CD70-CD27 interaction between tumor cells and immune cells in the tumor microenvironment can be used for therapeutic purposes. The expression of CD70 on cancer cells is linked to the induction of regulatory T cells, which may inhibit the immune system in the tumor microenvironment (17). Additionally, IMM40H can inhibit growth signals and/or limit the acquisition of T-cell immune regulatory function inside the tumor microenvironment, while removing CD70+ malignant cells through Fc-mediated effector activities. A chimeric CD27 receptor (CAR) expressing recombinant Jurkat cells (Jurkat-CAR-CD27) was tested in a bioassay to determine their target-blocking activity (Figure 4A). When Jurkat-CAR-CD27 bound to Raji (CD70+), it transduced a stimulatory signal, which upregulated the expression of CD69. IMM40H can block CD70-CD27 interaction, which in turn inhibits the expression of CD69. IMM40H has potent target-blocking activity (IC50 = 2.94 ng/mL), which is significantly better than that of cusatuzumab (IC50 = 12.69 ng/mL) and other competing CD70 mAbs (Figure 4B).

3.4 IMM40H exhibited potent antitumor effects through Fc-dependent effector functions *in vitro*

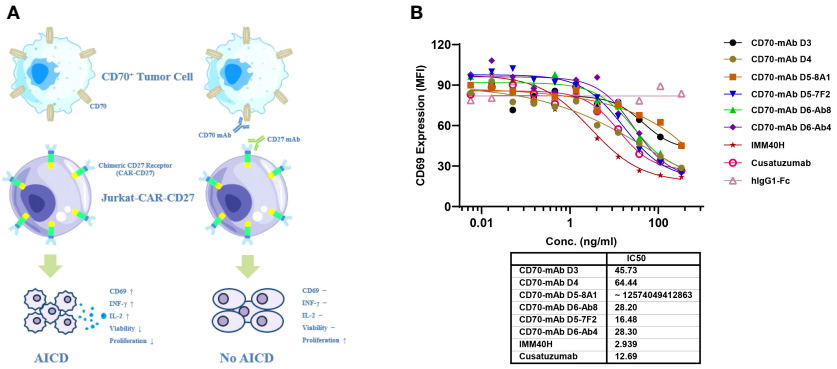
In healthy tissues and organs, expression of the CD70 protein is very low. IgG1 was prioritized while developing anti-CD70 therapeutic antibodies since it has a high affinity for binding and activating FcγRs and can elicit potent ADCC and ADCP against CD70+ tumor cells. IgG1 was selected for the formats of IMM40H, with the S298A, E333A, and K334A substitution in the Fc region to enhance the Fc-dependent effector functions. *In vitro* pharmacology studies showed that IMM40H can induce tumor cell lysis through ADCC, ADCP, and CDC in malignant cells expressing CD70. IMM40H exhibited stronger ADCC activity against cells associated with hematological malignancies (Raji, U266B1, Daudi, and Jeko-1 cell lines) compared to competing CD70 mAbs, while



	EC50
CD70-mAb D3-Ab4	41.99
CD70-mAb D4	335.5
CD70-mAb D5-8A1	7023
CD70-mAb D6-Ab8	108.6
CD70-mAb D5-7F2	129.6
CD70-mAb D6-Ab4	236.5
IMM40H	34.07
Cusatuzumab	547.9

	EC50
CD70-mAb D3	46.09
CD70-mAb D4	116.3
CD70-mAb D5-8A1	2123
CD70-mAb D6-Ab8	49.44
CD70-mAb D5-7F2	71.77
CD70-mAb D6-Ab4	126.5
IMM40H	21.31
Cusatuzumab	129.8

FIGURE 3
The binding of IMM40H and competing CD70 mAbs to CD70+ tumor cells was evaluated by FACS. **(A)** The binding of IMM40H and competing CD70 mAbs to Burkitt's lymphoma Raji cell. **(B)** The binding of IMM40H and competing CD70 mAbs to multiple myeloma U266B1 cells. The results showed that IMM40H had a higher binding activity to Raji and U266B1 cells than the competitor anti-CD70 antibodies. Competitor anti-CD70 antibodies sequence derived from patents data. Cusatuzumab, WO2012123586A1; CD70-mAb D3, US20120294863A1; CD70-mAb D4, WO2007038637A2; CD70-mAb D5–8A1&CD70-mAb D5–7F2, WO2013192360A1; CD70-mAb D6-Ab4&CD70-mAb D6-Ab8, WO2013043933A2.



	IC50
CD70-mAb D3	45.73
CD70-mAb D4	64.44
CD70-mAb D5-8A1	~ 12574049412863
CD70-mAb D6-Ab8	28.20
CD70-mAb D5-7F2	16.48
CD70-mAb D6-Ab4	28.30
IMM40H	2.939
Cusatuzumab	12.69

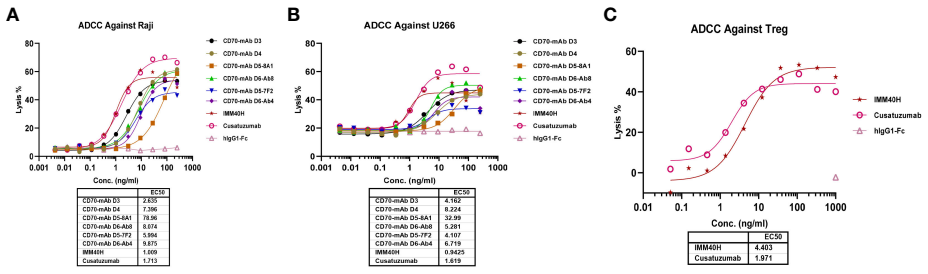
FIGURE 4
The target-blocking activity of IMM40H was characterized by bioassay using the Jurkat-CAR-CD27 cell line. **(A)** MOA of Jurkat-CAR-CD27 cell. Upon ligation with CD70+ positive tumor cell, the CAR-CD27 (extracellular CD27 domain that was linked to CD8α-hinge, CD28-TMD/ICD, and CD3ζ signal domains in a specific order) molecule transduces a stimulatory signal and activates Jurkat-CAR-CD27 cells. The activated Jurkat-CAR-CD27 cells up-regulate Fas and Fas ligand, upon interaction of Fas on one cell with Fas ligand on another cell, death signal will be transduced to Jurkat-CAR-CD27 cells, thus will undergo AICD. Which was coupled with an increase in the expression of CD69, INF-γ and IL-2. Antibodies specific for CD70 or CD27 can block the interaction of CD70 with extracellular CD27 domain and thus will block the CD70 induced AICD. **(B)** The target blocking activity of IMM40H was significantly better than that of the competing CD70 mAbs.

also exhibiting potent killing effects against solid tumor cells (A498) (Figures 5A, B, Supplementary Figures 5A-C). A significant proportion of human Tregs gain stable CD70 expression while losing CD27 after prolonged *in vitro* stimulation (18). Using commercially activated Tregs (Sailybio, Cat#XFB-nTreg-02BA) as target cells, the results showed that due to the low expression of CD70 in Treg cells, IMM40H was able to directly kill Tregs through ADCC (Figure 5C, Supplementary Figure 6). Besides enhancing the immune defense against tumors by disrupting the communication between CD70-CD27 and Tregs, IMM40H can reduce the ratio of activated Tregs and relieve immune suppression by directly killing them. Using flow cytometry, phagocytosis was evaluated in the monocytic cell line THP-1 (effector) against the target cell lines Raji, Daudi, U266B1, Jeko-1, and A498 labeled with CFSE by measuring the THP-1 green fluorescence following 2 h of incubation with IMM40H. IMM40H induced stronger ADCP against Raji and U266B1 than the competing CD70 mAbs, including cusatuzumab (Figures 6A, B). ADCP effects on THP-1 cells with an EC_{50} range of MFI of 0.035 nM in Raji, 0.0094 nM in U266B1, 0.0718 nM in Daudi, 0.0052 nM in Jeko-1, and 0.0508 nM in A498, respectively (Supplementary Figure 7). Cell lysis as a marker for CDC was determined via propidium iodide (PI) labeling by flow cytometry assays. The lysis EC_{50} for IMM40H was 0.395 nM in Raji cells (Supplementary Figure 8), whereas IMM40H had no CDC activity against other CD70+ tumor cell lines, including U266B1, Daudi, Jeko-1, and A498 (data not presented).

3.5 IMM40H exhibited strong antitumor efficacy *in vivo*

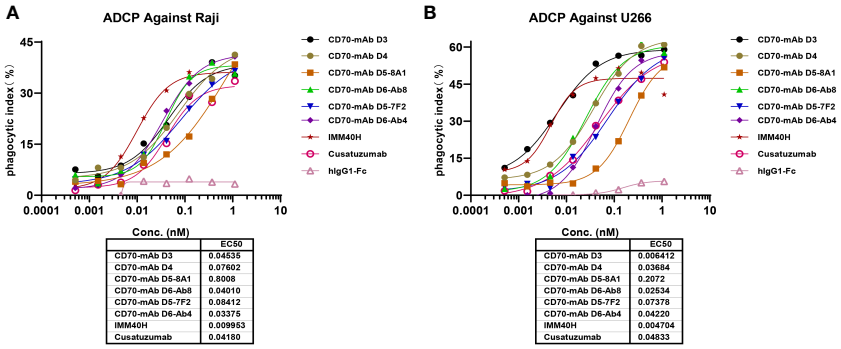
3.5.1 *In vivo* activity against multiple myeloma (U266B1) subcutaneous xenograft model

We examined the effect of IMM40H in subcutaneous xenograft models derived from the multiple myeloma cell line U266B1 compared to the effects of bortezomib and Cusatuzumab, which are commonly used for multiple myeloma treatment. IMM40H (IP, QW×4, 0.3, 1, 3 mg/kg), Bortezomib (IV, BIW×4W, 0.5 mg/kg), and Cusatuzumab (IV, QW×4, 1 mg/kg) were administered after grouping. Animals in all groups showed no significant abnormality during the study, without any remarkable body weight differences among animals in the 0.3, 1, and 3 mg/kg IMM40H groups. At the



	EC50
IMM40H	4.603
Cusatuzumab	1.971

FIGURE 5
The ADCC activity against CD70+ tumor cells and Tregs was measured using FcR-TANK. **(A)** IMM40H and the competing CD70 mAbs induced ADCC against Raji. **(B)** IMM40H and competing CD70 mAbs induced ADCC against U266. **(C)** IMM40H induced ADCC against Tregs. The ADCC-inducing activity of IMM40H was stronger than the competing CD70 mAbs and had similar activity to defucosylated cusatuzumab.



	EC50
CD70-mAb D3	0.04535
CD70-mAb D4	0.07602
CD70-mAb D5-8A1	0.8008
CD70-mAb D6-Ab8	0.04010
CD70-mAb D5-7F2	0.08412
CD70-mAb D6-Ab4	0.03375
IMM40H	0.009953
Cusatuzumab	0.04180

	EC50
CD70-mAb D3	0.006412
CD70-mAb D4	0.03684
CD70-mAb D5-8A1	0.2072
CD70-mAb D6-Ab8	0.02534
CD70-mAb D5-7F2	0.07378
CD70-mAb D6-Ab4	0.04220
IMM40H	0.004704
Cusatuzumab	0.04833

FIGURE 6
The ADCP activity against CD70+ tumor cells were measured using THP-1 cell. **(A)** IMM40H induced ADCP against Raji. **(B)** IMM40H induced ADCP against U266. The phagocytic index was determined by FACS and defined as the percentage of CFSE positive macrophages. The phagocytic index was calculated using the formula: phagocytic index % = (E+T+Ab) % CFSE positive cell − (E+T) % CFSE positive cell. IMM40H induced stronger ADCP against CD70+ tumor cells than the competing CD70 mAbs, including cusatuzumab.

endpoint of the study, all mouse tumors in the three IMM40H treatment groups were eliminated. The therapeutic effect was observed earlier with IMM40H (0.3 mg/kg) than with cusatuzumab (1 mg/kg) (Figure 7).

3.5.2 *In vivo* activity against Burkitt's lymphoma (Raji) orthotopic xenograft model

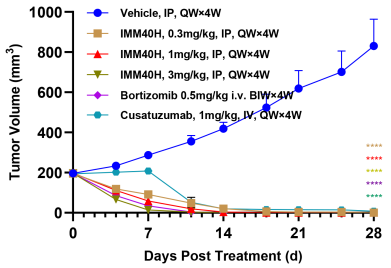
We further evaluated the antitumor activity of IMM40H *in vivo* via Raji orthotopic xenografts. IMM01 (SIRPα-Fc fusion protein) and IMM40H were tested in the study. IMM01 (0.3 mg/kg), IMM40H (1, 3, 10 mg/kg), and IMM01 (0.3 mg/kg) combined with IMM40H (3 mg/kg) were administered twice weekly via tail vein injection for three consecutive weeks. The antitumor activity confirmed that IMM40H substantially improved the survival time in a dose-dependent manner. Overall, 38% (3/8), 75% (6/8), and 75% (6/8) of mice in the three dosages groups (1, 3, and 10 mg/kg, respectively) survived at the endpoint. While CD47 blocker (IMM01) survived 88% (7/8) of the treated mice at the dose of 0.3 mg/kg, the combination of IMM01 (0.3 mg/kg) with IMM40H (3 mg/kg) survived 100% of the treated mice, suggesting a synergistic effect between CD47- and CD70-targeted therapy (Figure 8).

3.5.3 *In vivo* activity against renal carcinoma cell (A498) subcutaneous xenograft model

We also performed *in vivo* experiments using an A498 mouse xenograft model to evaluate the antitumor activities of IMM40H combined with IMM01 compared to the activities of IMM40H and IMM01 alone without affecting body weight. IMM01 (10 mg/kg), IMM40H (3, 10, 30 mg/kg), and IMM01 (5 mg/kg) combined with IMM40H (10 mg/kg) were administered twice weekly via intraperitoneal injection for four consecutive weeks. IMM40H at doses of 3–30 mg/kg showed a certain dose-dependent significant tumor inhibition efficacy. IMM40H exhibited a synergistic effect with CD47-targeted IMM01 in treating A498 xenograft renal carcinoma model. Tumor Growth Inhibition (TGI) percentage of Combo (5 + 10 mg/kg), IMM01 (10 mg/kg), and IMM40H (10 mg/kg) was 62.86%, 36.22%, and 39.52%, respectively (Figure 9).

3.6 IMM40H exhibited a favorable safety and tolerability profile *in vivo*

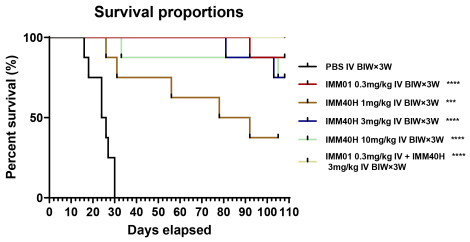
We used cynomolgus monkeys for pharmacokinetic and toxicological studies. The CD70 amino acid sequence was very



Groups	Tumor Volume(mm³)[a]	TGI (%)	*P* value[b]
Vehicle	830.22±133.84	--	--
IMM40H 0.3 mg/kg	0.00±0.00	100%	< 0.0001
IMM40H 1.0 mg/kg	0.00±0.00	100%	< 0.0001
IMM40H 3.0 mg/kg	0.00±0.00	100%	< 0.0001
Bortizomib 0.5 mg/kg	0.00±0.00	100%	< 0.0001
Cusatuzumab 1 mg/kg	8.11±8.11	99.05%	< 0.0001

TGI: Tumor Growth Inhibition Rate;
a, Mean ± SEM;
b, *p* value was calculated based on different groups of tumor volume using vehicle group as the control by T-Test.

FIGURE 7
In vivo activity against multiple myeloma (U266B1) subcutaneous xenograft model. IMM40H demonstrates much stronger tumor killing efficacy than Cusatuzumab. Therapeutic effect was observed earlier in IMM40H (0.3 mg/kg) group than in cusatuzumab (1 mg/kg) group.



Groups	Survival rate	Survival time (d)[a]	P value[b]
DPBS	0%	24.4±5.1	----
IMM01 0.3mg/kg	87.5%	106.0±5.7	< 0.0001
IMM40H 1mg/kg	37.5%	74.8±33.2	0.0005
IMM40H 3mg/kg	75%	104.0±9.5	< 0.0001
IMM40H 10mg/kg	75%	98.3±26.4	< 0.0001
IMM01 0.3mg/kg+ IMM40H 3mg/kg	100%	108.0±0.0	< 0.0001

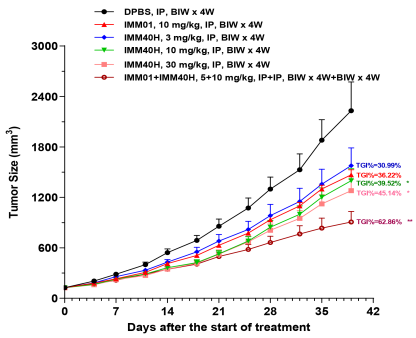
a, Mean ± SEM;
b, P values were calculated using Log-rank(Mantel-Cox) test and vehicle group as the control.

FIGURE 8
In vivo activity against Burkitt's lymphoma (Raji) orthotopic xenograft model. IMM40H substantially improved the survival time in a dose-dependent manner. The percentage of mice survived in the three dosage group (1, 3, 10 mg/kg) is 38% (3/8), 75% (6/8), and 75% (6/8) respectively. Interestingly, a combination of IMM01 (0.3mg/kg) with IMM40H (3mg/kg) showed 100% survival in the treated mice, which is superior to IMM40H or IMM01 alone, suggesting a synergistic effect between CD47- and CD70-targeted therapy.

similar in human and cynomolgus monkey (86.6%) but only slightly more similar between human and mouse (66.1%). IMM40H can bind to Cyno CD70, whereas mouse CD70 does not. The results of an affinity analysis revealed that IMM40H had an equivalent binding affinity for human and Cyno CD70, but the latter was slightly stronger, as determined by biolayer interferometry (BLI) (Supplementary Figure 9). In a GLP-compliant general toxicity study, the potential toxicity of IMM40H was evaluated when it was administered as a single-dose IV infusion to cynomolgus monkeys. All three tested doses of IMM40H (at 20, 50, and 100 mg/kg) were well-tolerated by the monkeys when administered intravenously. At different dosages of IMM40H (up to 100 mg/kg), there were no detectable changes in gross observations, urinalysis, coagulation, serum chemistry, hematological parameters, food intake, body weight, clinical observations, and survival (mortality/morbidity). Thus, IMM40H was well-tolerated at all doses tested, and a single IV infusion of 100 mg/kg was the maximum tolerated dose (MTD) in both male and female cynomolgus monkeys.

The PK analysis showed no differences between the sexes at all doses when the area under the serum concentration-time curve was compared from time zero to the last quantifiable concentration ($AUC_{0\text{-last}}$) and maximum observed concentration (C_{max}) in male and female cynomolgus monkeys. After a single IV infusion of different doses of IMM40H (0.5, 1.5, and 3 mg/kg), the IMM40H showed serum clearance (CL) of 0.00920, 0.00934, and 0.0123 mL/min/kg, respectively, and a half-life ($T_{1/2}$) of 97.3, 75.2, and 48.6 h, respectively. The volume of distribution at steady state (Vdss) was 0.0761, 0.0649, and 0.0711L/kg, respectively. The $AUC_{0\text{-last}}$ values were 634000, 2150000, and 3800000 ng·h/mL, respectively. The results showed that the systematic exposure ($AUC_{0\text{-last}}$ and C_{max}) of IMM40H increased proportionally as the dose increased from 0.5 mg/kg to 3 mg/kg (Figure 10, Table 1).

We further evaluated the potential toxicity of IMM40H to cynomolgus monkeys when it was administered by IV infusion once a week for five weeks. We also assessed the reversibility, persistence, and delayed occurrence of toxicity following a 42-day recovery period. We found that the administration of IMM40H resulted in IMM40H-related adverse but reversible microscopic changes consisting of mild mononuclear inflammation in the pulmonary interstitium and minimal or mild glomerulonephritis at ≥ 10 mg/kg/dose. No IMM40H-related abnormalities occurred in clinical observations, food consumption, body weight, ophthalmology, body temperature, safety pharmacology (electrocardiogram, blood pressure, respiratory and neurological examinations), urinalysis, immunophenotyping, and macroscopic observations at all doses. Therefore, the highest non-severely toxic dose (HNSTD) of IMM40H was considered to be 30 mg/kg. At this dose, C_{max} and $AUC_{0-169\ h}$ of IMM40H were 959,000 ng/mL and 66,400,000 h*ng/mL, respectively, in females, and 877,000 ng/mL and 57,500,000 h*ng/mL, respectively, in males on Day 22 (Table 2).



Groups	Tumor Volume(mm³)[a]	T/C (%)	TGI (%)	P value[b]
DPBS	2230.53±342.18	--	--	--
IMM01 10 mg/kg	1468.02±59.97	65.91	36.22%	0.076
IMM40H 3 mg/kg	1578.20±210.27	70.82	30.99%	0.158
IMM40H 10 mg/kg	1398.74±142.85	62.79	39.52%	0.049
IMM40H 30 mg/kg	1280.41±133.86	57.47	45.14%	0.027
IMM01+ IMM40H 5 mg/kg + 10 mg/kg	907.58±124.90	40.68	62.86%	0.005

TGI: Tumor Growth Inhibition Rate; T/C: Relative Tumor Volume Growth Rate.
a, Mean ± SEM;
b, *p* value was calculated based on different groups of tumor volume using vehicle group as the control by T-Test.

FIGURE 9
In vivo activity against renal carcinoma cell (A498) subcutaneous xenograft model. IMM40H at the doses of 3~30 mg/kg showed a significant tumor inhibition efficiency in a dose-dependent manner. IMM40H demonstrated synergistic effect with CD47-targeted IMM01 (SIRPα-Fc), which is superior to IMM40H or IMM01 alone.

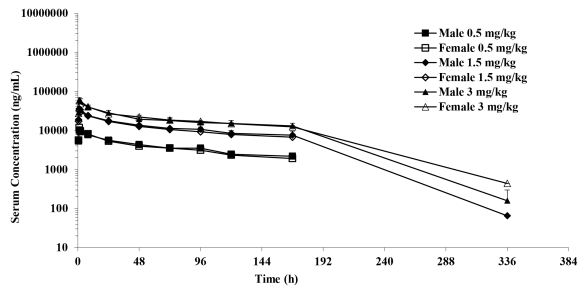


FIGURE 10
Mean serum concentration after single IV infusion at dose of 0.5,1.5 and 3.0 mg/kg to male and female cynomolgus monkeys.

4 Discussion

Cancer immunotherapy advanced considerably following the development of therapeutic antibodies that target critical immune checkpoints. In 2011, the U.S. Food and Drug Administration (FDA) authorized the use of ipilimumab for the treatment of melanoma that has spread to other parts of the body (19). Individuals suffering from unresectable or advanced melanoma who do not respond to prior therapy were provided approval to receive treatment by pembrolizumab on September 4, 2014 (20). Nivolumab was granted FDA approval on December 22, 2014, for the treatment of individuals with metastatic or unresectable melanoma whose illness progressed even after ipilimumab therapy, and in patients who tested positive for a BRAF V600 mutation following treatment with a BRAF inhibitor (21). Immune checkpoint blockers are a promising new option for treating malignancies that are beyond the scope of traditional treatment. Patient response rates are still low in most cases, indicating further or combinatorial targeting of immune checkpoints is needed.

Many different types of cancer, both hematologic and solid, have been linked to abnormal expression of CD70 and its receptor CD27. Tumor progression and immunosuppression are linked to the dysregulation of the CD70-CD27 axis in the tumor and its microenvironment (22). Since CD70 is normally expressed only by a small percentage of cells in the lymphoid compartment, therapies that specifically target this protein should have few unintended consequences. When tumor cells overexpress CD70, CD27 expression in tumor-infiltrating Tregs may facilitate immune evasion. Many different types of cancer, including renal cell carcinoma, glioblastoma, thymic carcinoma, nasopharyngeal carcinoma, T-anaplastic large-cell lymphoma, Waldenström's macroglobulinemia, and Hodgkin and non-Hodgkin lymphomas, may respond well to therapies that target CD70 (13, 15, 23–28).

Monoclonal antibodies, antibody-drug conjugates (ADCs), and CAR-T-based treatments have shown that CD70 is the optimal target due to its very limited expression pattern in certain blood cancers and solid tumors. Several drugs based on mAbs (SEA-CD70, MDX1411, and cusatuzumab), ADCs (ARX305, MDX-1203, AMG172, SNG-CD70, and SNG-75), and CAR-Ts (CTX130, CD70–001, ALLO-316, and GIMIIRB-20006) are currently being tested in clinical trials for the treatment of CD70-related diseases (29). Combining cisplatin and docetaxel with anti-CD70 treatment (Cusatuzumab) can boost antitumor immune responses in NSCLC patients, as shown by preclinical evidence (30). Defucosylated anti-CD70 monoclonal antibody cusatuzumab (ARGX-110) prevents tumor immune escape by blocking the survival of Tregs and restoring normal myeloid differentiation. Phase I dose-escalation study of cusatuzumab showed good tolerability, pharmacokinetics,

TABLE 1 PK Parameters in Cynomolgus Monkeys After Single IV Injection at dose of 0.5, 1.5, and 3 mg/kg of IMM40H.

IMM40H						
Dose Route	IV Infusion		IV Infusion		IV Infusion	
Dose level(mg/kg)	0.5 mg/kg		1.5 mg/kg		3 mg/kg	
PK Parameters	Mean	SD	Mean	SD	Mean	SD
Cmax (ng/mL)	11000	1120	34600	3070	58200	6380
Tmax (h)	1.17	0.408	1.17	0.408	1.00	0.00
T1/2 (h)	97.3	16.8	75.2	50.0	48.6	40.4
Vdss (L/kg)	0.0761	0.00590	0.0649	0.0135	0.0711	0.0112
Cl (mL/min/kg)	0.00920	0.00149	0.00934	0.00223	0.0123	0.00243
AUC0-last (ng•h/mL)	634000	68400	2150000	177000	3800000	440000
AUC0-inf (ng•h/mL)	925000	146000	2820000	747000	4220000	1000000

AUC0-inf, area under the curve to infinite time; AUClast, area under the serum concentration–time curve from time zero to the last quantifiable concentration; Cl, clearance; Cmax, maximum observed concentration; IV, intravenous; PK, pharmacokinetics; T1/2, half-life; Tmax, time of maximum observed concentration; Vdss, volume of distribution at steady state.

TABLE 2 TK Parameters in Cynomolgus Monkeys After Repeat-dose IV Infusion at dose of 3, 10, and 30 mg/kg of IMM40H.

Dose (mg/kg)	Study Day	Sex	Cmax (ng/mL)	Tmax (h)	AUC0-169h (h*ng/mL)
3	1	Male	63400 ± 16000	1.0 (1.0 - 1.0)	3970000 ± 390000
		Female	72700 ± 4240	1.0 (1.0 - 1.0)	4100000 ± 187000
	22	Male	58700 ± 29300	1.0 (1.0 - 1.5)	1610000 ± 1490000
		Female	81500 ± 20200	1.0 (1.0 - 1.5)	3720000 ± 3280000
10	1	Male	198000 ± 19400	1.0 (1.0 - 1.0)	11600000 ± 1640000
		Female	216000 ± 20300	1.0 (1.0 - 1.5)	13200000 ± 878000
	22	Male	209000 ± 72900	1.0 (1.0 - 1.5)	9810000 ± 10400000
		Female	300000 ± 79300	1.0 (1.0 - 1.5)	21400000 ± 12000000
30	1	Male	614000 ± 55200	1.0 (1.0 - 1.5)	37200000 ± 3780000
		Female	690000 ± 107000	1.0 (1.0 - 1.5)	38200000 ± 4450000
	22	Male	877000 ± 146000	1.0 (1.0 - 1.0)	57500000 ± 15200000
		Female	959000 ± 263000	1.0 (1.0 - 3.0)	66400000 ± 39500000

Data are presented as mean ± SD for Cmax and AUC0-169h values, and median (range) for Tmax. AUC0-169 = area under the serum concentration-time curve (AUC) from time zero to 168 hours post end of infusion (169 hours post start of the infusion, AUC0–169h); Cmax, maximum observed concentration; Tmax, time of maximum observed concentration.

and preliminary antitumor activity at all dose levels (0.1, 1, 2, 5, and 10 mg/kg) in patients with advanced CD70-positive malignancies (31). Cusatuzumab also inhibits LSC proliferation, reduces leukemic blast cells, and blocks CD70/CD27 signaling (8, 9). Patients with previously untreated AML who were ineligible for intense chemotherapy responded well to a combination of cusatuzumab and azacitidine, as determined by a Phase I/II study (10–12).

We obtained IMM40H through hybridoma screening and antibody humanization techniques. IMM40H specifically binds to the CD70 target and has higher affinity and stronger blocking activities compared to competitor anti-CD70 antibodies, including Cusatuzumab. IMM40H can interrupt the proliferation and activation of Treg cells by inhibiting CD70/CD27 signaling. Additionally, IMM40H also showed potent Fc-dependent effector functions (ADCC/CDC/ADCP) via S298A, E333A, and K334A substitution in the Fc region, resulting in a strong immune attack on hematologic malignancies and potent therapeutic efficacy. Our preclinical data also suggested that IMM40H has potent antitumor activity in the U266B1 multiple myeloma tumor model, eradicating subcutaneously established tumors even at a dose as low as 0.3 mg/kg. Moreover, IMM40H (0.3 mg/kg) showed a therapeutic effect faster than cusatuzumab (1 mg/kg). With cusatuzumab (1 mg/kg), tumors were cleared in five of the six mice. A strong synergistic effect of IMM01 (SIRPα-Fc fusion protein) and IMM40H was found on Burkitt's lymphoma Raji and renal carcinoma cell A498 tumor models. Synergistic antitumor activity between CD47-and CD70-targeted therapy acts as a foundation for their combined application in future clinical studies. IMM40H has a favorable safety and tolerability profile, considering that significant IMM40H-related toxic side effects were not observed. The HNSTD for repeat-dose toxicity and MTD for single-dose toxicity were up to 30 mg/kg and 100 mg/kg, respectively, in cynomolgus monkeys. We obtained IND approval for IMM40H from the NMPA and the FDA in August 2022, and we aim to initiate Phase I clinical studies.

5 Conclusions

Anti-CD70 targeted combinatorial therapy was effective in preclinical and clinical investigations. Although AML is the primary indication of monotherapy and combinatorial therapy, this strategy might be applied to other tumor types also. The CD70-CD27 axis was studied extensively for its role in tumor promotion and immune evasion in cancer, and new insights into its putative molecular processes emerged. Therefore, methods to suppress the signaling pathways implicated in the CD70-CD27 axis might offer attractive new therapeutic possibilities along with current techniques that rely on targeting CD70. Pre-clinical findings showed that IMM40H has a higher binding ability, stronger ability to block the interaction of CD70/CD27, and stronger ability to kill tumor cells than other CD70 competitors. We aim to investigate the safety and efficacy of IMM40H in clinical trials for hematomas and solid tumors. It might emerge as a novel, safe, and effective therapeutic option for treating cancers.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee (IACUC) of WuXi AppTec and Medicilon Biology. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WT designed and directed the study. WT and SL wrote the manuscript draft. SL and DC carried out the experiments. SL, DC, HG, DL, CY, RZ, TX, FZ, XB, YY, NS, WZ, LZ, GZ, LP, XT performed the experiments. All authors critically reviewed and approved the final manuscript.

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Conflict of interest

All authors were employed by the company ImmuneOnco Biopharmaceuticals Shanghai Inc. WT, SL, DC, and HG are inventors of the patent application on IMM40H.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1240061/full#supplementary-material

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Adjusting the dose of traditional drugs combined with immunotherapy: reshaping the immune microenvironment in lung cancer

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Immunotherapy is currently the most promising clinical treatment for lung cancer, not only revolutionizing second-line therapy but now also approved for first-line treatment. However, its clinical efficiency is not high and not all patients benefit from it. Thus, finding the best combination strategy to expand anti-PD-1/PD-L1-based immunotherapy is now a hot research topic. The conventional use of chemotherapeutic drugs and targeted drugs inevitably leads to resistance, toxic side effects and other problems. Recent research, however, suggests that by adjusting the dosage of drugs and blocking the activation of mutational mechanisms that depend on acquired resistance, it is possible to reduce toxic side effects, activate immune cells, and reshape the immune microenvironment of lung cancer. Here, we discuss the effects of different chemotherapeutic drugs and targeted drugs on the immune microenvironment. We explore the effects of adjusting the dosing sequence and timing, and the mechanisms of such responses, and show how the effectiveness and reliability of combined immunotherapy provide improved treatment outcomes.

KEYWORDS

lung cancer, immune checkpoint inhibitors, immune microenviroment, drug dose adjustment, immunothearpy

1 Introduction

According to the International Agency for Research on Cancer's World Cancer Statistical Report, approximately 1.8 million deaths occur annually due to lung cancer, followed by rectal cancer, liver cancer, and stomach cancer (1). Lung cancer is most prevalent among male patients and ranks second among female patients (2) due to its initial asymptomatic nature and difficulty in detection (3, 4). Currently, lung cancer holds the highest incidence and mortality rate globally (5). Smoking causes 80% of lung cancer deaths, while other risk factors include radon gas, asbestos, cumulative exposure to air pollution, polycyclic aromatic hydrocarbon emissions, and genetic factors (6).

Lung cancer is categorized based on histology into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) (7). NSCLC accounts for 80-85% of all lung cancer cases and includes adenocarcinoma, squamous carcinoma, and other histological subtypes (8). Poor prognosis usually follows an advanced NSCLC diagnosis (9), but new insights into the molecular mechanisms of disease progression and an increased understanding of the disease have allowed for the development of novel treatment options. These treatments include surgery, radiation therapy, chemotherapy, targeted therapy, immunotherapy, interventional therapy, and a combination of Chinese traditional and western medicine, as shown in Figure 1. Significantly improved survival rates have been observed in lung cancer patients with the continuous improvement of systemic therapy.

Lung cancer treatment has shifted from indiscriminate cytotoxic chemotherapy to more refined targeted agents. The development of small molecule drugs and monoclonal antibodies that target specific components of dysfunctional molecules or immune pathways, as well as mutated genes that target lung cancer, and the development of more personalized combinations based on different conditions, has led to more optimal treatment options for lung cancer (10). Currently, immunotherapy is the most promising clinical treatment for lung cancer (11, 12). The goal of cancer immunotherapy is to elicit a cellular immune response (6, 13). Immunotherapies for lung cancer include tumor-related vaccines, chimeric antigen receptor (CAR)-T, T cell receptor (TCR)-T cell therapy, tumor infiltrating lymphocytes (TILs) therapy, lysing viruses, targeted antibodies for lung cancer, and immune checkpoint inhibitors (ICIs) (6). Among them, ICIs are the most widely used in clinical practice,which have not only revolutionized second-line treatment, but they are now also approved for first-line treatment (14, 15). Cytotoxic T lymphocyte associated antigen 4 (CTLA-4) (3, 16) is the first antibody in immunotherapy to be approved by the U.S. Food and Drug Administration (17). Since the discovery of CTLA-4, several immune checkpoint proteins have been discovered, including programmed death-1 (PD-1), T-cell immunoglobulin and ITIM domain (TIGIT), T-cell immunoglobulin domain and mucin domain-containing molecule-3 (TIM-3), lymphocyte activation gene 3 (LAG-3), V-domain Ig suppressor of T cell activation, B and T cell lymphocyte attenuator and cluster of differentiation 200. Among the most common clinical treatments for NSCLC are PD-1 monoclonal antibodies, including nivolumab and pembrolizumab (18). However, their clinical effectiveness is not high and not all patients benefit from them. The response rate after second-line treatment with nivolumab is approximately 20%. First-line treatment with pembrolizumab is currently limited to patients with a PD-1 ligand (PD-L1) tumor percentage score above 50%, which accounts for approximately one-third of NSCLC patients, and has an response rate of 69% (15). The upregulation of PD-1 expression in TILs is one of the main immunosuppressive mechanisms in the tumor microenvironment (TME). The TILs interact with ligands (PD-L1 and PD-L2), leading to a decrease in $CD8^+$ T cells and an increase in regulatory T cells (Tregs), suppressing the function of $CD8^+$ T cells or causing immune escape in response to an adaptive response to interferon (IFN) signaling (17, 19). Other factors that affect the effectiveness of treatment include the absence of tumor expression of MHC class I molecules (20, 21), low numbers of $CD4^+$ T cell infiltrates in tumor tissue, high numbers of myeloid-derived suppressor cells (MDSC), and low expression of PD-L1 on cancer cells. Based on these potential mechanisms, the antitumor efficacy of immunotherapy can be boosted with the use of other types of treatment. Thus, finding the best combination strategy to expand anti-PD-1/PD-L1-based immunotherapy is currently a hot issue in lung cancer research (22).

In this paper, we review what is known about the current state of NSCLC research, with a particular emphasis on the TME, the current available drugs for treatment, and the potential for using combined therapies at appropriate doses to improve treatment response while minimizing treatment-related adverse events.

2 TME in lung cancer

Tumor cells and peripheral cells both coexist and compete with each other. Among the surrounding cells are intrinsic and specific immune cells, resulting in a unique environment that varies by tumor type and is highly adapted to tumor behavior; this is referred to as the TME (21). The TME includes tumor-associated macrophages (TAMs), cancer-associated fibroblasts, tissue-specific mesenchymal cells, endothelial cells, intrinsic and specific immune cells, TILs, neutrophils, eosinophils, MDSCs, cytokines and extracellular matrix (23–26) (Figure 2). The metabolic status of immune cells in the TME is a key factor affecting their immune response (27) and plays an important role in tumorigenesis, disease progression, and treatment response and prognosis (28). Under normal physiological conditions, innate and acquired immune cells capture and destroy cancer cells through immune surveillance (29, 30). However, in the pathological state, tumor cells can shape the immunosuppressive microenvironment through different mechanisms.

The tumor immune microenvironment consists of a diverse array of cell types, including $CD4^+$ T cells, $CD8^+$ T cells, B cells, TAMs, natural killer cells, $CD1c^+$ myeloid and $CD141^+$ myeloid dendritic cells, neutrophils, basophils, eosinophils, and mast cells (31). $CD4^+$ T cells have different subsets of cells (TH1, TH2, TH17, and Tregs) that perform specialized immunomodulatory functions and secrete different cytokines to enhance or suppress immune responses (32). Tregs are a functional subpopulation of suppressor T cells that express the transcription factor FOXP3 (33). $CD8^+$ T cells are activated to secrete IFN-γ and tumor necrosis factor after cell receptors on their

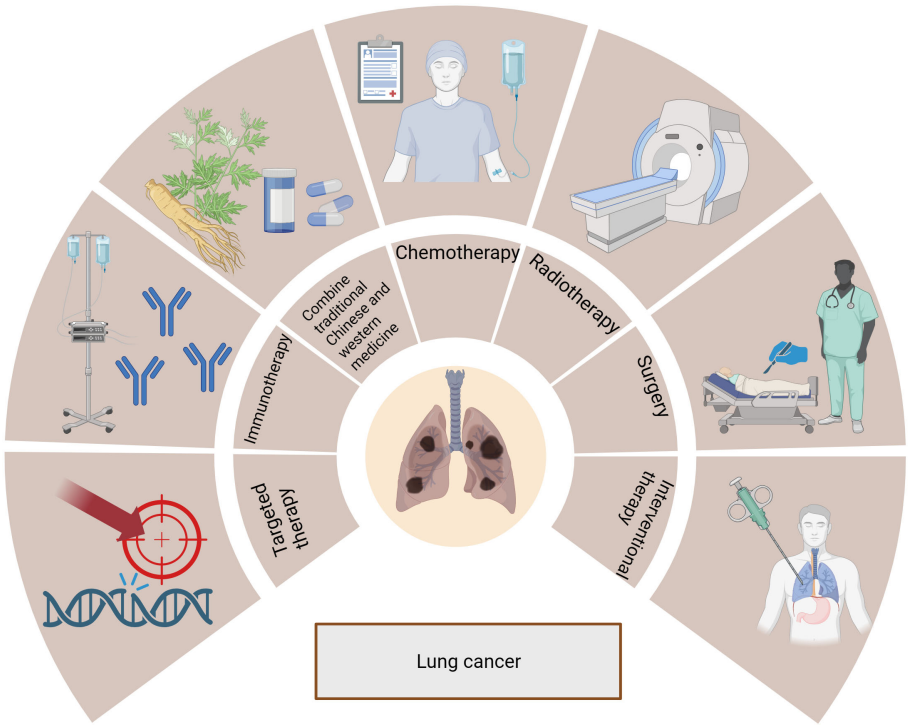

FIGURE 1
Treatments for lung cancer include surgery, radiotherapy, chemotherapy, targeted therapy, immunotherapy, interventional therapy, and combination of traditional Chinese and Western medicine.

surface destroy tumor cells (34). CD4 CTL and $CD8^+$ T cells express granzyme and perforin, which are effectors that mediate cytotoxic activity in target cells (32). The activation process of macrophages is highly plastic, and depending on signals in the TME, macrophages can be polarized into M1 or M2 functional phenotypes (35, 36). M1 macrophages secrete IFN, interleukin (IL), nitric oxide synthase, and reactive oxygen species to exert and enhance anti-tumor immunity (35, 37). M2 macrophages are associated with high expression of IL-10, IL-1β and vascular endothelial growth factor (VEGF) *in vivo*, and form a beneficial survival environment for tumor cells by suppressing immunity and promoting tumor angiogenesis, invasion and distant metastasis (35, 38).

Tumor cells can shape the immunosuppressive microenvironment through nutritional competition, secretion of cytokines, the release of metabolites and modulation of immune cell metabolism to affect immune cell growth, development and differentiation, thereby increasing the function of immune cells toward a pro-tumor phenotype, a process that helps promote the immune escape of tumor cells themselves (27). When PD-L1 on tumor cells binds to PD-1 on T cells, the T cells are unable to specifically recognize the tumor cells, and this also results in immunosuppression (39). If this immunosuppression caused by tumor cells is reversed by drugs, the immune cells can resume their normal function of recognizing and killing tumor cells.

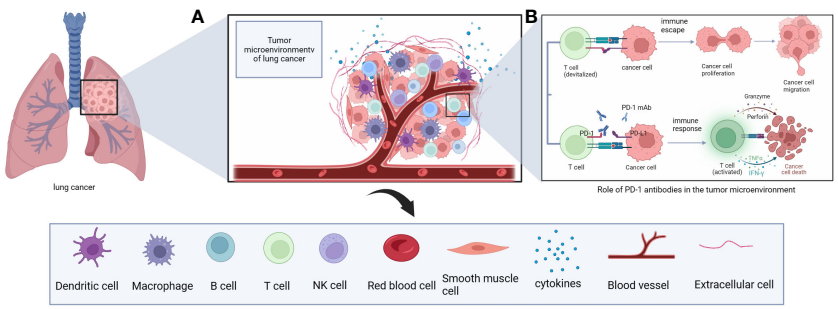

FIGURE 2
(A) The immune microenvironment of lung cancer includes immune cells such as T cells, B cells, NK cells, macrophages, DC cells and cytokines such as IL-2. **(B)** Principles of anti-tumor effects of PD-1 monoclonal antibodies in the immune microenvironment of lung cancer.

3 Low dose of chemotherapy drugs in combination with PD-1/L1 monoclonal antibody

More than 100 chemotherapeutic drugs have entered clinical use since 1948; they are divided into four main categories: 1) alkylating agents such as cyclophosphamide, cisplatin, and oxaliplatin; 2) antimetabolites such as pemetrexed, gemcitabine, and fluorouracil; 3) botanicals such as vincristine and paclitaxel; and 4) antibiotics such as doxorubicin and bleomycin. Chemotherapeutic drugs are thought to produce anti-proliferative or cytotoxic effects during cell division (40), selectively killing cells that are proliferating rapidly in the body. Thus, while killing tumor cells, bone marrow suppression may also occur with a decrease in neutrophils, lymphocytes, platelets and hemoglobin (41). In addition, adverse skin reactions to chemotherapy occur in 30-40% of patients (41). High doses of chemotherapy drugs can also cause significant liver and kidney damage and gastrointestinal complications, side effects that many patients do not tolerate well (42). Thus, chemotherapy combined with immunotherapy seems to be somewhat contradictory because chemotherapy can kill anti-tumor immune cells. To further improve the treatment efficacy in clinical treatment of lung cancer with a combination of chemotherapy drugs and PD-1 monoclonal antibody, the chemotherapy dose may be reduced to reduce the killing effect on immune cells (43).

Recent evidence indicates that some chemotherapy drugs at low doses have anti-angiogenic and sometimes even immunomodulatory effects (44). Several new studies have demonstrated that small doses of gemcitabine combined with cisplatin can not only cause immunogenic death of lung cancer tumor cells but can also directly activate NK cells and increase IFN-γ secretion, thus inhibiting tumor growth. The optimal antitumor outcome was observed in *in vivo* experiments when administering a low dose of gemcitabine (30 mg/kg) (45) (Table 1, Figure 3).

Conventional chemotherapy attempts to maximize efficacy and directly eradicate tumor cells using doses close to the maximum tolerated dose (MTD) and has been the standard of care in lung cancer treatment (51, 52). Even with the use of intensive chemotherapeutic agents, remission rates as well as survival remain poor for lung cancer patients. However, metronomic chemotherapy (MET) is dosed at one-tenth to one-third the MTD (52) and defined as (53) the rhythmic chemotherapy of low-dose cytotoxic drugs with short or no drug-free breaks over prolonged periods (51). MET have shown promising anti-angiogenic properties (53, 54), as well as anti-tumor immune activation, while limiting side effects (54–56). Zhong et al. conducted a study to investigate the effects of three different rhythmic chemotherapy regimens on tumor growth in C57BL mice. The researchers injected 10^6 cells into the right abdomen of the mice and after four days, administered different doses of cyclophosphamide for a period of three weeks. The three regimens used were MTD, which consisted of three doses of 150 mg/kg during the first week only; Met-1, which was 170 mg/kg given every six days; and Met-2, which was 25 mg/kg given every other day. The results of the study revealed that continuous administration of low-dose cyclophosphamide (Met-2) had a significant impact on the tumor microenvironment. Specifically, there was an increase in the number of $CD4^+$ and $CD8^+$ T cells, while the number of Tregs decreased. Low doses of cyclophosphamide also decreased the expression of transforming growth factor (TGF-β) receptor 2 by up-regulating the expression of E-calcineurin and down-regulating the expression of N-calcineurin (49) (Table 1, Figure 3). TGF-β can act as an immunosuppressive factor, and tumors with high expression of TGF-β can escape the surveillance of the immune system and inhibit expression of CD86 by TAMs. As CD86 is a tumor suppressor (57, 58), this implies that inhibition of TGF-β may enhance the antitumor immune response. In one study, co-administration of a Toll-like receptor 9 agonist and TGF-β2 inhibitor not only effectively exerted anti-tumor effects, but also led the TME to have T and NK cell enrichment and improved immunosuppression (59). In addition, sustained regular low-dose cyclophosphamide administration exerts anti-angiogenic effects by inhibiting the expression of VEGF, which can have a sustained tumor suppression effect and has the advantages of less toxic side effects and less drug resistance than conventional MTD administration. A prominent feature of this anti-angiogenic effect is tumor stabilization, not rapid tumor destruction (60).

In an *in vivo* study, rhythmical treatment with vincristine combined with Endo (Met NVB+Endo) gave better results than a maximum tolerated dose of vincristine combined with Endo (MTD NVB+Endo) in terms of anti-tumor responses, reduction of CD31, VEGF, HIF-1α and CEPS expression, as well as reduction of toxic side effects and induction of apoptosis. In this experiment, mice were administered vincristine at the MTD of 10 mg/kg, with the MET ranging from 1/10 to 1/3 of the maximum daily dose. In addition, the combination showed better antitumor effects than either drug alone (50). VEGF expression, detected by western blotting, indicated reduced VEGF expression levels in the Met NVB+Endo group and MTD NVB+Endo group (Table 1, Figure 3). The potent antitumor effect of MET in combination with anti-angiogenic drugs through enhanced inhibition of tumor-associated angiogenesis is consistent with previous findings (50, 61). Despite the observed positive outcomes in terms of tumor control and reduced side effects, conclusive Phase III trial results are yet to be established. Moreover, patient drug dosage and dosing intervals are currently determined empirically, and inter-individual variances necessitate a criterion for patient categorization (62).

Low-dose chemotherapy drugs combined with ICIs have a synergistic effect in the treatment of tumors (44). Li Zhou et al. (42) showed carboplatin activated the STING/TBK1/IRF3 signaling pathway and the STING-NF-κB signaling pathway, then experimentally verified that low dose of carboplatin could increase PD-L1 expression in lung cancer cells. In addition, a low dose (20 mg/kg) of carboplatin also increased the infiltration of cytotoxic $CD8^+$ T cells and the secretion of the chemokines CXCL10 and CCL5 compared to a high dose (75 mg/kg) (42) (Table 1, Figure 3). Low-dose carboplatin in combination with PD-1 monoclonal antibody significantly improved the anti-tumor effect

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TABLE 1 Modulation mechanisms of the anti-tumor immune effect induced by low-dose chemotherapy.

Drugs	Medication regimen	Adjusted ratio of chemotherapeutic agents (low / standard dose)	Immune cells impacted	Signaling pathway or target	Cytokines	References
Gemcitabine combined with cisplatin in small doses	low-dose (30 mg/kg) gemcitabine	25%	NK cell		IFN-γ, HMGB1	(45)
Low-dose carboplatin	20 mg/kg	26.7%	CD8+ T cell	STING/ TBK1/IRF3 STING-NF-κB	CXCL10, CCL5	(42)
Sub-lethal dose of pemetrexed/5-FU	100nM	35.7%	TIL	TS-ROS-NF-κB-PD-L1	IL-2	(46)
Low-dose gemcitabine with SRA737+ anti-PD-L1	gemcitabine (40 mg/kg, 1/7, first day of the week), SRA737+ (100 mg/kg, 2/7, first and second day of the week) and anti-PD-L1 (300 μg, 1/7, third day of the week)	33.3%	CD8+ T cell, DC, M1 macrophage, M2 macrophage, MDSC	STING/ TBK1/IRF3	Type 1 IFN, IFN-β, CCL5 and CXCL10	(47)
Low-dose high-density DTX or PTX	DTX (11 mg/kg) or PTX (11 mg/kg)	33.3%	APC, T cell	PI3K/AKT/ NF-κB	HMGB-1	(48)
Small doses of cyclophosphamide	25 mg/kg every other day	55.6%	CD4+T cell, CD8+ T cell, Treg		TGF-β	(49)
Rhythm Vincristine combined with Endo	1/10-1/3 of the maximum daily dose	10%~33.3%		HIF-1α and CEPS	CD31, VEGF	(50)

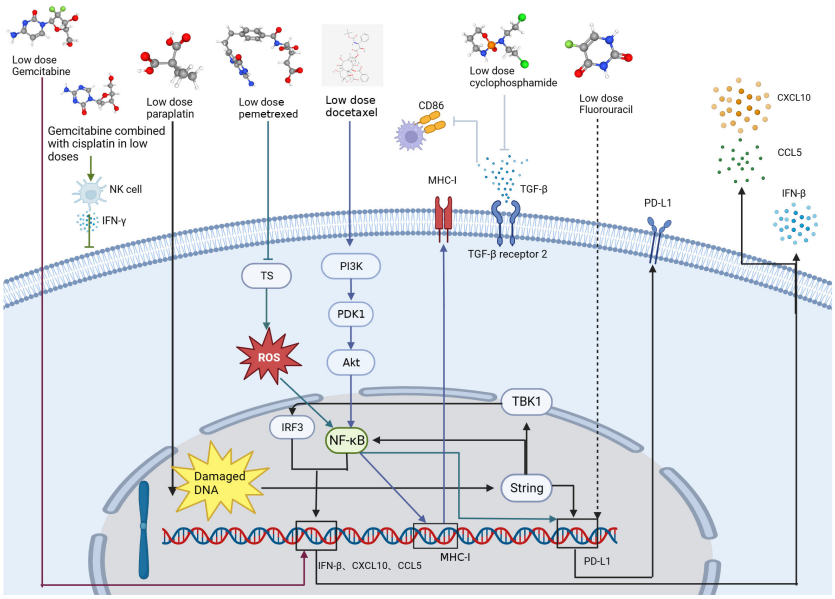

FIGURE 3
Mechanisms by which low-dose chemotherapeutic agents (gemcitabine, decitabine, carboplatin, pemetrexed, doxorubicin, cyclophosphamide, and fluorouracil) act on tumor cells and enhance antitumor immune function. The arrows of each color represent the pathway by which each drug acts on the lung cancer.

compared to both PD-1 monoclonal antibody alone and carboplatin alone without significant toxic side effects (42).

Lu et al. (46) found that sublethal doses of pemetrexed (100nM) and 5-fluorouracil (5-FU) could upregulate PD-L1 expression and regulate TIL activity in NSCLC cells, and found that pemetrexed or 5-FU elevated PD-L1 protein levels in a dose-dependent manner. *In vivo* experiments, the combination of pemetrexed (100 mg/kg) with a PD-1/PD-L1 blocker (3 mg/kg) further enhanced the antitumor immune response. The antimetabolite pemetrexed induced PD-L1 upregulation by activating the ROS-NF-κB signaling pathway through inactivation of thymidylate synthase and thus in combination with PD-1 monoclonal antibody activation of $CD4^+$ T cells and $CD8^+$ T cells provides a more favorable immune microenvironment for tumor growth inhibition (46) (Table 1, Figure 3).

Sen et al. found that low-dose gemcitabine (40 mg/kg, first day of the week) in combination with SRA737+ (100 mg/kg, first and second day of the week) and anti-PD-L1 (300 μg, third day of the week) combination therapy in the treatment of tumor-bearing mice had significantly better antitumor effects than single-agent or two-by-two dosing regimens. This triple therapy increased T-cell infiltration, decreased T-cell depletion, and significantly increased antigen-presenting cell subpopulations. This was demonstrated by a significant increase in the number of $CD8^+$ cytotoxic T cells, dendritic cells, and M1 macrophages and a significant decrease in the number of immunosuppressive M2 macrophages and MDSC. Triple therapy also increased the expression of the type 1 interferon gene, IFN-β, and the chemokines CCL5 and CXCL10 (47) (Table 1, Figure 3).

He et al. demonstrated that low-dose high-density DTX (11 mg/kg) or PTX (11 mg/kg) indirectly activated the killing effect of T cells on tumor cells by activating the PI3K/AKT/NF-κB signaling pathway, promoting the exposure of antigen on the surface of the tumor cells, and further activating the antigen-presenting function of antigen-presenting cells. Combined with PD-1/PD-L1 monoclonal antibody, it can increase the expression of type 1 macrophages, dendritic cells (DCs) and cytotoxic $CD8^+$ T cells (48) (Table 1, Figure 3).

In summary, low-dose chemotherapy drugs have vascular and immunomodulatory effects, and their combined application with ICIs has a synergistic effect. One of the important tasks ahead is to conduct more research to further determine the optimal dose, frequency, and sequence of chemotherapy drugs that achieve the best antitumor efficacy with ICIs.

4 Targeted drugs and lung cancer

Current targeted therapy for lung cancer includes the targets VEGF, EGFR, ALK, ROS1, MET, BRAF, NTRK, RET, and RAF (63). As research continues, many other oncogenic drivers, such as HER2 exon 20 insertion mutations are being identified, and the efficacy data of targeted therapies are constantly being updated (64). Targeted therapies are undoubtedly a milestone in the development of cancer therapy. They play a key role in early disease detection and increase our understanding of tumor evolution and treatment resistance. Targeted therapies represent one of the future directions of precision oncology approaches.

4.1 Current status of anti-angiogenic drugs for lung cancer

Angiogenesis involves several growth factors (65), among which the VEGF family consists of VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (66). VEGF-A is a major regulator of angiogenesis and is closely associated with angiogenesis in NSCLC, and VEGF receptor (VEGFR)-1 and VEGFR-2 are both receptors for VEGF-A (66, 67). VEGFR-1 binds VEGF-A with a higher affinity than VEGFR-2 (68). Anti-angiogenic drugs normalize local blood vessels (69) and can be divided into four categories: anti-VEGF monoclonal antibodies (mAb), anti-VEGFR mAb, induced VEGF-trap receptors, and VEGFR tyrosine kinase inhibitors (TKIs). In addition, endothelial inhibitors inhibit endothelial cell proliferation by inhibiting a series of angiogenic factors, such as recombinant human vascular endothelial inhibitor (Endo) (66, 69).

Since the approval of the first anti-angiogenic drug, bevacizumab, for the treatment of NSCLC, anti-angiogenic therapy has become a popular strategy for the treatment of advanced NSCLC (66). Bevacizumab is a recombinant humanized monoclonal antibody against VEGF-A, and patients treated with bevacizumab have improved OS. However, the addition of bevacizumab leads to increased toxicity, particularly neutropenia, thrombosis, hypertension, proteinuria, and bleeding events (15). Bevacizumab is not approved for the treatment of NSCLC due to the high risk of bleeding reported in early trials. This risk is associated with the central site of the disease, which often infiltrates the large mediastinal vessels (70). Ramucirumab, a recombinant human IgG1 monoclonal antibody targeting VEGFR-2, also blocks the activation of VEGFR-2 by ligands other than VEGF-A compared to bevacizumab (10, 15, 61, 71). Combination therapy with ramucirumab significantly improves progression free survival and overall survival, but adverse effects are also common (72).

Anti-angiogenic TKIs target VEGFR1-3 in addition to a variety of other kinases (15). The most common, anlotinib, was approved by the National Drug Administration on 8 May 2018 and 30 August 2019 for third-line treatment in patients with advanced NSCLC and SCLC, respectively (73). Despite the wide range of targets of TKIs, most TKIs appear to be only weakly effective in the treatment of NSCLC. Several clinical trials are investigating whether anti-angiogenic drugs can stimulate immunity, improve immunosuppression and thereby enhance antitumor immunity. New therapeutic targets including

metabolic intermediates of vascular endothelial cells and cellular components of the TME may lead to the discovery of new novel targets beyond the VEGF family (28).

4.2 Immunomodulatory effects of anti-angiogenic drugs

Clinical studies have shown that VEGF can affect immune cells (74, 75), including inhibiting the differentiation of thymic hematopoietic progenitors to $CD8^+$ and $CD4^+$ T cells, suppressing the proliferation cytotoxic activity of effector T cells through binding to VEGFR2, reducing the activity of natural killer cells, increasing Tregs and MDSC in the TME (68, 76) and up-regulating a variety of immune checkpoints, such as PD-L1, CTLA-4, TIGIT, TIM-3 and LAG-3 (77, 78) (Figure 4).VEGF blockade has been shown to reduce VEGF-mediated inhibition of DC maturation (79) (Figure 4),which can be reversed by anti-angiogenic drugs targeting VEGF-A-VEGFR. Given these results, the association of anti-angiogenic molecules with immunomodulatory agents with suppressive checkpoints may be of particular interest in VEGF-A producing tumors. The combination of bevacizumab and atezolizumab reverses the immunosuppression produced by VEGF and lifts PD-L1-mediated immunosuppression (80). In experimental studies, anti-angiogenic agents bevacizumab and sorafenib (polytyrosine kinase VEGFR2 inhibitors) reversed VEGF-mediated inhibition of monocyte differentiation to DCs *in vitro* (81).

In a study involving experimental animals with lung adenocarcinoma, a reduction in the infiltration of $CD8^+$ T cells into tumors was observed in the anti-VEGFR2 antibody (DC101) group receiving a low-dose (10 mg/kg). However, there was no notable variation in the percentage of T cells that underwent *in vitro* treatment with a combination of medium-dose (20 mg/kg) and high-dose (40 mg/kg) DC101 and anti-PD1 antibody. Combining low-dose anti-VEGFR2 antibody with anti-PD1 antibody treatment resulted in a delay in tumor growth and extended the survival time of mice afflicted with tumors (82).VEGF-A upregulates both LAYN and immune receptors in human $CD8^+$ T cells such as TIGIT (82). LAYN is a key gene in the regulation of immunity, and a bioinformatics analysis showed that LAYN is associated with prognosis and the level of immune infiltration of $CD8^+$ T cells, $CD4^+$ T cells, macrophages, neutrophils, and DCs in patients with several cancers, especially colon and gastric cancers. In addition, LAYN expression may contribute to the regulation of TAMs, DCs, T-cells, and Tregs in colon and gastric cancers (83). However, no experiments have been performed to verify this conclusion. In a human tissue lymphocyte transcriptional atlas, analyzing RNA-SEQ data from colorectal and NSCLC tumors along with normal colon and lung samples, high expression of Tregs' cellular signature genes, such as LAYN, MAGEH1 and CCR8, in whole tumor samples was associated with poor prognosis. This finding highlights the specific expression pattern of immune checkpoints and their ligands in tumor-infiltrating Tregs and effector cells and suggests that their functional relevance should be studied directly at the tumor site (84). PD-1 combined with its ligand PD-L1 allows tumor cells to escape recognition by T cells, achieving immune escape and exerting immunosuppressive effects; the combination of LAYN and its ligand has the potential to stimulate Tregs and further suppress effector T cells. Bioinformatic analysis of data is a resource that can generate and validate hypotheses to increase our understanding of tumor-infiltrating Tregs biology and identify immune targets (84).

4.3 Anti-angiogenic drugs combined with chemotherapy and immunotherapy exert powerful anti-tumor effects in lung cancer

In NSCLC, VEGF-A is overexpressed, and the progression of NSCLC is closely associated with angiogenesis. The larger the tumor

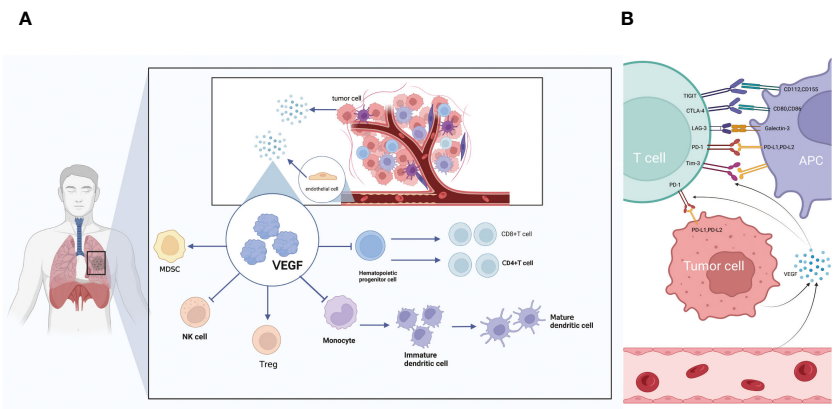

FIGURE 4
(A) The relationship between VEGF and immune cell action. VEGF secreted by tumor cells and endothelial cells can promote immunosuppressive MDSC and Treg cells. Inhibit the differentiation of monocytes into mature DC, inhibit the differentiation of T cells and inhibit NK cells. **(B)** The relationship between VEGF and immune checkpoints. VEGF secreted by tumor cells and endothelial cells can promote multiple immune checkpoints including TIGIT, PD-L1, LAG-3, CTLA-4 and TIM-3.

size of NSCLC or the more advanced the tumor, the more likely it is to undergo excessive, abnormal angiogenesis (66). In one study, angiogenesis was found to be more abundant in lung squamous carcinoma than in lung adenocarcinoma (85). In addition, high levels of circulating VEGF-A are associated with poor prognosis in NSCLC, so using circulating levels of VEGF-A expression to predict patient prognosis may be useful (86). Chinnasamy et al. developed anti-VEGFR2 CAR T cells in a mouse model in which T cells cotransduced with anti-VEGFR-2 CAR and IL-12 infiltrated, expanded and were maintained in tumor tissues for longer. The altered immunosuppression of the TME by anti-VEGFR-2 CAR can lead to effective tumor regression. This is effective in mice, but further efficacy and safety assessments are needed for humans (87).

Chemotherapy combined with anti-angiogenesis drugs such as bevacizumab is more effective against NSCLC than chemotherapy alone. In one study (88), median survival in the chemotherapy plus bevacizumab group for NSCLC was 12.3 months compared with 10.3 months in the chemotherapy alone group. Median progression free survival was 6.2 and 4.5 months for the chemotherapy plus bevacizumab group and chemotherapy alone group, respectively, and the effective rates were 35% and 15%, respectively. Clinically significant bleeding rates were 4.4% and 0.7%, respectively. There were 15 treatment-related deaths in the chemotherapy plus bevacizumab group, including 5 deaths from pulmonary hemorrhage (88). Thus, bevacizumab combined with chemotherapy can significantly improve PFS and effectiveness compared to chemotherapy alone, but the former has an increased rate of bleeding.

In a subsequent study, the dosage and duration of drug use was adjusted to see if side effects, such as bleeding could be reduced while retaining effectiveness. The toxicity, biology and antitumor activity of the rhythmic chemotherapy combined with the bevacizumab regimen were investigated by treating a group of 114 patients, 86 of whom were treated with split-dose cisplatin and oral etoposide plus bevacizumab; 28 patients were treated with split-dose cisplatin and oral etoposide. These patients had no significant toxicity or delay associated with toxicity during the chemotherapy course, and no toxic deaths, bleeding, or serious infections occurred (70). Rhythmic chemotherapy is an emerging approach to the treatment of cancer patients based on the long-term use of low-dose cytotoxic drugs (89). This approach allows higher dose intensities of cytotoxic drugs to be achieved compared to conventional chemotherapy regimens, avoiding dangerous blood concentration spikes (90). The reduction of VEGF and IL-17A levels in the tumor tissue of the 86 patients in the combined treatment group was paralleled by an increase in the percentage of peripheral blood central memory T cells, activated $CD62L^+$ cytotoxic T cells and the expansion of activated myeloid-derived DCs expressing CD83 and CD80, activating the immune system; this result was ascribed as partly related to the rhythmic method of chemotherapy administration and partly to the maintenance dosing of bevacizumab (70). This suggests changes in the dosage of chemotherapeutic agents can lift immunosuppression and in combination with anti-angiogenic agents can further activate the immune system. Thus, it is hypothesize that adding immunotherapy on this basis for chemotherapy plus anti-angiogenic agents may maximize the effect of each treatment modality and simultaneously circumvent the side effects due to single drug dose requirements, allowing for long-term treatment to improve survival and quality of life.

Chemotherapy aims to inhibit the over-proliferation of cancer cells but may not effectively control two of the most important conditions in the tumor-permissive environment: neo-angiogenesis and tolerogenic immunity. This conjecture was tested in a prospective randomized trial that included patients with advanced, unresectable pancreatic, non-small cell lung, or prostate cancer (91). One group of patients was given standard chemotherapy and served as a control group; the other group was treated with chemotherapy plus an anti-angiogenic and anti-tumor immune-inducing regimen. The latter group had significantly longer survival, lower blood levels of neovascularization and immune tolerance mediators, and higher levels of anti-angiogenic and anti-tumor immune mediators than the control group (91). The anti-angiogenic effect was monitored by detecting VEGF and vasopressor levels, and the anti-tumor immunomodulation was determined by assessing the number and presence of Tregs and DCs. Several antitumor immune induction regimens are possible, including low-dose rhythm cyclophosphamide, high-dose COX-2 inhibitors, granulocyte colony-stimulating factor, sulfhydryl donors, and blood derivatives containing autologous tumor antigens released into the blood from the patient's tumor. Whether this antitumor immune induction regimen is equally or more effective if replaced with low-dose rhythm chemotherapy plus ICI or immune inducer plus ICI requires additional study. In an *in vivo* trial, triple combination therapy, i.e., radiation combined with PD-L1 monoclonal antibody and anlotinib, was used to improve the tumor microenvironment and to counteract the immunosuppressive effects of radiation on the tumor microenvironment in Lewis lung cancer mice. Compared with radiation-combined immunotherapy, anlotinib was able to promote infiltration of $CD8^+$ T cells and M1-type macrophages and reduce the number of MDSCs and M2 macrophages. In addition, IFN-γ and IL-18 levels were higher, while IL-23, IL-13, IL-1β, IL-2, IL-6, and IL-10 levels were significantly lower. Triple combination therapy also promoted the anti-tumor effects of radioimmunotherapy by downregulating the expression of NF-κB, MAPK and AKT signaling pathways (92) (Table 2). The anti-angiogenic and immune-activating effects of anlotinib provide a strong theoretical basis for the clinical treatment of lung cancer (92).

5 Current status of other targeted drugs for lung cancer and immunomodulatory effects of low doses on lung cancer

With the identification of alterations in the targeted oncogene, advanced lung cancer can be treated with greater precision (64). Targeted agents are increasingly available as a first-line choice of lung cancer treatment and have improved prognosis and reduced toxicity compared to chemotherapy (97). EGFR mutations and ALK fusions are the most common targeted alterations (98, 99). In the analysis of specific cell populations, different TME modifications

TABLE 2 Regulatory mechanisms of anti-tumor immune effects induced by different targeted drugs.

Drug	immune cells impacted	Cytokines	Signaling pathway or target	References
Bevacizumab	DC, CTL Treg	IL-17	PD-L1, TIGIT, LAG-3, TIM-3, CTLA-4	(70, 77, 78)
Anlotinib	CD8+ T cells, M1 macrophages, MDSCs, M2 macrophages	IFN-γ, IL-18, IL-23, IL-13, IL-1β, IL-2, IL-6, IL-10	NF-κB, MAPK and AKT	(92)
EGFR-TKI	CD8+ T cells, DC FOXP3+ Tregs M2 macrophages	IFN-γ, IL-10	STAT3 pathway, PD-L1	(29, 93)
ALK-TKI DNA-PK inhibitors	T cells CD8+ T cell	IFN-γ TGFβ	PD-L1 PD-L1	(94, 95) (96)

were detected in EGFR-positive and ALK-positive tumors compared to EGFR/ALK-negative tumors: TME in EGFR-positive tumors had decreased numbers of $CD8^+$ T cells; TME in ALK-positive cases had increased numbers of Tregs. This suggests that in the development of lung cancer different immune cell responses occur (98, 100). In addition, targeted oncogenic alterations were also associated with PD-L1 expression, and upregulated by activation of MAPK, PI3K-AKT-mTOR and JAK-STAT3 signaling pathways in NSCLC cells with altered KRAS, EGFR and ALK activating genes or PD-L1 expression (19, 101–106).

5.1 EGFR

EGFR is one of the most common mutation driver genes and is considered an oncogenic factor (107). As a representative of precision medicine, EGFR-TKI therapy significantly alleviates the development of activating mutant EGFR-driven NSCLC (108). EGFR-TKI drugs include erlotinib, afatinib, gefitinib, and osimertinib (63). Madeddu et al. (29) found that EGFR-TKI enhanced MHC class I and class II antigen presentation in response to IFN-γ, increased $CD8^+$ T cell and DC levels, eliminated $FOXP3^+$ Tregs, inhibited proliferation and differentiation of M2 macrophages, and decreased PD-L1 expression. In another clinical trial of EGFR-TKI combined with ICI therapy, the use of an EGFR-TKI afatinib inhibited $CD8^+$ T cell proliferation and a time-related modulation of $CD8^+$ T cell proliferation was found in NSCLC patients who received afatinib-targeted therapy for up to 48 weeks during treatment. In the early phase of treatment, afatinib inhibited $CD8^+$ T cell proliferation, and in the late phase of treatment, $CD8^+$ T cells responded adaptively to afatinib treatment (93). In contrast, the results of several clinical trials of EGFR-TKI combination immunotherapy showed no additional benefit in the treatment of lung cancer (69, 72, 97, 109). The different results of these studies may be related to the different types, specificity and doses used of the EGFR-TKI drugs.

5.2 ALK

Immunogenic cell death (ICD) was originally discovered in the context of chemotherapy, but only a small fraction of chemotherapeutic agents can trigger ICD, which is related to their clinical long-term efficacy against cancer and their ability to inhibit DNA-to-RNA transcription (94). In one study, small doses of ALK inhibitors, crizotinib (≤5 μM) and ceritinib, induced ICD when ALK was activated due to chromosomal translocations, suggesting a targeting effect to promote ICD (94) (Table 2). In a co-culture system of tumor cells and DC-cytokine-induced killer cells, PD-L1 expression in NSCLC cell lines was associated with EGFR mutations and ALK fusion genes, and ALK fusion protein overexpression increased PD-L1 expression (80). In contrast, Mu et al. (95) found that ALK fusion proteins downregulated PD-L1 expression (Table 2). No synergistic effect of the combination of ALK-TKI and PD-1 monoclonal antibody against tumor cells was observed using *in vivo* experiments. One possible explanation is that in ALK-positive NSCLC, ALK-TKI may have a similar role in disrupting PD-1/PD-L1 interactions as anti-PD-1 antibodies, but no additional role. The trial was conducted at the cellular level only, and further *in vivo* experiments are needed to explore the results more accurately. However, the use of crizotinib in combination with cisplatin, followed by PD-1 monoclonal antibody, not only induced ICD but also greatly improved the cure rate in TC1 lung cancer mice (110). There are few studies on ALK-TKI combined with immunotherapy, and results of the studies available to date are not yet convincing.

5.3 DNA-dependent protein kinase

Radiation therapy is commonly used in the treatment of lung cancer, but the development of radiation combination therapy is still limited (111, 112). The anti-tumor activity of radiation therapy is mainly derived from the production of double-strand breaks (DSB) in DNA, which, if not repaired, can induce cancer cell death through a variety of mechanisms. Therefore, targeting DSB repair mechanisms in tumors might optimize the effect of radiotherapy. Peposertib (also known as M3814) is a potent and selective DNA-PK inhibitor that effectively inhibits the repair of radiation-induced DNA DSBs, which largely enhances the efficacy of radiotherapy (96). In addition, DNA-PK inhibitor substantially enhanced PD-L1 expression in irradiated cancer cells, providing a clear rationale for combination with PD-L1 targeted immunotherapy (96). Given its

critical function, DNA-PK has been targeted in cancer therapy in concert with DNA-damaging agents (113, 114). In addition, DNA-PK inhibitors significantly enhanced the secretion of TGF-β in the tumor microenvironment and the expression of PD-L1 in irradiated cancer cells, providing a theoretical basis for the combination of DNA-PK inhibitors with immunotherapy. Moreover, the addition of M3814 resulted in a significant enhancement of activity in the less immunogenic B16F10 and immune-excluded 4T1 mouse models, a result that was correlated with increased $CD8^+$ T cell infiltration in tumors in addition to increased TGF-β secretion as well as PD-L1 expression. In addition to melanoma as well as breast cancer, in lung cancer it has been shown that M3814 alone or in combination with cisplatin enhances the efficacy of anti-PD-L1 monoclonal antibodies (115). However, the clinical use of DNA-PK inhibitors has not been well studied, and there are some unresolved issues, such as short serum half-life due to metabolic instability and unclear optimal doses for combination with immunotherapy.

6 Discussion

Immunotherapy is a complex and intriguing area of cancer research (116). How to optimize it is currently a hot topic in cancer research, including how to further improve response rates, expand the population that can benefit, and reduce the incidence of treatment-related adverse events (92). Increasingly, studies indicate that reducing the dose of chemotherapy and adjusting the dosing regimen can not only lead to better anti-tumor effects but can also reduce drug toxicities and regulate the immune microenvironment by modifying the number of immune cells and cytokines to further achieve anti-tumor immunity. Chemotherapeutic drugs can upregulate PD-L1 expression to create favorable conditions for combining PD-1/PD-L1 monoclonal antibodies and allow low-dose chemotherapy combined with immunotherapy to work better. Currently, low-dose chemotherapy combined with immunotherapy has been validated in both *in vitro* and *in vivo* experiments, but clinical studies are limited to date, and more research is needed. Similarly, reducing the dose of anti-angiogenic drugs and adjusting the dosing regimen can improve the anti-tumor effect and activate immunity, which has also been demonstrated in some animal studies. Low-dose anti-angiogenic agents are able to modulate multiple immune checkpoints other than PD-L1, such as TIGIT and LAYN, but all lack substantial experimental data to support this. In a clinical study, the trinity of low-dose chemotherapy plus anti-angiogenic plus immune inducer was found to be more effective than the combination of any two drugs or a single drug (91). For targeting oncogenes, the most common are EGFR mutations and ALK fusions. Studies have shown that normal doses or high doses of EGFR-TKI and ALK-TKI can alter the immune microenvironment in lung cancer, but whether immune activation or immune suppression occurs needs to be further explored.

In conclusion, for the long-term treatment of lung cancer patients, an appropriate dose and targeted combination and dosing regimen based on individual patient differences and tolerance to the drug that provides the best combination strategy to expand anti-PD-1/PD-L1-based immunotherapy can greatly improve the prognosis and quality of life for a patient.

Author contributions

LW: Writing – original draft, Writing – review & editing. ZW: Writing – review & editing. LC: Writing – review & editing. CD: Writing – review & editing BJ: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Relapsed refractory multiple myeloma with CNS involvement successfully treated with Elranatamab: first reported case

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Central nervous system (CNS) involvement in multiple myeloma (MM) is a rare and challenging complication associated with poor prognosis and limited treatment options. Emerging T-cell directing therapies, such as bispecific antibodies (bsAbs) and chimeric antigen receptor T cells (CAR-T), have shown remarkable success in treating MM, but their efficacy in CNS involvement remains unclear. Elranatamab, a humanized bispecific antibody targeting B-cell maturation antigen (BCMA) and CD3-expressing T cells, has demonstrated promising results in relapsed refractory MM. However, its efficacy in treating CNS-MM has not been reported. We present a case of a 37-year-old male MM patient with CNS involvement who has been successfully treated with Elranatamab.

KEYWORDS

Elranatamab, multiple myeloma, bispecific Ab, BCMA, CNS involvement

Introduction

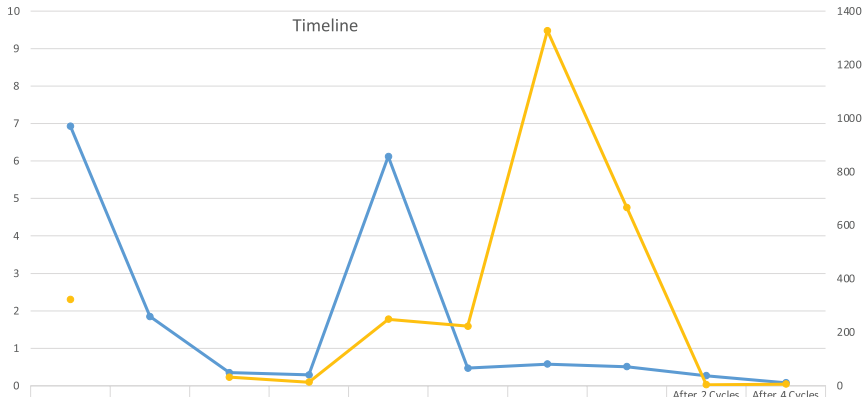
Emerging T-cell directing therapies, such as bispecific antibodies (bsAbs) and chimeric antigen receptor T cells (CAR-T), have demonstrated remarkable responses and outcomes in extensively treated and treatment-resistant patients (1). Despite significant advances in the treatment of MM, central nervous system (CNS) involvement remains a challenging and rare complication that can lead to severe neurological symptoms and impact patient outcomes. Elranatamab is a humanized bispecific antibody that targets both B-cell maturation antigen (BCMA)-expressing multiple myeloma (MM) cells and CD3-expressing T cells (2). Elranatamab has shown a promising overall response rate of 70% in heavily pretreated myeloma patients. However, there is currently limited or no available data regarding its use and efficacy specifically for CNS involvement in multiple myeloma. Here we report early and effective use of Elranatamab for relapsed refractory Multiple Myeloma patient with CNS involvement.

Case description

A 37-year-old male patient presented with a sudden onset back pain and fatigue. Laboratory test results revealed normochromic normocytic anemia, elevated total protein, and an increased serum creatinine level indicating renal failure. The diagnosis of IgG lambda Multiple Myeloma ISS III and R-ISS II was confirmed with an increased number of plasma cells in the bone marrow aspiration and biopsy. Cytogenetic analysis showed 46 XY karyotype with no additional myeloma specific molecular abnormalities including 17 p deletion, translocation t(11,14), t(14,16), t(4,14) and amp/gain 1q. 30 gene next generation sequences analyses of bone marrow at the time of diagnosis showed KRAS mutation with VAF (variant allele frequency) 37.2% and CALR mutation 48% VAF. PET CT (Positron Emission tomography) scan showed multiple lytic lesions and spinal bone-derived plasmacytomas. Bortezomib, Cyclophosphamide, Dexamethasone (VCD) was initiated as a first-line treatment, and after the normalization of renal functions, the patient proceeded with Bortezomib, Lenalidomide and Dexamethasone (VRD). Very good partial response (VGPR) response according to M protein level was reached after 4 cycles of induction therapy however, PET CT scan showed persistent Fluorodeoxyglucose (FDG) avid solitary lesion paravertebrally located in the spinal cord. 3-Gray (Gy) involved-field radiation therapy (IFRT) was applied before preceding to transplantation. Although maintenance therapy initiated after the stem cell transplantation because of the high-risk features such as extramedullary nature of the disease, patient was in VGPR only 6 months. Due to increased number of extramedullary lesions and increased FDG uptake while on maintenance therapy Carfilzomib, Cyclophosphamide and Dexamethasone (KCd) was started and after 2 cycles of therapy patient underwent second autologous transplantation due to prolonged cytopenia. Cytopenia was resolved after transplantation but no improvement observed regarding to disease. Patient was on KCd as a consolidation therapy after the transplant. PET-CT, which was obtained in three months after transplantation revealed increased lytic lesions and bone derived plasmacytomas. Due to extra medullary predominant nature of the disease, Proteasome inhibitor combined chemotherapy regimen, Carfilzomib plus RD-PACE initiated. After two cycles of therapy patient progressed with new plasmacytomas. Daratumumab, Bortezomib, Dexamethasone combination therapy started. After 3 cycles of therapy PET-CT revealed progressive bone lesions. Patient presented with newly onset diplopia, headache, and eye movement abnormalities. Diagnostic lumbar puncture and cranial Magnetic resonance imaging (MRI) were performed in order to exclude Multiple Myeloma involvement in CNS. Cranial MRI did not show any myeloma related cranial lesions or leptomeningeal findings but flow-cytometric analyses of cerebrospinal fluid showed increased clonal CD138 positive plasma cells. CNS involvement was confirmed and weekly Elranatamab 76 mg subcutaneous started by compassionate use of the drug. Treatment schema and response assessment of the patient are detailed in Figure 1. Daratumumab continued as scheduled two weeks apart and Dexamethasone given 20 mg weekly. Grade 2 cytokine release syndrome (CRS) fever with low flow oxygen need were required at day 3. One dose of Tocilizumab therapy initiated. No other adverse events observed including ICAN (Immune Effector Cellular Therapy Associated Neurotoxicity), after two cycles of Elranatamab, CNS findings showed significantly increased clonal plasma cells (Figure 2). Neurological symptoms regressed. PET CT scan showed complete remission after 4 cycles of therapy (Figure 3). Patient is still in remission and has been following up since April 2023.

Discussion

Soft-tissue plasmacytomas indicate an aggressive form of MM, characterized by autonomous growth of a clone and/or sub clone independent of the bone marrow microenvironment. This condition is associated with high-risk genetic features, increased proliferation, resistance to apoptosis, and treatment resistance (3,



	Time of Diagnosis	After 2 Cycles of VCd	After 2 Cycles of VRd	After First ASCT	After VR Maintenance	After KCd and 2nd ASCT	After K-RD-PACE and KCd Consolidation	After DVd	After 2 Cycles of Elranatamab plus Daratumumab	After 4 Cycles of Elranatamab plus Daratumumab
Serum M Protein (g/dL)	6.93	1.85	0.35	0.29	6.12	0.47	0.58	0.51	0.27	0.08
Serum Free Kappa Light Chain (mg/L)	7.2		9.2	8.9	8.2	1.2	0.7	0.7	0.7	0.7
Serum Free Lambda Light Chain (mg/L)	2320		294	119	2040	267	929	466	2.6	4.3
Serum Free Lambda/Kappa Ratio	322.222		31.957	13.371	248.780	222.500	1327.143	665.714	3.714	6.143

FIGURE 1
Timeline of patient treatment schema.

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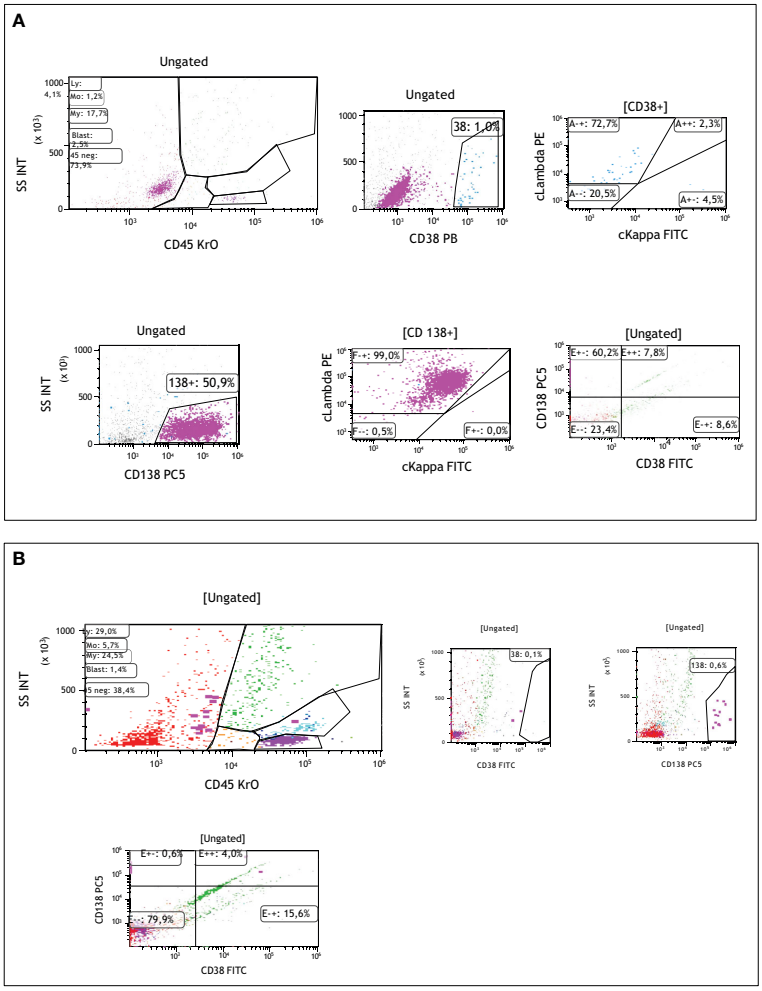

FIGURE 2
(A) Flowcytometric analyses of CNS fluid at the time of diagnosis showing CD138 positive lambda clonal plasma cells and decreased CD38 positivity after Daratumumab therapy. **(B)** Flowcytometric analyses of CNS fluid after 2 cycles of Elranatamab showing decreased CD138 positive lambda clonal plasma cells.

4). Although CNS involvement is extremely rare, the prognosis is even more dismal than extramedullary disease (EMD) in other locations, particularly with leptomeningeal involvement. A multicenter retrospective cohort study investigating CNS involvement in Multiple Myeloma reported a median overall survival of 7 months from the time of CNS involvement (5). In this study, untreated and treated patients had median OS of 2 and 8 months, respectively. While there is no standard of care treatment for CNS involvement in MM, systemic treatment alone or in combination with either intrathecal chemotherapy or radiotherapy has shown a significant improvement in survival when compared to no systemic treatment (5).

Optimal therapy for CNS involvement in MM is not very well established due to small numbers of reported patients with CNS involvement and heterogeneity of their treatments. Traditional approaches such as IT chemotherapy and radiation therapy can lead to dismal survival of 1-2 months (6). The efficacy of new drugs in CNS involvement has been documented, but their full potential remains uncertain. Clinical studies for CNS-MM treatment are scarce, making it a challenging area to address. One of the reasons for this difficulty is the presence of the blood-brain barrier (BBB), which acts as a natural defense, restricting the entry of numerous drugs into the central nervous system (7). The dilemma is whether the BBB is intact and acts as a barrier for drugs but when increased vascular permeability with in the tumor happens it causes transferability or some molecules are able to cross the intact BBB (8). There is limited data on MM agents' transferability to CSF and their effectivity. A literature review on the cerebrospinal fluid (CSF) transferability of drugs for MM showed that IMIDs and Daratumumab can cross the BBB (9–11). No data are available about other monoclonal antibodies such as Isatuximab and Elotuzumab (12). Previous trials have demonstrated that BCMA CAR-T cells are associated with manageable toxicity and remarkable effectiveness in patients with relapsed/refractory multiple myeloma (R/R MM) (13). However, their potential and suitability for CNS MM treatment have not been determined yet. Yiyun et al. reported 4 CNS MM cases who had been treated with BCMA CART cell therapy and they identified the presence of BCMA CAR-T cells in CSF, and found that BCMA CAR-T cells are safe and effective in treating CNS MM, but the duration of

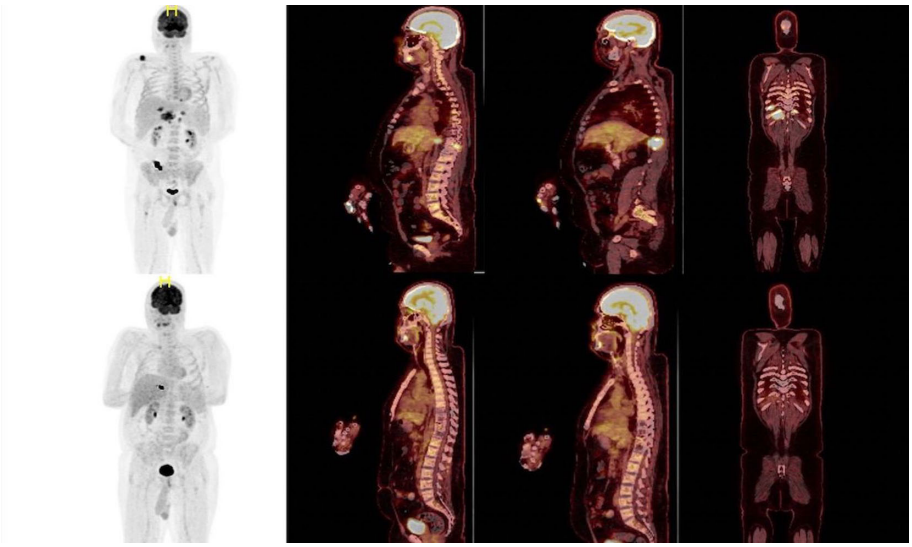

FIGURE 3
PET CT images are showing a complete remission before (on the top) and after (bottom) Elranatamab therapy.

remission may demand improvement (14). Elranatamab is one of the promising bispecific antibodies and has been shown promising results in the setting of relapsed refractory MM, however its effectiveness in CNS involvement remains unclear.

Conclusion

Elranatamab is one of the promising bispecific antibodies and has been shown promising results in the setting of relapsed refractory MM, however its effectiveness in CNS involvement remains unclear. This is the first CNS-MM case who had been treated with Elranatamab successfully.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The patients provided a written informed consent to participate in this study. Written informed consent was obtained from the patient for the publication of any potentially identifiable images or data included in this article. Written informed consent was obtained from the participant/patient(s) for the publication of this case report.

Author contributions

YM: Writing – original draft. SY: Writing – review & editing. SM: Writing – review & editing. EM: Writing – review & editing. ZB: Writing – review & editing. LK: Writing – review & editing. OS: Writing – review & editing, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Immune-related adverse events of anti-PD-1 immune checkpoint inhibitors: a single center experience

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Objectives: Immune checkpoint inhibitors (ICIs) stimulate antitumor immune responses and, in parallel, they might trigger autoimmune and other immunopathological mechanisms eventually leading to immune-related adverse events (irAE). In our study, we assessed patients with malignancies who underwent anti-PD-1 treatment at the University of Debrecen, Clinical Center.

Patients and methods: Between June 2017 and May 2021, 207 patients started ICI treatment at our university. A total of 157 patients received nivolumab and 50 were treated with pembrolizumab. We looked for factors associated with the development of irAEs. In addition to correlation studies, we performed binary logistic regression analysis to determine, which factors were associated with irAEs. We also performed Forward Likelihood Ratio (LR) analysis to determine independent prognostic factors.

Results: At the time of data analysis, the mean duration of treatment was 2.03 ± 0.69 years. ROC analysis determined that 9 or more treatment cycles were associated with a significantly higher risk of irAEs. A total of 125 patients received ≥9 treatment cycles. Three times more patients were treated with nivolumab than pembrolizumab. Of the 207 patients, 66 (32%) developed irAEs. Among the 66 patients who developed irAEs, 36 patients (55%) developed one, 23 (35%) developed two, while 7 (10%) developed three irAEs in the same patient. The most common irAEs were thyroid (33 cases), dermatological (25 cases), pneumonia (14 cases) and gastrointestinal complications (13 cases). Patients who developed irAEs received significantly more treatment cycles (21.8 ± 18.7 versus 15.8 ± 17.4; p=0.002) and were younger at the start of treatment (60.7 ± 10.8 versus 63.4 ± 10.1 years; p=0.042) compared to patients without irAEs. Pembrolizumab-treated patients developed more but less severe irAEs compared to those receiving nivolumab.

Conclusion: ICI treatment is very effective, however, irAEs may develop. These irAEs might be related to the number of treatment cycles and the type of treated malignancy.

KEYWORDS

immune-checkpoint inhibitors, immune-related adverse events, anti-PD-1, nivolumab, pembrolizumab, Central-Eastern Europe, Hungary

Introduction

Immune checkpoints are cellular proteins that regulate immune responses. When the B7-1/CD80 molecule on antigen-presenting cells (APC) antigen binds to the T-cell CD28 antigen, positive costimulation starts, and the T lymphocytes become activated. On the other hand, if the B7-2/CD86 or the programmed death ligand 1 (PD-L1) molecule on the surface of APC binds to cytotoxic T-lymphocyte antigen 4 (CTLA4) or T-cell PD-1 receptor, respectively, a negative coinhibitory signal is generated, T lymphocyte anergy develops, and antitumor immune responses will be attenuated (1–3). Immune-checkpoint inhibitors (ICI) block CTLA4- or PD-1-mediated coinhibition and thus may restore antitumor immunity (1–4). ICI therapy has become a significant breakthrough in oncology. Numerous CTLA4 (ipilimumab), PD-1 (nivolumab, pembrolizumab) and PD-L1 inhibitors (atezolizumab, durvalumab, cemiplimab, avelumab) have been approved for the treatment of various malignancies (4–8).

Based on the mode of action of ICIs described above, the stimulation of antitumor immune responses may, in parallel, result in the enhancement of autoimmune and other immunological pathways and thus the possible development of immune-related adverse events (irAE) of these drugs (3, 5, 7–12). Such irAEs occur in up to 40% of cases receiving ICI monotherapy (5, 10). While anti-CTLA4 + anti-PD-1 combination therapy result in higher response rates and longer progression-free survival than either agent alone, combination therapy has been associated with more frequent irAEs (up to 95%) (4, 10, 13). Usually irAEs with anti-PD-1 antibodies are less frequent than those with anti-CTLA4 (12).

The irAEs typically start within the first 3 months after the initiation of ICI therapy (5, 10–12, 14). They include endocrine (thyroid, pituitary, diabetes), gastrointestinal (colitis), respiratory (pneumonitis), musculoskeletal (arthritis, manifest autoimmune rheumatic diseases), dermatologic (rash, itching), neurologic (polyneuropathy, aseptic meningitis, demyelination, Guillain-Barré syndrome) and, more rarely, renal (nephritis), hepatobiliary (hepatitis, cholangitis) and ophthalmologic (uveitis, keratitis, retinopathy, dacryoadenitis) manifestations (3, 5, 10–12). These irAEs might have a significant negative impact on the patient's performance status, which is also a very important factor in treatment planning (5, 10). Among general symptoms, fatigue is the leading complaint with a rate of 16-37% (5, 10). Interestingly, the occurrence and the severity of certain irAEs have been associated with better efficacy of ICI treatment (15).

There have been several recommendations for the monitoring and management of ICI irAEs. IrAEs associated with anti-PD-1 therapeutic agents are generally reversible and well tolerated (5, 16, 17). It is also possible that patients who previously received ICI therapy would develop late-onset irAEs (5, 10, 17). The management of such irAEs highly depends on the grade (G) of severity. In mild cases (Grade 1), except for cardiac and neurologic side effects, only symptomatic treatment (NSAIDs, corticosteroids) is required, and ICI treatment could be continued. In cases of moderate (Grade 2) irAEs, oral corticosteroid treatment is necessary with close monitoring of the symptoms. Grade 3 and 4 irAEs might occur in 20-25% of patients undergoing anti-PD-1 treatment and respiratory, and gastrointestinal irAEs are the most frequent among serious events. In cases of severe (Grade 3) irAEs, ICI therapy needs to be temporarily interrupted along with administering parenteral corticosteroids. ICI therapy may be restarted when the symptoms resolved to Grade 1. Finally, ICI therapy should be terminated permanently in more severe and life-threatening cases (Grade 4), and high-dose parenteral corticosteroids or even synthetic or biologic immunosuppressive drugs can be initiated. The management of these irAEs also require a multidisciplinary approach and consultations with other medical specialties, as well as health professionals and advocacy experts (5, 14, 16–22).

The present study assessed irAEs in patients with malignant solid tumors with anti-PD-1 therapy, either nivolumab or pembrolizumab treatment between 2017 and 2021 at the University of Debrecen. We evaluated the frequency of irAEs, compared these irAEs in nivolumab- versus pembrolizumab-treated patients, and investigated the determinants of irAE development in these patients. To the best of our knowledge, this is the first Hungarian cohort where ICI irAE data were collected and systematically analyzed.

Patients and methods

Patients

Between June 2017 and May 2021, ICI treatment was initiated for 207 patients at the Departments of Oncology and Pulmonology, University of Debrecen. Patient characteristics are included in Table 1. Among the 207 patients, there were 138 males and 69

females. Their mean age was 64.6 ± 8.2 years and their age at the initiation of ICI therapy was 62.6 ± 9.8 years (Table 1). Eventually 157 patients received nivolumab and 50 received pembrolizumab (Table 1). At the time of ICI treatment, patients did not receive any additional chemotherapy or radiotherapy. All patients underwent regular follow-ups until the date of data cut, December 31, 2021.

Data collection and statistical analysis

During data collection, we reviewed the charts of all patients and logged all necessary data into an Excel sheet. Statistical analysis was performed using SPSS version 26 (IBM, Armonk, NY, USA) software. Data are expressed as the mean ± SD for continuous variables and percentages for categorical variables. The distribution of continuous variables was evaluated by the Kolmogorov-Smirnov test. As the distribution of data was not normal, non-parametric tests were used. Continuous variables were compared between groups by the Mann-Whitney test, while nominal variables were compared using the χ^2 or Fisher's exact test, as appropriate. Correlations of any two continuous variables were determined by the Spearman's test. Binary logistic regression analysis was performed to assess prognostic factors for irAEs. Moreover, we analyzed Forward Likelihood Ratio (LR) to determine independent prognostic factors. Receiver Operating Characteristic (ROC) curves show the sensitivity and specificity for every possible cut-offs for a test. P values < 0.05 were considered statistically significant.

Results

Descriptive details of ICI therapy

In our cohort, nivolumab and pembrolizumab were initiated in 157 and 50 patients, respectively (p<0.01; Table 1). Among the 207 patients, ICI was started as 1st, 2nd or ≥3rd line of treatment in 29, 159 and 19 patients, respectively. At the time of the data cut, the mean treatment duration was 2.03 ± 0.69 years. Altogether 152 patients received anti-PD-1 therapy in the past (73%), while the treatment was still ongoing in 55 patients (27%) (Table 1). Among patients who received former anti-PD1 therapy, the reasons for discontinuation or switch were disease progression (105 cases; 69% of patients treated in the past), death (29 cases; 19%), complete remission (6 cases; 4%), irAEs (6 cases; 3%); on patient's request (3 cases; 2%) or unknown reason (3 cases, 2%) (Table 1). Until the data cut, the patients received a mean of 16.6 ± 13.7 cycles of therapy. Altogether 125 patients received 9 treatment cycles or more. The types of malignancies are included in Figure 1. The most frequent malignancies were lung (n=127), renal (n=34), tonsillo-pharyngeal (n=14) and urinary bladder cancers (n=11) (Figure 1).

TABLE 1 Clinical characteristics and efficacy results.

	All	Treatment		
		Nivolumab	Pembrolizumab	p value
Number of patients, n	207	157	50	
Female : male ratio	69:138	50:107	19:31	p=0.422
Age, years*	64.6 ± 8.2	64.4 ± 9.9	65.2 ± 11.3	p=0.209
Age at treatment initiation, years*	62.6 ± 9.8	62.3 ± 10.1	63.4 ± 11.2	p=0.145
Treatment duration, years*	2.03 ± 0.69	2.13 ± 0.90	1.86 ± 0.86	p=0.051
Mean number of cycles, n*	16.6 ± 13.7	18.9 ± 19.3	13.9 ± 12.2	p=0.120
Number of patients with cycles ≥ 9, n (%)	125 (60)	97 (62)	28 (56)	p=0.466
Line of treatment, n (%)				***p<0.01***
1st	29 (14)	4 (2)	25 (50)	
2nd	159 (77)	138 (88)	21 (42)	
3rd or more	19 (9)	15 (10)	4 (8)	
Ongoing or past treatment, n (%)				
Ongoing	55 (27)	40 (25)	15 (30)	p=0.554
Past	152 (73)	117 (75)	35 (70)	p=0.549
Discontinuation or switch of the first ICI therapy, n (%)				
Progression	105 (69)	86 (74)	19 (54)	
Complete remission	6 (4)	5 (4)	1 (3)	
Death	29 (19)	19 (16)	10 (29)	
irAE	6 (4)	3 (2)	3 (8)	
Patient's request	3 (2)	2 (2)	1 (3)	
Unknown	3 (2)	2 (2)	1 (3)	
All	152 (100)	117 (100)	35 (100)	p=0.078
PFS after ICI (months)	16.6 ± 16.0	16.7 ± 16.4	16.1 ± 14.8	p=0.677

*Data are expressed as mean ± SD. Significant differences between the nivolumab versus pembrolizumab groups are in **bold italics**. ICI, immune-checkpoint inhibitor; PFS, progression-free survival.

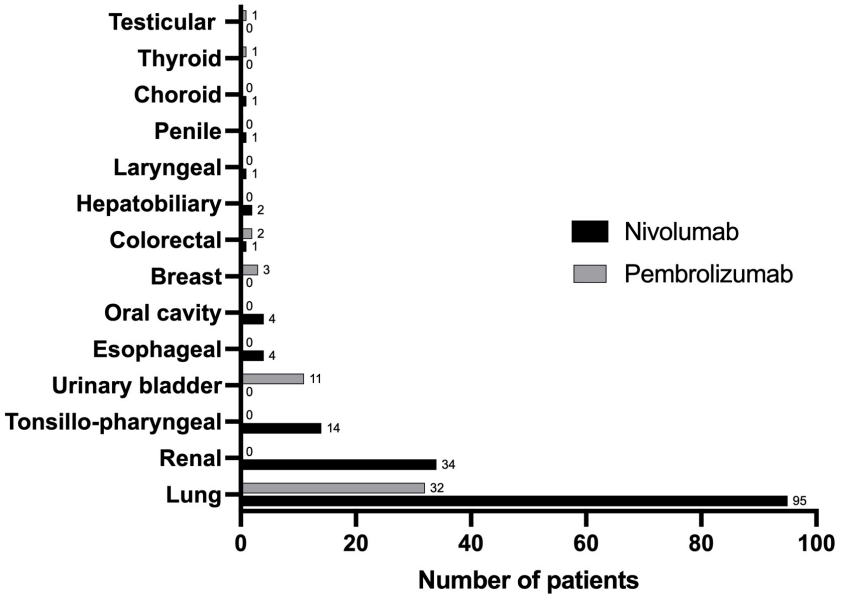

FIGURE 1
The distribution of indications for nivolumab and pembrolizumab treatment. Numbers show the number of patients with the given type of malignancy.

In the nivolumab group, the male:female ratio was 107:50. The mean age was 64.4 ± 9.9 years, while that at treatment initiation was 62.3 ± 10.1 years. Among the 157 patients, nivolumab was initiated as 1st, 2nd or ≥3rd line of treatment in 4, 138 and 15 patients, respectively. The mean treatment duration was 2.13 ± 0.90 years. Altogether 117 patients (75%) earlier received nivolumab therapy, while this treatment was still ongoing in 40 cases (25%) (Table 1). Among patients who received nivolumab therapy in the past, the reasons for discontinuation or switch were disease progression (86 cases; 74% of patients treated in the past with nivolumab), death (19 cases; 16%), complete remission (5 cases; 4%), irAEs (3 cases; 2%), on patient's request (2 cases; 2%) or unknown reason (2 cases, 2%) (Table 1). Our patients received a mean 18.9 ± 19.3 cycles of therapy. Altogether 97 patients received ≥9 treatment cycles (Table 1). Among patients receiving nivolumab, the most frequent malignancies were lung (n=95), renal (n=34), tonsillo-pharyngeal (n=14), esophageal (n=4) and oral cavity malignancies (n=4) (Figure 1).

In the pembrolizumab group, the male:female ratio was 31:19. The mean age was 65.2 ± 11.3 years, while that at treatment initiation was 63.4 ± 11.2 years. Among the 50 patients, pembrolizumab was initiated as 1st, 2nd or ≥3rd line of treatment in 25, 21 and 4 patients, respectively. The mean treatment duration was 1.86 ± 0.86 years. Altogether 35 patients received pembrolizumab treatment in the past (70%), while this therapy was still ongoing in 15 patients (30%) (Table 1). Among patients who earlier received pembrolizumab treatment in the past, the reasons for discontinuation or switch were disease progression (19 cases; 54% of patients treated in the past with pembrolizumab), death (10 cases; 29%, complete remission (1 case; 3%), irAEs (3 cases; 8%), on patient's request (1 case; 3%) or unknown reason (1 case, 3%) (Table 1).

Patients received a mean of 13.9 ± 12.2 cycles of therapy. Altogether 28 patients received 9 or more treatment cycles (Table 1). Among patients receiving pembrolizumab, the most frequent tumors were lung (n=32) and urinary bladder tumors (n=11) (Figure 1).

Considering treatment outcomes, progression-free survival (PFS) rates were calculated in all, as well as nivolumab- and pembrolizumab-treated patients. After anti-PD1 therapy, PFS was observed for 16.6 ± 16.0 months. In the nivolumab- and pembrolizumab-treated subset, PFS durations were 16.7 ± 16.4 and 16.1 ± 14.8, respectively (Table 1).

Finally, we analyzed and compared the nivolumab and pembrolizumab groups. There were three times more patients treated with nivolumab than with pembrolizumab. There were also statistically significant differences in the line of treatment as 88% of nivolumab-treated patients received this ICI in 2nd line, while pembrolizumab was used as 1st line treatment in 50% and 2nd line treatment in 42% of the cases ($p<0.01$). There were no significant differences between the nivolumab- and pembrolizumab-treated patients with respect to genders, age, age at treatment initiation, treatment duration, number of cycles, the number of patients receiving ≥9 cycles, whether anti-PD-1 treatment was in the past or ongoing, the reasons for discontinuation and PFS (Table 1). Regarding the types of malignancy, 75% of lung and all 34 renal, 14 tonsillo-pharyngeal, 4 esophageal and 4 oral cavity cancer patients received nivolumab. On the other hand, only 25% of lung, as well as all 11 bladder and 3 breast cancer patients, were treated with pembrolizumab (Figure 1).

Descriptive data on irAEs

Table 2 includes important information for ICI-related irAEs. Among all 207 patients, 66 (32%) developed altogether 103 irAEs

(Table 2). Thirty-six patients (55% of patients with irAE) developed one, 23 (35%) developed two, while 7 (10%) developed three different irAEs (Table 2). The most frequent irAEs were thyroid (33 cases; 50% of patients with irAE), dermatological (25 cases; 38%), pneumonitis (14 cases; 21%) and gastrointestinal (13 cases; 20%). In addition, nephropathy (7 cases; 11%), hepatopathy (6 cases; 9%), conjunctivitis (2 cases; 3%), pancreatitis (1 case; 1.5%), polyneuropathy (1 case; 1.5%) and polyarthritis (1 case; 1.5%) also occurred (Table 2).

Among the 157 nivolumab-treated patients, 45 (29% of nivolumab-treated patients) patients developed 68 irAEs (Table 2). In this cohort, 26 patients (58% of nivolumab-treated patients with irAE) developed one, 15 (33%) developed two, while 4 (9%) developed three different irAEs (Table 2). In this group, the most frequent IRAEs were thyroid (23 cases; 30% of all nivolumab-treated patients with irAE), dermatological (17 cases; 38%), gastrointestinal (11 cases; 24%) and pneumonitis (9 cases; 20%). We also observed hepatopathy (3 cases; 7%), nephropathy (2 cases; 4%), conjunctivitis (2 cases; 4%) and polyarthritis (1 case; 2%) (Table 2).

In the pembrolizumab-treated subgroup, among 50 patients, 21 (42%) developed 35 irAEs (Table 2). Here 10 patients (48% of pembrolizumab-treated patients with irAE) developed one, 8 (38%) developed 2, while 3 (14%) developed 3 different irAEs (Table 2). In this group, the most frequent irAEs were thyroid (10 cases; 48% of all pembrolizumab-treated patients with irAE), dermatological (8 cases; 38%), nephropathy (5 cases; 24%) and pneumonitis (5 cases; 24%). We also observed hepatopathy (3 cases; 14%), gastrointestinal toxicity (2 cases; 10%), pancreatitis (1 case; 5%) and polyneuropathy (1 case; 5%) (Table 2).

IrAEs developed after a mean of 10.0 ± 10.4 cycles in anti-PD-1-treated patients, which occurred after 12.0 ± 11.8 cycles with nivolumab and 7.0 ± 5.7 cycles with pembrolizumab (p=0.034). If more than one irAEs occurred, the time for the first irAE to appear was calculated (Table 2).

With respect to irAE severity, the percentage of Grade 1, 2 or 3 irAEs in all anti-PD-1-treated, nivolumab-treated or pembrolizumab treated patients were 60%-35%-5%, 50%-46%-4% and 80%-14%-6%, respectively (Table 2). Most irAEs were well-controlled by NSAIDs, corticosteroids or immunosuppressants (data not shown in detail). As discussed above, only six irAEs (3% of all patients) resulted in treatment discontinuation, three in the nivolumab and three in the pembrolizumab group. Treatment discontinuation was needed in one Grade 3, three Grade 2 and two Grade 1 irAE events (Tables 1 , 2).

When comparing nivolumab- and pembrolizumab-treated patients, we did not find significant differences in the proportion of patients with irAEs (p=0.078) and in the relative number of different irAEs (p=0.566). When assessing the specific irAEs,

TABLE 2 Immune-related adverse events.

	All	Treatment		
		Nivolumab	Pembrolizumab	p value
Number of patients, n	207	157	50	
Number of patients with irAE, n	66	45	21	p=0.078
Number of patients with				
1 irAE (%)	36	26	10	
2 irAEs (%)	23	15	8	
3 irAEs (%)	7	4	3	
Total number of irAEs, n	103	68	35	p=0.566
Number of treatment cycles before the first irAE, n*	10.0 ± 10.4	12.0 ± 11.8	7.0 ± 5.7	***p=0.034***
Severity of irAEs				
Grade 1, n (%)	62 (60)	34 (50)	28 (80)	
Grade 2, n (%)	36 (35)	31 (46)	5 (14)	
Grade 3, n (%)	5 (5)	3 (4)	2 (6)	
Mean severity in Grade*	1.53 ± 0.63	2.00 ± 0.61	1.35 ± 0.65	***p=0.027***
irAE subtypes, n (% of patients with irAE)				
All	**66 (100)**	**45 (100)**	**21 (100)**	
Thyroid	33 (50)	23 (30)	10 (48)	
Skin (rashes)	25 (38)	17 (38)	8 (38)	
Pneumonitis	14 (21)	9 (20)	5 (24)	
Gastrointestinal	13 (20)	11 (24)	2 (10)	
Nephropathy	7 (11)	2 (4)	5 (24)	
Hepatopathy	6 (9)	3 (7)	3 (14)	
Conjunctivitis	2 (3)	2 (4)	-	
Pancreatitis	1 (1.5)	-	1 (5)	
Polyneuropathy	1 (1.5)	-	1 (5)	
Polyarthritis	1 (1.5)	1 (2)	-	

*Data are expressed as mean ± SD. Significant differences between the nivolumab versus pembrolizumab groups are in **bold italics**. Abbreviation: irAE, immune-related adverse event.

nephropathy was significantly more frequent in the pembrolizumab group (p=0.010). Otherwise, there were no differences in the various organ-specific irAEs between the two subgroups. Moreover, irAEs developed after significantly more cycles with nivolumab compared to pembrolizumab (p=0.034). Finally, while nivolumab-associated irAEs were almost equally Grade 1 and 2, pembrolizumab treatment resulted in Grade 1 irAEs in 80% of the cases (p=0.027) (Table 2).

Factors associated with the development of irAEs

When comparing patients with (n=66) and without IRAEs (n=141), patients with irAEs received significantly more treatment cycles (21.8 ± 18.7 versus 15.8 ± 17.4; p=0.002) and were younger at treatment initiation (60.7 ± 10.8 versus 63.4 ± 10.1 years; p=0.042). The number of IRAEs correlated with the number of treatment cycles in a certain patient (R=0.227; p=0.001).

In the simple Spearman's correlation analysis, the development of irAEs positively and significantly correlated with the length of PFS (R=0.264; p<0.001), the total number of ICI cycles administered (R=0.273; p<0.001) and recent (ongoing) ICI treatment (R=0.183; p=0.008). The number of irAEs also correlated with PFS (R=0.263; p<0.001), the number of ICI cycles (R=0.276; p<0.001) and recent ICI treatment (R=0.193; p=0.005). Finally, the number of ICI cycles administered before the first irAE developed also correlated with PFS (R=0.603; p<0.001) (Table 3).

We performed binary regression analysis to determine possible prognostic factors for the development of irAEs. As defined by the ROC analysis (Figure 2), 9 or more treatment cycles as cut-off resulted in an increased risk for irAEs with an Odds ratio (OR) of 3.328 (95%CI: 1.008-1.042; p=0.004), a sensitivity of 72% and a specificity of 54%. The forward LR method also confirmed the same with an OR of 3.578 (95%CI: 1.875-6.831; p<0.001). Nivolumab and pembrolizumab were also compared with respect to the frequency of irAEs. In the binary logistic regression analysis, there was a non-significant tendency showing that pembrolizumab treatment was more frequently associated with irAEs compared to nivolumab (OR: 1.878 [95% CI: 0.980-3.599]; p=0.058). However, in the Forward LR analysis, this difference was statistically significant with an OR of 2.169 (95%CI: 1.089-4.321; p=0.028).

TABLE 3 Results of Spearman's correlation analysis: significant correlations.

Parameter 1	Parameter 2	R value	p value
Development of irAE	PFS	0.264	<0.001
	Number of ICI cycles	0.273	<0.001
	Ongoing ICI therapy	0.183	0.008
Number of irAEs	PFS	0.263	<0.001
	Number of ICI cycles	0.276	<0.001
	Ongoing ICI therapy	0.193	0.005
Number of ICI cycles before first irAE	PFS	0.603	<0.001

ICI, immune-checkpoint inhibitor; irAE, immune-related adverse event; PFS, progression-free survival.

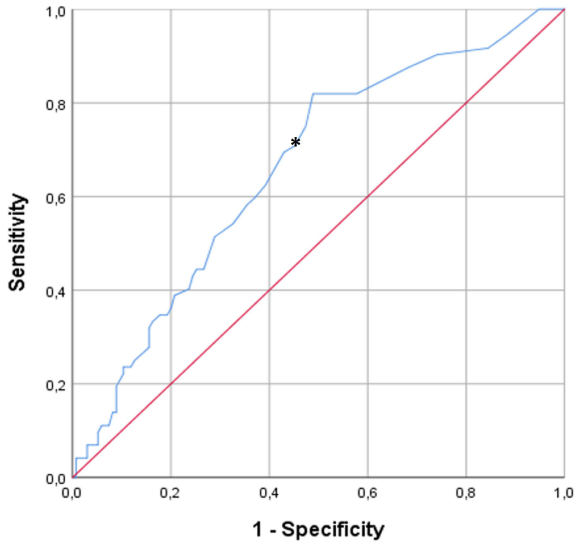

FIGURE 2
ROC analysis of the association of treatment cycles and the development of irAEs. The asterisk indicates the cut-off of 9 cycles. Nine or more treatment cycles as cut-off resulted in an increased risk for irAEs with an Odds ratio (OR) of 3.328 (95%CI: 1.008-1.042; p=0.004), a sensitivity of 72% and a specificity of 54%.

Concerning the specific irAEs, in binary comparisons, patients with thyroid irAEs received more treatment cycles than those without thyroid irAEs (23.0 ± 18.8 versus 16.8 ± 17.7; p=0.04). Patients with pneumonitis also received more treatment cycles (23.1 ± 12.0 versus 17.3 ± 18.3; p=0.022) and had a longer duration of treatment compared to those without pneumonitis (2.5 ± 1.2 versus 2.0 ± 0.9 years; p=0.032). We could not identify any associations between dermatological, gastrointestinal or other specific irAEs or other factors studied.

Discussion

ICIs have become a significant breakthrough in the treatment of numerous malignancies (4–8). However, due to their mode of action, irAEs may develop during therapy due to the stimulation of anti-cancer immune responses [reviewed in (3, 5, 7, 10–12, 21)]. As there have been few reports in this field in the Central-Eastern European (CEE) region including Hungary, we aimed to share our experience collected on a relatively large cohort of 207 patients treated with PD-1 inhibitors, either nivolumab or pembrolizumab at the Clinical Center of the University of Debrecen.

In our cohort, only 6 patients needed treatment termination due to irAEs. Eventually one-third of the patients developed at least one

irAE, after a mean 10 treatment cycles. IrAEs can occur early, while late-onset irAEs are difficult to predict with available tools and, consequently, are hard to prevent (12). Half of these patients had only one irAE, while one-third of them had two and only 10% had three. In accordance with the literature (3, 5, 7, 10–12, 21), the most frequent irAEs were thyroid, skin, diseases, pneumonitis and gastrointestinal conditions. We did not observe any myocarditis (23) or neurotoxicity (24) except for one case of polyneuropathy. In general, 60% of the patients developed Grade 1 irAEs. Most irAEs could be well-controlled using internationally accepted oncology and rheumatology protocols (5, 14, 16, 18–21) and national recommendations (5) and did not require treatment discontinuation. Indeed, irAEs with anti-PD-1 are less frequent than those with anti-CTLA4 (12) and in clinical trials the discontinuation rates are 3-8% (12).

When comparing the two anti-PD-1 agents, in our study, pembrolizumab was twice more often associated with irAEs compared to nivolumab. On the other hand, regarding severity, nivolumab treatment was associated with relatively less Grade 1 but more Grade 2 irAEs compared to pembrolizumab suggesting that pembrolizumab treatment results in milder irAEs. In most systematic reviews, meta-analyses, and comparative assessments, nivolumab and pembrolizumab had similar safety and tolerability profiles (25–28). Therefore, the differences found in our study suggesting that pembrolizumab might cause irAEs more often but these irAEs are milder might be due to other conditions. For example, three times more patients were treated with nivolumab compared to pembrolizumab in this cohort. In our study, pembrolizumab was used earlier, more often in 1st line. Moreover, there were major differences in treatment indications. For example, pembrolizumab was administered to mostly patients with lung cancer. It has not been established, how the underlying malignancy type influences irAE development, severity, and outcomes (3, 5, 7, 10, 11, 21). Thus, it is difficult to directly compare these two ICIs due to heterogeneity in the treatment environment.

Our results confirmed those from others, suggesting that irAEs might show associations with ICI efficacy (12, 15, 29, 30). Moreover, pneumonitis has been suggested to be predictive of favorable outcomes in patients receiving anti-PD-1 antibodies (12, 31). In other studies, risk factors include pre-existing autoimmune diseases, especially those that are active at the time of ICI initiation. In addition, treatment-related factors, such as the type of ICI (anti-PD-1 versus anti-CTLA4), combination of ICIs, as well as intrinsic factors including tumor and genetic heterogeneities, cancer type, tumour microenvironment and the microbiota might also influence the development of autoimmune irAEs (12, 32, 33).

There have been numerous recommendations for the management and possible prevention of autoimmune irAEs (5, 14, 16, 18–21, 34). In our cohort, 60% of irAEs were Grade 1 and most irAEs were easy-to-control and only very few patients required the discontinuation of ICI therapy. As discussed above, many irAEs occur relatively early, in our case, after a mean of 10 treatment cycles. Several preventive strategies and pretreatment assessments of target organ function have been implemented in preventing chemotherapy-related toxicities, which are more predictable than irAEs. With respect to irAEs, no evidence-based algorithms for active surveillance of such events have become available. Most proposed strategies have been based on expert opinion (5, 12, 16, 20). Very few of our patients required cessation of ICI therapy. In most cases, rechallenge after ICI discontinuation is safe and do not lead to repeated irAEs (35).

The strength of our study is that it might be the largest CEE cohort with respect to irAEs associated with ICI therapy. Moreover, we could include a relatively high number of patients from one center and perform multiple analyses to understand the determinants of irAEs. Of course, this study might also have limitations including its single center nature and the solely clinical approach to these issues.

Conclusions

In our cohort of 207 patients treated with nivolumab or pembrolizumab, we achieved a 16-month PFS with both anti-PD-1 agents. One-third of patients developed irAEs, mostly in Grade 1 and did not require treatment discontinuation in all but 6 cases. There were no major differences between the two drugs in general. However, pembrolizumab seemed to be associated with irAEs more frequently, but these irAEs were less severe compared to those of nivolumab, which could be explained by differences in indications, patient numbers, and other factors. Finally, our results also suggest a close relationship between ICI efficacy as determined by PFS and irAEs. Despite the possible limitations of our study, we collected and analyzed data in the CEE region and provided more information on ICI-related irAEs for practicing physicians (34).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by University of Debrecen Central Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin because We only used patient data but patients could not be identified so we did not need consent.

Author contributions

ES, ÉS, ZS: conceptualization, drafting and finalization of manuscript. NM, LB, AM, IB, ZO, PÁ, AV: patient recruitment,

patient examination, obtaining data GT: laboratory assessments, data provider KH: statistical analysis, data curation All authors work on behalf of the Hungarian OncoRheumatology Network (HORN) initiative. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Combinatorial blockade for cancer immunotherapy: targeting emerging immune checkpoint receptors

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The differentiation, survival, and effector function of tumor-specific $CD8^+$ cytotoxic T cells lie at the center of antitumor immunity. Due to the lack of proper costimulation and the abundant immunosuppressive mechanisms, tumor-specific T cells show a lack of persistence and exhausted and dysfunctional phenotypes. Multiple coinhibitory receptors, such as PD-1, CTLA-4, VISTA, TIGIT, TIM-3, and LAG-3, contribute to dysfunctional CTLs and failed antitumor immunity. These coinhibitory receptors are collectively called immune checkpoint receptors (ICRs). Immune checkpoint inhibitors (ICIs) targeting these ICRs have become the cornerstone for cancer immunotherapy as they have established new clinical paradigms for an expanding range of previously untreatable cancers. Given the nonredundant yet convergent molecular pathways mediated by various ICRs, combinatorial immunotherapies are being tested to bring synergistic benefits to patients. In this review, we summarize the mechanisms of several emerging ICRs, including VISTA, TIGIT, TIM-3, and LAG-3, and the preclinical and clinical data supporting combinatorial strategies to improve existing ICI therapies.

KEYWORDS

immune checkpoint inhibitors, combinatorial immunotherapies, PD-1, CTLA-4, VISTA, TIGIT, TIM3, LAG3

Abbreviations: ICR, Immune checkpoint receptor; ICI, Immune checkpoint inhibitor; CTL, Cytotoxic T cell; APC, Antigen presenting cell; MDSC, Myeloid-derived suppressor cell; TCR, T cell receptor; MHCII, Major histocompatibility complex II; TILs, Tumor infiltrating lymphocytes; PD-1, Programmed death-1; CTLA-4, Cytotoxic T lymphocyte-associated protein 4; VISTA, V domain immunoglobulin suppressor of T cell activation; TIGIT, T-cell immunoreceptor with Ig and ITIM domains; TIM-3, T-cell immunoglobulin and mucin domain-containing protein 3; LAG-3, Lymphocyte activation gene 3.

Introduction

The cancer-immunity cycle refers to the process wherein tumor antigen-reactive T cells undergo successful priming and differentiate into cytotoxic killer T cells that infiltrate tumor tissues and eliminate cancer cells (1). The differentiation, expansion, survival, and effector function of these tumor-specific cytotoxic T cells (CTLs) is regulated by the collective signaling effects of the T-cell receptor, costimulatory/coinhibitory receptors, and cytokine receptors, which culminate in transcriptional and epigenetic programs to guide T-cell fate. Unlike in acute viral infections where effector CTLs and memory T-cell responses develop properly, tumor-specific CTLs exhibit dysfunctional states in response to chronic stimulation and a myriad of immunosuppressive factors in the tumor microenvironment (TME). These T cells progressively lose proliferative capacity, memory potential, and effector functions, and enter an "exhausted" state. Exhausted T cells upregulate the expression of multiple ICRs, including PD-1, CTLA-4, VISTA, TIGIT, TIM-3, and LAG-3, which sustain dysfunctional antitumor T-cell responses (2, 3).

Immune checkpoint inhibitors (ICIs) are antibodies or small molecules that bind and block the function of ICRs, thereby reducing tumor-induced T-cell exhaustion and restoring anticancer immunity. Ipilimumab, the monoclonal antibody (mAb) blocking cytotoxic T lymphocyte antigen 4 (CTLA-4), was the first ICI therapy approved by the Food Drug Administration (FDA) in 2011. Currently, several mAbs targeting CTLA-4, PD-1, and PD-L1 have been approved for clinical applications. However, despite revolutionizing the field of oncology, the major challenge of existing ICI therapies is the overall low response rate. Understanding the unique molecular and cellular mechanisms of each ICR may support the development of novel combinatorial therapies that optimally restore antitumor immunity.

This review summarizes updated literature regarding the established and emerging ICRs: PD-1, CTLA-4, VISTA, TIGIT, TIM-3, and LAG-3. Due to the scope limitation, we omit discussions of additional emerging ICRs such as B7-H3, B7-H4, HHLA2, and butyrophilin-like 2 (BTNL2), which have been reviewed elsewhere (4). Herein, we provide an overview of each ICR's structure, expression, signaling mechanisms, and current preclinical and clinical data. We also elaborate on the concept that multiple ICRs operate concurrently to impair the expansion, survival, and effector functions of tumor-reactive cytotoxic T cells (Figure 1), as well as control the maturation and function of dendritic cells (DCs), macrophages, and myeloid-derived suppressor cells (MDSCs) (Figure 2). Given the frequent coexpression and functional crosstalk of these ICRs, we affirm the concept that combinatorial targeting of ICRs may achieve synergistic therapeutic outcomes compared to monotherapies.

Programmed death -1

PD-1 structure and expression

Programmed death -1 (PD-1, CD279) belongs to the B7/CD28 family of receptors, which are type-I transmembrane proteins consisting of an immunoglobulin variable (IgV) domain, a transmembrane domain, and a cytoplasmic tail with signaling capacities. PD-1 engages the ligands PD-L1 and PD-L2 and acts as a coinhibitory receptor that regulates both the adaptive and innate arms of the immune system (4, 5).

PD-1 expression is detected in activated T cells, Foxp3$^+$ regulatory T cells (Tregs), natural killer (NK) cells, innate lymphoid cells (ILC2s), B lymphocytes, macrophages, DCs, and monocytes. In T cells, PD-1 gene expression is induced by TCR

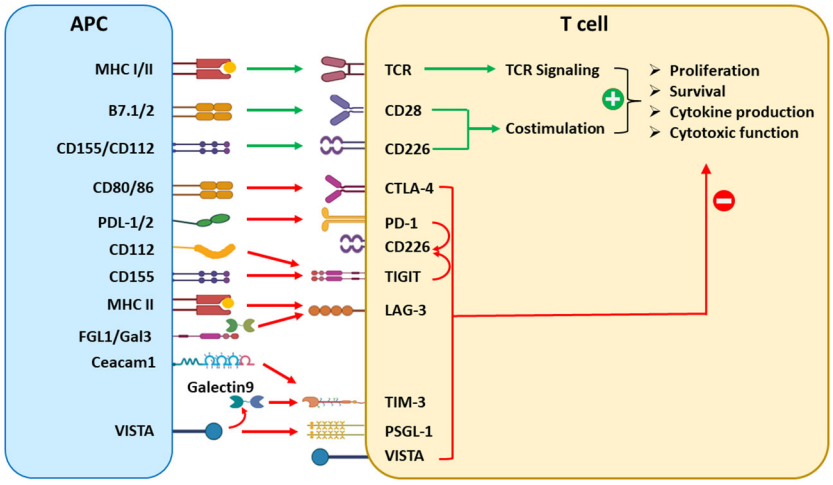

FIGURE 1
Overview of coinhibitory ICRs and their effects in conventional T cells. T-cell activation requires TCR recognition of cognate antigens presented on APCs and costimulation provided by B7/CD28 or CD115/CD226 interactions. On the other hand, many coinhibitory ligand/receptor pathways are activated to dampen T-cell responses. The B7/CTLA-4 and PD-L1/2/PD-1 pathways are the cornerstones of the immune checkpoint paradigm. Emerging inhibitory ICRs, including TIGIT, LAG-3, TIM-3, and VISTA, each recognized by multiple ligands, play nonredundant yet convergent roles as the "brakes" of T-cell responses.

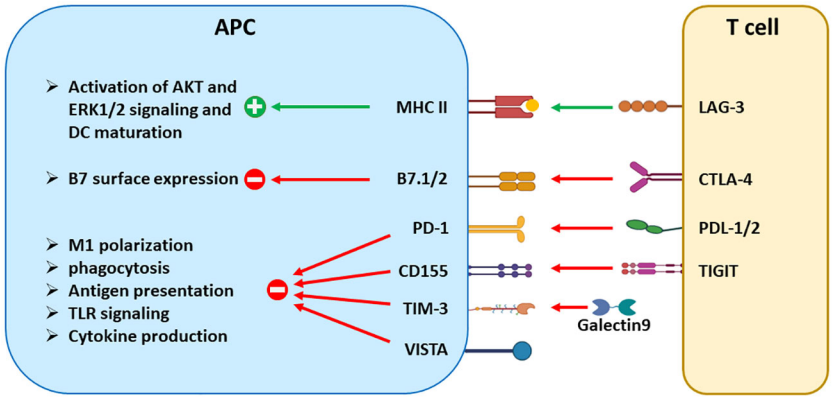

FIGURE 2
The signaling effects of ICRs in antigen-presenting cells. Aside from suppressing T-cell activation, many ICRs regulate the maturation, antigen presentation, cytokine production, and other effector functions of DCs and tumor-associated macrophages. CTLA-4 reduces the surface expression of B7 molecules through trans-endocytosis. LAG-3 and TIGIT trigger signaling in a reverse direction by engaging their respective binding partners MHCII and CD155. On the other hand, PD-1, TIM-3, and VISTA are expressed in APCs and transmit inhibitory signals to inhibit the effector functions of APCs, including phagocytosis, antigen presentation, and cytokine production. Both PD-1 and VISTA are also expressed in tumor-driven MDSCs and contribute to the differentiation and suppressive function of MDSCs.

signaling and positively regulated by multiple transcription factors including AP-1, NFATc1, FoxO1, NF-$_K$B, Notch, STAT, and IRF9 (5). In cancers and chronic viral infections, PD-1 expression in exhausted T cells is significantly higher than in T cells from healthy hosts (3). The expression of PD-1 and its ligand PD-L1 on immune cells and cancer cells may serve as an indicator of disease progression and poor prognosis in a wide range of cancers (6).

Molecular mechanisms of PD-1

The intracellular domain of PD-1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) (5). In T cells, the engagement of PD-1 by its ligand PD-L1 results in the recruitment of the tyrosine-protein phosphatases SHP1 and SHP2, which downregulate the phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK), and mammalian target of rapamycin (mTOR) pathways. CD28 can be directly dephosphorylated by SHP2 and is the major target of PD-1 inhibitory signaling (7). At the cellular level, the consequences of the PD-1 pathway are multifaceted, resulting in altered T-cell metabolism with impaired glycolysis and augmented fatty acid oxidation, reduced cell expansion and effector cytokine production, and impaired T-cell mobility (3, 4).

In addition to the canonical PD-L1/PD-1 interactions, PD-L1 binds to CD80, which is expressed on antigen-presenting cells (APCs) and activated T cells (8). Trans-interactions of PD-L1 on APCs and CD80 on T cells could transmit inhibitory signaling to T cells and impair antitumor immunity (8, 9). On the other hand, cis-interactions of PD-L1/CD80 on APCs reduced PD-L1/PD-1 interactions and CD80/CTLA4 interactions, without affecting interactions between CD80 on APCs and CD28 on T cells (10–12). Blocking cis-interaction of PD-L1/CD80 reduced CD80 expression on APCs and impaired antitumor immune responses (11). An anti-CD80 antibody blocking PD-L1/CD80 cis-interactions augmented PD-L1/PD-1 interactions and alleviated autoimmune disease (13).

In addition to T cells, PD-1 is expressed in tumor-associated macrophages and inhibits their phagocytic function, which in turn controls antitumor immune responses (14). Furthermore, PD-1 plays a role in regulating tumor-driven emergency myelopoiesis. PD-1 deletion in myeloid progenitors reduced the accumulation of GMPs and MDSCs, which may be the result of elevated ERK1/2 and mTORC1 signaling and metabolic reprogramming (15). In preclinical models and cancer patients, blocking interactions of PD-1 with PD-L1 augments the effector function of PD-1$^+$ exhausted CTLs, and induces the expansion of TCF1$^+$ progenitor-like exhausted T cells with self-renewal capacity (16). On the other hand, blocking PD-1 may trigger hyperproliferation and suppressive function of Tregs and contribute to hyperprogressive diseases (17).

Targeting the PD-1/PD-L1 axis for cancer immunotherapy

Monoclonal antibodies specific for PD-1 (nivolumab, pembrolizumab), and PD-L1 (durvalumab, atezolizumab, and avelumab) have proven to be clinically effective and gained FDA approval across a wide range of cancers, such as skin cancer, lung cancer, Hodgkin lymphoma, renal cell carcinoma (RCC), head and neck cancer, bladder cancer, colorectal cancer, liver cancer, gastric cancer, triple negative breast cancer, and cervical cancer (18, 19). Additional antibodies blocking PD-1, such as cemiplimab, camrelizumab, sintilimab, toripalimab, tislelizumab, zimberelimab, prolgolimab, and dostarlimab, have been approved for cancer applications worldwide. A meta-analysis of randomized controlled trials has concluded that anti-PD-1/PD-L1 inhibitors are more advantageous for treating advanced and metastatic cancers

than conventional therapies, with better overall survival and progression-free survival particularly in male patients with younger age, without central nervous system or liver metastasis, no EGFR mutations, and with higher PD-L1 expression (18).

While PD-L1/PD-1 inhibitors are approved for treating an expanding list of cancers, their use as monotherapies generated an overall low response rate, due to mechanisms of primary and acquired resistance (20, 21). To improve the response rate to ICIs, numerous combination strategies have been studied in preclinical and clinical trials, including combining PD-L1/PD-1 inhibitors with chemotherapeutics such as cyclophosphamide, radiotherapy, targeted therapy, agonistic costimulatory antibodies targeting CD134, CD137 or ICOS, innate immune stimulators such as STING agonists, epigenetic modulators, and cancer vaccines such as oncolytic viruses (19, 22, 23). On the other hand, these combinatorial regimens fail to address the roles of other non-overlapping ICRs that constitute one of the dominant resistance mechanisms to PD-1/PD-L1 inhibitors. In the rest of this review, we will summarize studies of emerging ICRs (i.e., VISTA, TIGIT, TIM-3, and LAG-3) and demonstrate the rationales for combinatorial therapies targeting non-redundant ICRs together with PD-1/PD-L1 inhibitors.

CTLA-4

CTLA-4 structure and expression

Cytotoxic T lymphocyte-associated protein 4 (CTLA-4, CD152), together with CD28, represents the B7 family of receptors. Similar to PD-1, CTLA-4 contains an extracellular IgV-domain, a transmembrane domain, and a cytoplasmic tail with motifs for intracellular signaling (24, 25). CTLA-4 is constitutively expressed on Foxp3+ regulatory T cells (Tregs) and is inducible upon activation in conventional T cells. In addition, CTLA-4 expression has been detected in natural killer cells, B cells, dendritic cells, and myeloid cells (26–31).

In T cells, CTLA-4 gene expression is induced by Foxp3 and NFAT (32). The stability of CTLA-4 mRNA is regulated post-transcriptionally, by microRNAs such as miR-145 and miR-155 (33, 34). In resting T cells, a majority of CTLA-4 resides intracellularly within endosomes and relocalizes to the cell surface upon TCR stimulation (27, 31, 35–37). CTLA-4 protein localization is dynamically regulated by clathrin-mediated endocytosis and endosomal recycling, which is dependent upon the tyrosine phosphorylation status of its cytoplasmic domain (38).

Molecular mechanisms of CTLA-4

CTLA-4 inhibits the expansion, cytokine production, and differentiation of conventional T cells and contributes to the development and function of Foxp3$^+$ Tregs. CTLA-4 exerts inhibitory effects by competing against CD28 due to its higher affinity for B7 molecules, as well as by recruiting phosphatases SHP2 and PP2A, which in turn downregulate signaling of TCR and CD28 (39–42). In addition to T-cell intrinsic mechanisms, CTLA-4 indirectly suppresses T-cell responses by modulating dendritic cells: CTLA-4 downregulates the surface expression of B7 molecules through trans-endocytosis (43) or induces the expression of indoleamine 2,3-dioxygenase (IDO), which in turn impairs T-cell proliferation (44). CTLA-4 also reverses the stop signal in activated T cells and reduces the contact time between T cells and APCs, leading to decreased cytokine production and T-cell proliferative responses (45).

The mechanisms of CTLA-4-mediated immunosuppression in cancers are distinct from PD-1 and potentially synergistic with PD-1 (46): although both receptors act on activated conventional T cells, PD-1 controls effector T-cell function at a later stage, mainly within peripheral tissue sites and the tumor microenvironment, while CTLA-4 intercepts T-cell priming in the lymph nodes and governs the function of Tregs (47, 48). CTLA4 is constitutively expressed in Foxp3$^+$ Tregs and CTLA-4-specific antagonistic antibodies not only augment effector T-cell activation but also induce ADCC-mediated depletion of tumor-infiltrating Tregs (49–51). On the other hand, unlike PD-1 and PD-L1, CTLA-4 is not expressed in myeloid cells and does not directly regulate suppressive myeloid cells within the TME. These functional distinctions provide mechanistic rationales for developing combination therapies targeting both axes.

Combinatorial blockade of PD-1 and CTLA-4

Studies have shown that while CTLA-4 and PD-1 blockade each boosts antitumor T-cell responses, dual blockade results in stronger therapeutic outcomes in preclinical models and human patients (52–54). ICI monotherapies induced the expansion of different tumor-infiltrating T cells (TILs), i.e., PD-1 blockade expanded exhausted-like CD8$^+$ CTLs, whereas CTLA-4 blockade expanded both ICOS$^+$ Th1-like CD4 effectors and exhausted CD8$^+$ CTLs. In contrast, the combined blockade induced the expansion of terminally differentiated effector CD8+ CTLs that are not seen in monotherapies and further increased Th1-like CD4$^+$ effector T cells (52, 53). Similar findings have been shown in human melanoma patients treated with ipilimumab and nivolumab therapy. In addition to melanoma, dual blockade of CTLA-4 and PD-1 was studied in a murine breast cancer model (53). While monotherapies showed modest effects, combination therapy led to complete tumor regression in a majority of mice. The synergistic efficacy was due to the anti-CTLA-4 antibody-induced expansion of the T-cell receptor (TCR) repertoire and augmented functionality of TILs, accompanied by intratumoral Treg depletion. Taken together, these studies have demonstrated the mechanisms of synergy with dual ICI therapy that may guide clinical applications.

Ipilimumab (Yervoy) was the first FDA-approved monoclonal antibody for cancer immunotherapy, owing to robust clinical responses for metastatic melanoma (55, 56). We summarize recent clinical trials that have advanced PD-1 and CTLA-4 combinatorial therapy; comprehensive overviews of other clinical trials involving ipilimumab can be found in other reviews (57, 58).

As a monotherapy, the effect of ipilimumab is not as strong as that of the PD-1 antibody nivolumab (Opdivo) for resected stage III or IV melanoma and showed shorter survival and higher toxicity for patients than the PD-1 antibody pembrolizumab (Keytruda) (59, 60). However, when ipilimumab was given concurrently with PD-1 antibody, dual blockade therapy demonstrated significantly improved outcomes in clinical studies. The advantages of dual ICI therapy were first noted in a Phase I dose-escalation study using nivolumab and ipilimumab administered together, which led to better response rates and progression-free survival compared to previously reported results from either monotherapy (61). A subsequent phase III study highlighted better responses and survival with combinatorial therapy when used for metastatic melanoma patients with PD-L1 negative tumors compared to either nivolumab alone or ipilimumab alone, despite the higher occurrence of grade 3 or 4 treatment-related adverse events (62). Follow-up studies showed durable responses and sustained benefits for survival in these patients across multiple years (63–65). Treatment-naive patients with advanced melanoma also benefited from nivolumab-plus-ipilimumab treatment, once again producing higher objective-response rates and progression-free survival with acceptable safety profiles compared to ipilimumab alone (57).

Current research continues to advance PD-1 and CTLA-4 combinatorial immunotherapy in the treatment of other cancers. Beyond melanoma, FDA approval of anti-PD-1 and anti-CTLA-4 dual therapy has expanded to hepatocellular carcinoma (HCC), unresectable pleural mesothelioma, RCC, metastatic non-small cell lung cancer (NSCLC), and advanced or metastatic esophageal squamous cell carcinoma (66–68). Combinatorial ICI therapy in the neoadjuvant setting has also shown promise, with tolerance and strong pathological responses for late-stage melanoma, early-stage colon cancers, and late-stage urothelial cancer (69, 70). Dual blockade of CTLA-4 and PD-1 is currently being evaluated in numerous clinical trials for advanced solid tumors, such as head and neck squamous cell carcinoma (HNSCC) and glioblastomas (NCT04080804, NCT04606316). For testing combined treatment with pembrolizumab (anti-PD-L1), a randomized, double-blind phase III KEYNOTE-598 study (NCT03302234) showed that in patients with metastatic NSCLC, adding ipilimumab to pembrolizumab did not improve efficacy and exhibited greater toxicity than pembrolizumab monotherapy (71). Another phase I expansion trial (NCT02089685) evaluated the efficacy and safety of pembrolizumab combined with a reduced dose of ipilimumab in patients with advanced melanoma and RCC and showed manageable toxicity profile and robust antitumor activity (72).

VISTA

VISTA structure and expression

V-domain immunoglobulin suppressor of T-cell activation (VISTA, alias Gi24, Dies-1, PD-1H, DD1α) is homologous to B7 family receptors and acts as a negative regulator of antitumor immunity and autoimmunity (73–78). VISTA is a type I transmembrane protein containing a single IgV-like extracellular domain (ECD), a transmembrane segment, and a cytoplasmic tail that does not contain ITAM, ITIM, or ITSM motifs. Structural studies have revealed unique features of the VISTA ECD that are distinct from those of other Ig superfamily members, including two additional disulfide bonds, the insertion of an unstructured C-C' loop, the striking enrichment of histidine residues within the ECD, and an extra H β-strand that forms an intramolecular clamping disulfide bond (79, 80). Mutagenesis studies have demonstrated that these structural features contribute to the surface orientation and suppressive function of VISTA (79, 80).

VISTA expression in mice is largely restricted within the hematopoietic compartment, with the highest expression on $CD11b^+$ myeloid lineages such as monocytes, macrophages, granulocytes, and dendritic cells (73, 74). VISTA is also expressed in lymphocytes including NK cells, TCRγδ T cells, naïve $CD4^+$ and $CD8^+$ TCRαβ T cells, and $Foxp3^+$ Tregs. A similar expression pattern of VISTA is seen in human peripheral blood monocytic cells. VISTA gene expression is positively regulated by the transcription factors P53, HIF1-α, and STAT3 (81–83). However, whether VISTA exerts any impact on the functions of HIF1-α and STAT3 remains unknown. VISTA expression is also regulated by TGF-β/Smad3 signaling in T cells and myeloid cells (84).

In human cancer tissues, VISTA expression was mostly enriched in tumor-infiltrating myeloid cells and T cells (75, 85). In addition to immune cells, VISTA expression was detected in mesothelioma (86), gastric cancer (87), and AML (83, 88, 89). VISTA expression has been associated with resistance to immunotherapy and poor patient survival in many cancers, including prostate cancer, lymphoma, bladder cancer, melanoma, breast cancer, and AML (88, 90–95),

Molecular mechanisms of VISTA

VISTA impairs antitumor immunity through its ligand activity in myeloid cells and T cell-intrinsic activity. Although it has been speculated that VISTA also acts as an inhibitory receptor (96), the signaling mechanism is unclear and it remains possible that T cell-intrinsic activity may rely on *cis* interactions with other signaling partners. At the molecular level, several partners, such as PSGL-1, VSIG3, and galectin-9, have been identified to engage VISTA (97–99). While PSGL-1 was suggested as an inhibitory receptor for VISTA, VSIG3 was considered a ligand. Galectin-9 binds VISTA and forms a protein complex that promotes galectin-9-mediated apoptotic signaling. At the cellular level, VISTA regulates the development and function of macrophages, MDSCs, neutrophils, TCRγδ T cells, and $CD4^+$/$CD8^+$ conventional T cells (74, 75, 78, 100, 101). In macrophages, VISTA impairs TLR signaling by regulating the ubiquitination and stability of TRAF6 (102). Blocking VISTA synergizes with a TLR-agonistic vaccine by augmenting the activation of DCs and macrophages, increasing the production of stimulatory cytokines such as IL-12 and IL-27, and promoting the effector function of tumor-specific CTLs. VISTA also contributes to the suppressive function of MDSCs, although the exact molecular mechanisms remain undefined (82, 102).

Combinatorial blockade of VISTA and PD-1

In preclinical models, genetic deletion of VISTA or treatment with anti-VISTA mAb delayed tumor regression by inducing DC maturation, reducing the abundance of adaptive Foxp3$^+$ Tregs, reducing the abundance of MDSCs, and augmenting the effector function and abundance of CTLs (73, 76).

Studies led by Liu et al. first established the nonredundant and synergistic role of VISTA and PD-1 in mounting immune responses against self and tumor antigens (103). In both B16 melanoma and CT26 colon tumor models, combinatorial treatment with anti-VISTA and anti-PD-L1 mAbs resulted in tumor regression and long-term survival in comparison to monotherapies (103, 104). A separate VISTA-blocking mAb, SG7, suppressed the interaction between VISTA and VSIG3 or PSGL-1 and showed efficacy in combination with PD-1 blockade in the MC38 colon tumor model (105). Finally, a unique role of VISTA in promoting naive T-cell quiescence has been identified (106). Accordingly, a study in a CT26 tumor model showed that a triple blockade of VISTA/PD-1/CTLA-4 could improve the efficacy of PD-1/CTLA-4 dual blockade by promoting antigen-presentation in myeloid cells and reducing the quiescent state of CTLs (107).

Several clinically relevant VISTA-blocking agents have been developed and entered clinical trials. VSTB112 (Janssen Inc) was the first anti-VISTA mAb tested in the clinic (NCT02671955). CA-170 (Curis Inc) is an orally available small molecule that has dual targeting activities against PD-L1/L2 and VISTA. In preclinical models, CA-170 rescued T-cell function similarly to PD-1 antagonists and inhibited the growth of B16 melanoma, CT26, and MC38 murine tumor models (108, 109). CA-170 was tested in a phase I trial (NCT02812875) and a phase II trial (Clinical Trials Registry-India CTRI/2017/12/011026) (110). CA-170 showed an excellent safety profile and encouraging clinical activity in classic Hodgkin lymphoma and advanced NSCLC (109). HMBD-002 (Hummingbird Bioscience) is a human VISTA-specific mAb that binds to the C-C' loop of VISTA and blocks its interaction with VSIG3 (111). Studies of murine and humanized mouse models showed the effects of HMBD-002 in reducing MDSCs and augmenting T-cell responses. The phase I trial of HMBD-002 is ongoing (NCT05082610). W0180 (Pierre Fabre Inc) is a human VISTA-specific mAb being tested in a phase I trial (NCT04564417). The NCT05082610 and NCT04564417 trials will both test VISTA inhibitors in combination with pembrolizumab. KVA12123 (Kineta Inc) is a third human VISTA-targeting mAb that has recently been granted FDA acceptance for testing in phase I/II trials.

TIGIT

TIGIT structure and expression

T-cell immunoreceptor with Ig and ITIM domains (TIGIT) is an ICR that contains an IgV-like ECD, a type I transmembrane domain, and a cytoplasmic domain with ITIM and ITT motifs (112). TIGIT is expressed on NK cells, CD4$^+$/CD8$^+$ conventional T cells, and Foxp3$^+$ Tregs. In T cells, TIGIT expression is upregulated following TCR activation and is sustained with increased exhaustion (112).

In human cancers, TIGIT gene expression was found to be upregulated in tumors and correlated with poor prognosis for KIRC, KIRP, LGG, and UVM cancers (113). TIGIT protein expression is abundant in CD4$^+$/CD8$^+$ TILs and Tregs from a wide range of cancer types and is often correlated with poor clinical outcomes or resistance to ICI therapies (114). Coexpression of TIGIT and PD-1 on CD8+ TILs, which is associated with dysfunctional antitumor immune responses, has also been observed in cancers such as HCC, glioblastoma (GBM), acute myeloid leukemia, NSCLC, and melanoma (114).

Molecular mechanisms of TIGIT

TIGIT binds to three ligands CD112, CD113, and PVR (CD155), out of which CD155 exhibits the highest affinity (115, 116). The TIGIT/CD155 interaction inhibits the functions of NK cells, T cells, and APCs. Phosphorylation of both the ITT and ITIM domains is required for the inhibitory signaling of TIGIT in NK cells and T cells, partly by recruiting the adaptors Grb2 and SHIP1, which in turn dampen the PI3K, MAPK, and NF-$_K$B signaling pathways (117, 118). TIGIT also outcompetes CD226 for binding to CD155 and disrupts the costimulatory signal from CD226 in T cells (119). In addition to effector T cells, TIGIT is expressed in Foxp3$^+$ Tregs and plays a role in promoting their differentiation, stability, and suppressive function (120–122).

In APCs such as DCs and macrophages, CD155 is phosphorylated upon engaging TIGIT and subsequently inhibits MAPK signaling, resulting in tolerogenic APCs that produce elevated levels of IL-10 but reduced levels of IL-12, and fail to properly stimulate cognate T cells (123). Another recent study demonstrated that leukemia-associated macrophages express TIGIT and that blocking TIGIT drives M1-like phenotypes and increases phagocytosis (124).

Combinatorial blockade of TIGIT and PD-1

The efficacy of the dual blockade of TIGIT and PD-L1 has been demonstrated in murine breast and colon carcinoma models (112). The combination therapy rejuvenated tumor-specific CD8$^+$ CTLs by augmenting their expansion, effector functions, and the development of memory responses (112). A recent study has shown that the PD-1 and TIGIT pathways converge to regulate CD226, as both receptors impair the phosphorylation of CD226 (125). Furthermore, when CD8$^+$ TILs from human liver cancers were treated with TIGIT and PD-1-blocking mAbs, the coblockade of TIGIT and PD1 significantly improved the expansion, cytokine production, and cytotoxicity of CD8$^+$ TILs compared with single PD-1 blockade (126). Similar results were seen in an adoptive T-cell transfer study to treat human lung cancer, where dual blockade of

TIGIT/PD-1 or TIM-3/PD-1 resulted in greater tumor control than PD-1 monotherapy (127). Together, these studies provide a strong rationale for blocking both the PD-1 and TIGIT pathways to allow optimal CD226-dependent costimulatory signaling in $CD8^+$ T cells.

Currently, there are approximately > 50 clinical trials in the US testing several TIGIT-targeted mAbs, either as monotherapy or in combination with PD-L1/PD-1 inhibitors (clinicaltrials.gov). Bispecific antibodies targeting both TIGIT and PD-1 are also being tested in these trials. In a phase II clinical trial sponsored by Roche (NCT03563716), anti-TIGIT mAb (Tiragolumab) was granted breakthrough therapy designation and was tested in combination with anti-PD-L1 (atezolizumab) in metastatic NSCLC (128). The combination treatment has improved the overall response rate, progression-free survival, and overall survival, over atezolizumab alone (128). Notably, the benefit of the combination treatment was mainly observed in patients with high PD-L1 expression (> 50%) (128, 129). Another TIGIT antibody Vibostolimab (MK-7684) was evaluated in a phase I trial (NCT02964013) with and without combination with pembrolizumab for advanced solid tumors, including NSCLC, and showed promising antitumor activity (130). Additional TIGIT inhibitors, such as BMS-986207 (NCT04570839) (131), ASP8374 (NCT03260322, NCT04826393) (132), Domvanalimab (AB154) (NCT04262856) (133), BGB-A1217 (NCT04047862) (134), and Etigilimab (OMP-313M32) (NCT04761198) (135) are under investigation as single agents and in combination with PD-1/PD-L1 inhibitors in solid tumors.

TIM-3

TIM-3 structure and expression

T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3), along with TIM1 and TIM4, belongs to the TIM family of immunoregulatory proteins. The ECD of TIM-3 contains an immunoglobulin variable domain that binds to several ligands: galectin 9, phosphatidylserine, CEACAM1, and HMGB1 (136). Following the ECD is a mucin domain, a transmembrane domain, and a cytoplasmic domain that does not contain canonical inhibitory signaling motifs such as ITIM or ITSM motifs.

TIM-3 is expressed on subsets of activated $CD4^+$ and $CD8^+$ conventional T cells, Foxp3+ Tregs, NK cells, myeloid cells, and mast cells (136). TIM-3 can be cleaved into a soluble form by ADAM10 and ADAM17 (137). TIM-3 expression in T cells is coregulated with other ICRs including PD-1, TIGIT, and LAG-3 (138). Cytokines such as IL-12, IL-27, and IFN-β can upregulate TIM-3 expression (139, 140). In human cancers, TIM-3 is highly expressed in terminally exhausted $CD8^+$ CTLs, $Foxp3^+$ Tregs, tumor-associated macrophages, and MDSCs. TIM-3 expression levels have been shown to correlate with resistance to immunotherapies and poor prognosis in many cancer types such as melanoma, HCC, prostate cancer, RCC, colon cancer, bladder cancer, cervical cancer, gastric cancer, and esophageal squamous cell carcinoma (122, 141–149).

Molecular mechanisms of TIM-3

In conventional T cells, TIM-3 is recruited to the immune synapse upon TCR activation (150). Y256 and Y263, two of the five tyrosines on the cytoplasmic tail of TIM3, bind BAT3, a protein involved in the TCR signaling pathway (151). Bound BAT3 recruits LCK, a major upstream player in the TCR signaling pathway (152). However, engagement with galectin 9 results in the phosphorylation of Y256 and Y263 by interleukin-2-inducible T-cell Kinase (ITK), which releases BAT3 and impairs TCR signaling (153, 154). Another ligand, CEACAM1, binds TIM-3 *in cis* to promote the stability of TIM-3, while the *trans* interaction induces similar signaling outcomes as galectin-9 (154). The Galectin 9/TIM-3 axis induces apoptosis of effector Th1 cells and $CD8^+$ CTLs (152, 155, 156). In $Foxp3^+$ Tregs, TIM-3 signaling drives an effector-like phenotype and enhances suppressive function (157).

TIM-3 is also expressed in DCs, where its ligation induces the activation of Bruton's tyrosine kinase and c-Src, which inhibit NF-kB activation and subsequently reduce DC activation (158). In macrophages, TIM-3 has been reported to promote M2-like polarization by inducing SOCS1 (159). In monocytes and DCs, TIM-3 inhibits the cellular responses to TLR signaling and reduces the production of proinflammatory mediators (160). In a breast cancer model, blocking TIM-3 augmented the production of a key chemokine CXCL9 from $CD103^+$ DCs, thereby improving the antitumor immune responses when combined with chemotherapy (161).

Combinatorial targeting of TIM-3 and PD-1

In preclinical models, dual blockade of TIM-3 and PD-1 restored the function of both $CD4^+$ and $CD8^+$ T cells and led to complete tumor regression whereas either monotherapy was not effective (162, 163). A recent study has shown that PD-1 binds galectin-9 and that PD-1/TIM-3/galectin-9 may crosslink and form a lattice. As such, PD-1 functions to attenuate galectin-9/TIM-3-induced apoptosis (164). It should be noted that VISTA also binds to galectin-9 and augments the inhibitory effects of TIM-3 (99). Thus, these findings may provide a rationale for future studies to test the combined blockade of PD-1, TIM-3, and VISTA, to improve the persistence and functions of tumor-reactive $PD\text{-}1^+$ $TIM\text{-}3^+$ CTLs.

In human cancers, TIM-3 and PD1 are often coexpressed on $CD8^+$ T cells and mark the most dysfunctional T cell subsets. An earlier study of advanced melanoma showed that NY-ESO-1-specific $PD\text{-}1^+CD8^+$ TILs upregulate TIM-3 expression, which is correlated with dysfunctional phenotypes (165). Blocking TIM-3 augmented cytokine production and proliferation of T cells, while combined blockade of both TIM-3 and PD-1 showed synergistic effects. Similar findings were reported in colorectal cancer, where $TIM\text{-}3^+PD\text{-}1^+CD8^+$ TILs represented the predominant fraction of TILs and targeting both TIM-3 and PD-1 enhanced cell expansion, cytokine production, and cytotoxic activity (166). Recent studies of diffuse large B-cell lymphoma found that $TIM\text{-}3^+PD1^+$ TILs

exhibited a transcriptomic signature of T-cell exhaustion, reduced proliferation, and impaired cytokine production, but these dysfunctions were restored by the blockade of PD1 or TIM-3 (167, 168). Although there have not been any FDA-approved therapeutics targeting TIM-3, the pipelines for novel TIM-3 inhibitors are expanding: several TIM-3-specific antibodies (i.e., cobolimab, MBG453, Sym-023, BMS-986258, AZD7789, INCAGN02390, etc.) or TIM-3/PD-1 bispecific antibodies are being tested in clinical trials (169). A phase I/II trial (NCT02608268) evaluated MGB453 (anti-TIM3) in combination with PDR001 (anti-PD-1) in advanced solid cancers such as melanoma and NSCLC and showed excellent safety profile and preliminary antitumor activity (170). Similar encouraging results were shown by trials (NCT02817633 and NCT03680508) that evaluated TSR-022 (anti-TIM3) in combination with PD-1 inhibitors (171, 172). In addition, a phase Ia/b trial evaluated the safety, pharmacokinetics, and efficacy of LY3321367 (anti-TIM3) plus LY3300054 (Anti-PD-L1) and showed modest antitumor activity (173).

LAG-3

LAG-3 structure and expression

Lymphocyte activation gene 3 (LAG-3, CD223) is an Ig superfamily ICR and is homologous to CD4 (174, 175). The ECD of LAG-3 contains four IgV or IgC-like domains that are involved in ligand binding. The cytoplasmic domain of LAG-3 contains a serine phosphorylation site, the conserved KIEELE motif, and the glutamate-proline dipeptide repeat motif that is involved in its inhibitory signaling (176).

LAG-3 is expressed in many immune cell types including activated conventional $CD4^+$/$CD8^+$ T cells, $Foxp3^+$ Tregs, TCRγδ T cells, NK cells, dendritic cells, and B cells (175). In T cells, LAG-3 expression is induced upon TCR stimulation or by cytokines such as IL-12, IL-2, IL-15, IL-7, IL-6, and IL-8 (177–179). LAG3 expression is promoted by transcription factors such as TOX, NFAT, and NR4A, while suppressed by T-bet (180–186). Studies of human cancers have shown that LAG-3 expression is abundant in TILs and associated with T cell dysfunction or insensitivity to PD-1 blockade. These include breast cancer (187), kidney renal clear cell carcinoma (188), melanoma (189), NSCLC (190, 191), HCC (192, 193), and B-cell lymphoma (194). LAG-3 expression in peripheral blood lymphocytes is also associated with resistance to ICI therapies in patients with melanoma and urothelial carcinoma (195). Furthermore, the clinical resistance to PD1 blockade may be correlated with reduced shedding of LAG-3 in $CD4^+$ conventional T cells due to reduced expression of the protease ADAM10 (196).

Molecular mechanisms of LAG-3

LAG-3 is recognized by multiple ligands including MHCII (197–199), fibrinogen-like protein 1 (FGL-1) (200), galectin-3 (201), and liver sinusoidal endothelial cell lectin (LSECtin) (202). In conventional T cells, LAG-3 signaling suppresses T cell activation, proliferation, cytokine secretion, and cytotoxic functions (203). LAG-3 interacts *in cis* with the TCR/CD3 complex and inhibits TCR signaling by promoting local acidification and Lck dissociation (204). LAG-3 and PD1 interact *in cis* and cluster with pLck at the immunological synapse and recruit SHP1/2, thereby exerting inhibitory effects on T-cell signaling (205). LAG-3 also promotes the activation and suppressive function of $Foxp3^+$ Tregs (206). Soluble LAG-3 acts as an MHCII agonist and induces tyrosine phosphorylation and activation of the AKT and ERK1/2 signaling pathways, thereby inducing DC maturation and improving antitumor T-cell responses (207, 208).

Combinatorial targeting of LAG-3 and PD-1

Preclinical studies have established that LAG-3 cooperates with PD-1 in controlling antitumor immunity (175, 209). The striking synergy between LAG-3 and PD-1 has been demonstrated in murine melanoma, colon cancer, and ovarian tumor models, where the dual blockade against LAG-3 and PD-1 effectively controlled tumor progression that was resistant to respective monotherapies (205, 210). A study in the MC38 colon cancer model has shown that PD-L1 blockade elevated the expression of both costimulatory receptors (ICOS) and coinhibitory receptors (LAG3 and PD-1) in TILs, thereby providing a new mechanistic rationale for coblocking LAG3 (211).

In human ovarian cancer, NY-ESO-1-specific $CD8^+$ TILs demonstrated impaired effector function and enriched coexpression of the inhibitory molecules LAG-3 and PD-1. Dual blockade of LAG-3 and PD-1 during T-cell priming efficiently augmented proliferation and cytokine production by NY-ESO-1-specific $CD8^+$ T cells (212).

These preclinical and clinical studies have provided the backbone for combinational treatment strategies. Currently, numerous clinical trials are exploring the therapeutic benefits of simultaneously targeting LAG-3 and PD-1 (209, 213). LAG-3 targeted agents include soluble LAG-3, LAG-3-specific mAbs, or bispecific antibodies recognizing both LAG-3 and PD-1. Relatlimab (anti-LAG-3) in combination with nivolumab received FDA approval in March 2022 for treating unresectable or metastatic melanoma (214). Favezelimab (MK-4280) in combination with pembrolizumab was tested in a phase III trial (NCT02720068) for colorectal cancer and showed promising antitumor activity in PD-L1-positive tumors (213, 215). Ieramilimab (LAG525) was tested in a phase I/II study (NCT02460224) in combination with spartalizumab (PDR001, anti-PD-1) in advanced/metastatic solid tumors such as melanoma and TNBCs, demonstrating a good toxicity profile but moderate antitumor activity (216). Fianlimab (REGN3767, anti-LAG3) is being tested in combination with cemiplimab (anti-PD-1) in a phase I dose-escalation study (NCT03005782) in advanced melanoma patients and showed clinical activities (217). Eftilagimod alpha, a soluble LAG-3 fusion

protein, is being tested along with pembrolizumab for treating recurrent or metastatic head and neck squamous cell carcinoma (NCT03625323) (208). An ongoing phase I/II study (NCT04370704) is testing retifanlimab (INCMGA00012, Anti–PD-1), INCAGN02385 (Anti–LAG-3), and INCAGN02390 (Anti–TIM-3) triple combination therapy in patients with advanced solid tumors (218). Multiple trials tested BI-754111 (anti-LAG3) combined with BI-754091 (anti-PD-1) in patients with advanced solid tumors but no significant antitumor activity was reported (219). Lastly, bispecific antibodies targeting PD-1/LAG3, including tebotelimab (MGD013, NCT04212221) and RO7247669 (NCT04140500) are under early-stage clinical investigations (220).

Conclusions

Since the first FDA approval of ICIs in 2011, significant progress has been made toward optimizing existing ICI therapies. Taking the lessons from existing ICIs that target PD-1, PD-L1, and CTLA-4, current efforts in the field focus on identifying and targeting nonredundant ICRs that may potentially synergize with existing therapies. VISTA, TIGIT, TIM-3, and LAG-3 represent such candidates in the pipeline. Recent advances in understanding the converging role of ICRs in driving the dysfunction of both APCs and T cells (Figures 1, 2) have set the conceptual foundation for developing combinatorial therapies targeting these ICRs. Based on the frequent coexpression of ICRs in tumor tissues and the distinct yet convergent mechanisms of action (Table 1), it is expected that combined blockade of these emerging ICRs with PD-L1/PD-1 will result in additive or synergistic outcomes. Indeed, many novel ICI combination therapies are being investigated in early-stage trials (Table 2). To advance this concept into clinical applications, the field still faces some challenges, such as defining the molecular pathways and hierarchy of emerging ICRs, identifying the optimal ICR combinations for distinct cancer types and discrete biomarkers, and developing better preclinical models that present the full extent of immune-related toxicities as seen in human patients. In

TABLE 1 Blocking individual ICRs augments antitumor immune responses by convergent cellular and molecular mechanisms.

Effect in immune cell	PD-1 blockade	CTLA-4 blockade	VISTA blockade	TIGIT blockade	TIM3 blockade	LAG3 blockade
Conventional T cells	Augment CD28-mediated costimulation; enhance the proliferation and effector function of CTLs; Expand progenitor-like exhausted CTLs.	Expand ICOS+Th1-like CD4+ effector T cells; expand terminally differentiated effector CD8+ CTLs; expand CTL TCR repertoire; enhance CTL effector function; Improve T cell stop signal and interaction with DCs; combined blockade with anti-PD1 obtain synergistic effects	Enhance CTL cell proliferation, cytokine production and cytotoxic function; reduced CTL quiescence; combined blockade with anti-PD1 obtain synergistic effects.	Enhance CTL cell proliferation, cytokine production and cytotoxic function; combined blockade with anti-PD1 obtain synergistic effects.	Enhance CTL cell proliferation, cytokine production and cytotoxic function; improve T cell survival; combined blockade with anti-PD1 obtain synergistic effects	Enhance CTL cell proliferation, cytokine production and cytotoxic function; combined blockade with anti-PD1 obtain synergistic effects
FOXP3+ Tregs	Induces hyper-expansion of Tregs and contribute to hyper-progressive diseases.	Reduce intratumoral Tregs.	Reduce the differentiation of adaptive Tregs and their suppressive functions	Reduce Treg stability and suppressive function		Reduce the suppressive activity of Tregs
Antigen presenting cells (APCs)	Augment macrophage phagocytosis and M1 polarization.	Increase surface expression of B7 on APCs; reduce IDO expression	Promotes antigen presentation in DCs and macrophages; promote TLR-mediated activation and cytokine production of DCs and macrophages	Promotes M1 polarization of macrophages and DC activation; increase the production of chemokine Cxcl9 and cytokines	Promotes M1 polarization of macrophages; TLR signaling; DC activation	Soluble LAG-3 acts as a MHCII agonist and induces DC activation
Myeloid derived suppressor cells (MDSCs)	Reduce the expansion of tumor driven GMP and MDSCs; augment ERK1/2 and mTORC1 signaling; metabolic reprogramming in myeloid progenitors.		Reduce the abundance and suppressive function of MDSCs.			

This table summarizes the multitudinous effects of blocking each ICR, including PD-1, CTLA-4, VISTA, TIGIT, TIM-3, and LAG-3, in regulating antitumor immune responses. The relevant effector cell types include effector T cells, Foxp3$^+$ Tregs, APCs, and MDSCs.

TABLE 2 Clinical trials testing combined targeting of ICRs.

ICI combinations	Agents	Company	Clinical trials	Cancer types
CTLA4 + PD-1/PD-L1	Ipilimumab+ Nivolumab	Bristol-Myers Squibb	FDA approval	HCC, pleural mesothelioma, metastatic melanoma, colon cancer, urothelial cancer, metastatic NSCLC, RCC
	Ipilimumab+ Nivolumab	Bristol-Myers Squibb	NCT04080804 NCT04606316	HNSCC Glioblastoma Results: recruiting
	Ipilimumab+ Pembrolizumab	Merck Sharp & Dohme	NCT02089685 NCT03302234 NCT03873818	Metastatic melanoma, RCC Results: showed tolerability and antitumor activity NSCLC Results: combination therapy failed to improve efficacy over monotherapy. Metastatic melanoma (recruiting)
VISTA + PD-1/PD-L1	CA170 (dual activity)	Curis	NCT02812875 CTRI/2017/12/011026	Hodgkin lymphoma, NSCLC No results
	HMBD-002 + Pembrolizumab	Hummingbird	NCT05082610	Advanced solid tumors, TNBC, NSCLC No results
	W0180 + Pembrolizumab	Pierre Fabre	NCT04564417	Locally advanced or metastatic solid tumors, No results
	KVA12123 + pembrolizumab	Kineta	NCT05708950	Advanced solid tumors, Recruiting
TIGIT + PD-1/PD-L1	Tiragolumab + Atezolizumab	Roche	NCT03563716	Metastatic NSCLC Results: show improved ORR and PFS
	Vibostolimab (MK-7684) + Pembrolizumab	Merck Sharp & Dohme	NCT02964013 NCT04725188 NCT04738487 NCT05665595 NCT02625961 NCT05298423 NCT05845814	Advanced solid tumors, including NSCLC, melanoma, bladder cancer, urothelial carcinoma Results: recruiting
	BMS-986207 + Nivolumab+ Ipilimumab	Bristol-Myers Squibb	NCT05005273	NSCLC Results: terminated
	BMS-986207 + Nivolumab+ COM701 (anti- PVRIG)	Bristol-Myers Squibb	NCT04570839	Advance solid tumors No results
	ASP8374 + Pembrolizumab	Astellas	NCT03260322 NCT04826393	Advance solid tumors Recurrent glioma No results
	Domvanalimab (AB154) + Zimberelimab (AB122, anti-PD-1)	Arcus Bioscience	NCT04262856	Metastatic NSCLC Results: improved ORR and PFS in combination therapy.
	BGB-A1217 + Tislelizumab (anti-PD-1)	Beigene	NCT04047862	metastatic squamous NSCLC Results: recruiting
	Etigilimab + Nivolumab	Mereo BioPharma	NCT04761198	Advanced solid tumors, cervical cancer, uveal melanoma, ovarian cancer, NSCLC. Results: showed early efficacy
TIM3 + PD-1/PD-L1	Cobolimab (TSR-022) + Nivolumab or TSR-042 (anti-PD-1)	Tesaro	NCT02817633 NCT03680508	Advanced solid tumors such as NSCLC, melanoma, HCC, Results: showed clinical efficacy
	Sabatolimab (MBG453) + Spartalizumab (PDR001, anti-PD-1)	Novartis	NCT02608268	Advanced solid cancers such as melanoma and NSCLC Results: preliminary antitumor activity
	Sym023 + Sym-021 (anti-PD-1)	Symphogen	NCT03311412	Advanced solid tumors, lymphomas, No results.
	LY3321367 + LY3300054 (Anti-PD-L1)	Eli Lilly	NCT03099109	Advanced solid tumors, Results: modest antitumor activity.

(Continued)

TABLE 2 Continued

ICI combinations	Agents	Company	Clinical trials	Cancer types
	BMS986258 + Nivolumab	Bristol-Myers Squibb	NCT03446040	Advanced solid tumors, Recruiting
LAG3 + PD-1/PD-L1	Relatlimab + Nivolumab	Bristol-Myers Squibb	FDA approval	Unresectable or metastatic melanoma
	Favezelimab (MK-4280) + Pembrolizumab	Merck Sharp & Dohme	NCT02720068 NCT03598608 NCT05064059	Colorectal cancer, Lymphomas, Recruiting
	Ieramilimab + Spartalizumab (PDR001, anti-PD-1)	Novartis	NCT02460224	Advanced solid tumors, melanoma, TNBCs, mesothelioma, Results: modest antitumor activity
	Fianlimab + Cemiplimab (anti-PD-1)	Regeneron	NCT03005782	Advanced melanoma, Results: preliminary antitumor activity, ongoing biomarker analysis
	Eftilagimod alpha + Pembrolizumab	Immutep	NCT03625323	Metastatic NSCLC and HNSCC, Results: showed antitumor activity
	Encelimab (TSR-033) + Dostarlimab (TSR-042, anti-PD-1)	Tesaro	NCT03250832	Advanced solid tumors, No results.
	BI-754111 + BI-754091 (anti-PD-1)	Boehringer Ingelheim	NCT03156114 NCT03433898 NCT03697304 NCT03780725	Advanced solid tumors, NSCLC, Results: manageable safety profile but no improved antitumor activity
	Sym-022 + Sym-021 (anti-PD-1)	Symphogen	NCT03311412 NCT03489369 NCT03489343	Advanced solid tumors, lymphomas, Results: preliminary antitumor activity
LAG3 + TIM3 +PD-1	INCAGN02385 (anti-LAG3) + INCAGN2390 (anti-TIM3) + Retifanlimab (INCMGA00012, Anti–PD-1)	Incyte	NCT04370704	Advanced solid tumors Results: recruiting

conclusion, we emphasize that antitumor immunity is controlled by multiple nonredundant ICRs that together maintain immune dysfunction. Recent preclinical and early clinical data strongly support the rational design of novel ICI combinations to achieve synergistic therapeutic efficacies with manageable toxicities.

Author contributions

DR: Writing – original draft, Writing – review & editing. CG: Writing – original draft, Writing – review & editing. SP: Writing – original draft, Writing – review & editing. LW: Writing – original draft, Writing – review & editing, Conceptualization, Funding acquisition, Project administration, Supervision, Validation.

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Conflict of interest

LW is an inventor involved with the commercial development of VISTA with ImmuNext Inc Corporation Lebanon, NH.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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A first-in-human, open-label, dose-escalation and dose-expansion phase I study to evaluate the safety, tolerability, pharmacokinetics/pharmacodynamics, and antitumor activity of QL1604, a humanized anti–PD-1 mAb, in patients with advanced or metastatic solid tumors

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Background: QL1604 is a humanized immunoglobulin G4 monoclonal antibody against programmed cell death protein 1. This first-in-human, open-label phase I study aimed to investigate the safety and tolerability and to identify the recommended doses of QL1604 for future studies. Pharmacokinetics/pharmacodynamics (PK/PD) and preliminary antitumor activity were also assessed.

Methods: Patients with advanced or metastatic solid tumors who failed or had no standard therapies available were recruited. In the dose-escalation phase, patients were treated with QL1604 at 0.3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg intravenously once every 2 weeks (Q2W) in an accelerated titration with a traditional 3 + 3 design, followed by a dose-expansion phase at 3 mg/kg Q2W, 3 mg/kg once every 3 weeks (Q3W), 10 mg/kg Q2W and a fixed dose of 200 mg Q3W. Dose-limiting toxicities (DLTs) were assessed during the first 28 days after the first dose of study drug. Adverse events (AEs) were graded per National Cancer Institute Common Terminology Criteria for Adverse Events version 5.0, and antitumor activity of QL1604 was evaluated by investigators on the basis of Response Evaluation Criteria in Solid Tumors version 1.1.

Results: A total of 35 patients with advanced or metastatic solid tumors were enrolled. DLTs were reported in one patient at the dose level of 3 mg/kg Q2W (grade 3 immune-mediated myositis and myasthenia gravis), and maximum tolerated dose was not reached. The most frequent treatment-related AEs (≥10%) were fatigue (37.1%), anemia (22.9%), increased blood thyroid-stimulating hormone (17.1%), increased aspartate aminotransferase (AST) (17.1%), increased alanine aminotransferase (ALT) (14.3%), decreased white blood cell (WBC) count (11.4%), rash (14.3%), and pruritus (14.3%). AEs leading to discontinuation of QL1604 occurred in three of the 35 patients (8.6%). Partial responses (PRs) occurred in seven patients, resulting in an objective response rate of 20.0% (7/35). Single dose of QL1604 exhibited a dose-dependent increase in the exposure ranging from 0.3 mg/kg to 10 mg/kg. Mean receptor occupancy (RO) for QL1604 at the dose of 3 mg/kg (Q2W and Q3W) and 200 mg (Q3W) was greater than 80% during cycle 1 after one infusion.

Conclusion: QL1604 monotherapy exhibited favorable safety, PK, and signal of antitumor activity in patients with advanced or metastatic solid tumors, and the results supported further clinical studies of QL1604. On the basis of the safety, PK, and RO data, the recommended dosage for further clinical trials is 3 mg/kg or a fixed dose of 200 mg given every 3 weeks.

Clinical Trial Registration: https://classic.clinicaltrials.gov/ct2/show/NCT05649761?term=QL1604&draw=2&rank=1, identifier NCT05649761.

KEYWORDS

QL1604, anti-PD-1 mAb, advanced solid tumors, tolerability, first-in-human

Introduction

According to the latest statistics from The Global Cancer Observatory (GLOBOCAN), there were an estimated 19.3 million new cases of cancer and nearly 10 million deaths worldwide in 2020, and 90% of cancer is solid tumors (1). Cancer is one of the leading causes of death worldwide and the disease burden has increased over time. The therapeutic approach to solid tumors has changed profoundly over the past 30 years (2). With the breakthrough success of antibodies targeting immune checkpoints cytotoxic T-lymphocyte–associated antigen-4 and programmed death receptor 1/ligand 1 (PD-1/PD-L1) in clinical practice, immunotherapy has brought about a shift in tumor treatment paradigm, activating pathways or combined with other strategies to improve immune response to tumor (3, 4).

The PD-1/PD-L1–based pathway is of great value in tumor immunotherapy. It is a critical immune checkpoint that controls the induction and maintenance of immune tolerance in the tumor microenvironment. Blocking the binding of PD-1/PD-L1 with an immune checkpoint inhibitor allows the T-lymphocytes to kill tumor cells (5, 6). In the past decade, various PD-1/PD-L1 inhibitors have been approved worldwide for the treatment of various tumor types (7). PD-1/PD-L1 inhibitors, alone or in combination with conventional chemotherapy, radiotherapy, or targeted therapy, exhibit a manageable safety profile and durable antitumor activity, improving survival in patients with advanced or metastatic solid tumors (8–10). As PD-1/PD-L1 inhibitors have been widely used in cancer therapy and population of cancer patients is still large, new treatment option targeting PD-1/PD-L1 is still necessary.

QL1604 is a highly selective, humanized immunoglobulin G4 monoclonal antibody (mAb) against PD-1 immune checkpoint signaling. QL1604 remains an investigational drug with at least three clinical trials in solid tumors, including QL1604 monotherapy for unresectable or metastatic mismatch repair–deficient or high–microsatellite instability solid tumors (NCT04326829), and QL1604 plus chemotherapy versus chemotherapy in patients with stage IVB, recurrent, or metastatic cervical cancer (NCT04864782) (11).

Here, we report the results of a first-in-human, open-label, phase I study of QL1604 in patients with advanced or metastatic solid tumors. The primary objective of this study was to observe the safety and tolerability of single and multiple dosing of QL1604 and to determine the recommended doses for future clinical studies. The secondary objectives were to characterize the pharmacokinetics (PK)/pharmacodynamics (PD) and immunogenicity and to evaluate the preliminary antitumor activity of QL1604.

Methods

Study design

This study was an open-label, phase I study (Clinicaltrials.gov identifier: NCT05649761) designed to evaluate the safety, tolerability, PK/PD, and antitumor activity of QL1604 in patients

with advanced or metastatic solid tumors. The study conducted at the two centers in China was initiated on 29 May 2019. This study included dose-escalation and dose-expansion phases. For dose escalation, an accelerated titration combined with a 3 + 3 dose-escalation design was used. The planned doses were 0.3 mg/kg, 1 mg/kg, 3 mg/kg, and 10 mg/kg once every 2 weeks (Q2W). The 0.3 mg/kg cohort planned to enroll one patient, and 3 + 3 dose-escalation method was used for other cohorts. For expansion phase, 3 mg/kg Q2W, 3 mg/kg once every 3 weeks (Q3W), 10 mg/kg Q2W and 200 mg of fixed dose Q3W were planned doses.

The study protocol and all amendments were approved by the Ethics Committee of each center and conducted in compliance with the Declaration of Helsinki and the international standards of Good Clinical Practice. Written informed consent was obtained from all patients before start of any study procedure.

Patients

The study enrolled patients aged 18–70 years with a histologically or cytologically confirmed advanced or metastatic solid tumors that failed standard treatment or had no standard therapies available. Additional key eligibility criteria included at least one measurable lesion as assessed by the Response Evaluation Criteria in Advanced Solid Tumors (RECIST) version 1.1; an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; a life expectancy of at least 12 weeks (3 months); and adequate hematologic, renal, and liver functions. Patients were excluded if they had an active autoimmune disease requiring systemic treatment; prior use of corticosteroids (>10 mg/daily of prednisone or equivalent) or immunosuppressive medication within 14 days before the start of study treatment; had clinically significant cardiovascular or cerebrovascular disease within 3 months; had grade ≥2 [National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE), version 5.0] arrhythmia or heart failure, atrial fibrillation, or clinically significant supraventricular or ventricular arrhythmia requiring treatment or intervention; had received radiotherapy, chemotherapy, hormonal therapy, surgery, or molecular targeted therapy within 4 weeks prior to first dose of study treatment; known hypersensitivity to any mAb, QL1604 and/or any of its excipients; had received a live antitumor vaccine; and a known additional malignancy within 5 years before study start.

Procedures

In the dose-escalation part, patients received QL1604 via intravenous infusion at a dose level assigned according to the sequence of enrollment. Each treatment cycle lasted for 28 days. Treatment was continued until progression of the disease (PD), unacceptable toxicity, confirmed complete response (CR), loss to follow-up, or patient or investigator decision, whichever occurred first. Dose-limiting toxicities (DLTs) were observed during the 28-day period after the first dose of study drug at each dose level and included: grade ≥2 uveitis; grade ≥2 interstitial pneumonitis (grade 2 interstitial pneumonitis lasting for >7 days after glucocorticoid treatment); grade ≥3 non-hematologic adverse reactions (except for transient electrolyte abnormalities, diarrhea, nausea, and vomiting recovered to ≤grade 2 within 3 days after best support care, and asthenia recovered to ≤grade 2 within 7 days after best support care); grade ≥2 cardiac insufficiency; grade 4 thrombocytopenia or grade 3 thrombocytopenia with obvious bleeding tendency; grade 4 neutropenia lasting for ≥3 days or grade 3 neutropenia with ≥38.3°C fever; and other grade 4 hematologic toxicities. Decisions on dose escalation in this phase were made on the basis of the incidence of DLTs seen during the DLT observation period. The expansion phase for 3 mg/kg Q2W and 3 mg/kg Q3W cohorts started after the DLT observation period was finished for the last patient in 3 mg/kg cohort in dose-escalation phase. The expansion phase for 200 mg of fixed dose Q3W started after the DLT observation period was finished for the last patient in 10 mg/kg cohort in dose-escalation phase.

Safety and efficacy assessments

Adverse events (AEs) were assessed and graded according to the CTCAE (version 5.0) throughout the study and up to 90 days after the last dose, including incidence and severity of treatment-emergent AEs (TEAEs). Antitumor activity of QL1604 was evaluated by investigators on the basis of Response Evaluation Criteria in Solid Tumors (RECIST 1.1). Tumor responses were performed by computed tomography or magnetic resonance imaging at screening and every 6 weeks during the first 6 months and every 12 weeks thereafter.

Pharmacokinetics, pharmacodynamics and immunogenicity assessments

For PK studies, blood samples were collected at the following time points during single-dose phase (cycle 1): −0.5 h (pre-dose), 5 min (min), 2 h, 6 h, 24 h, 48 h, day 8 (D8), D15, and D22 after end of infusion. After entering multiple-dose phase, for Q2W cohort, blood samples were collected on D1 and D15 at 0.5 h prior to infusion and within 5 min after end of infusion each treatment cycle from cycle 2 (except for cycle 5). In cycle 5, blood samples were collected at −0.5 h (pre-dose), 5 min, 2 h, 6 h, 24 h, 48 h, and D8. For the Q3W cohorts, blood samples were collected on D1 at 0.5 h prior to infusion and within 5 min after end of infusion each treatment cycle from cycle 2 (except for cycle 6). In cycle 6, blood samples were collected at −0.5 h (pre-dose), 5 min, 2 h, 6 h, 24 h, 48 h, D8, and D15 after end of infusion. The single-dose plasma PK parameters included area under the concentration–time curve (AUC), maximum observed plasma concentration (C_{max}), time to peak plasma concentration (T_{max}), terminal elimination half-life ($t_{1/2}$), AUC from time zero (pre-dose) to the time of the last measurable concentration (AUC_{0-t}), and AUC from time zero (pre-dose) to infinity ($AUC_{0-\infty}$). In multiple ascending-dose study, degree of fluctuation, minimum plasma steady-state concentration (Css, min), and maximum plasma steady-state concentration (Css, max) were also analyzed.

For PD-1 receptor occupancy (RO), QL1604 binding to PD-1 molecules was detected by flow cytometry. Blood samples were collected at the at the following time points during single-dose phase (cycle 1): −0.5 h (pre-dose), 5 min, D2, D3, D8, D15, and D22 (for patients in the Q3W dose group, blood samples were not collected on D22) after end of infusion. After entering multiple-dose phase, for Q2W cohort, blood samples were collected on D1 and D15 at 0.5 h prior to infusion each treatment cycle from cycle 2 (except for cycle 5). Blood samples were collected at −0.5 h (pre-dose), 5 min, D2, D3, D8, D15, and D22 after end of infusion in cycle 5. For Q3W cohorts, blood samples were collected on D1 at 0.5 h prior to infusion each treatment cycle from cycle 2 (except for cycle 6). Blood samples were collected at −0.5 h (pre-dose), 5 min, D2, D3, D8, and D15 after end of infusion in cycle 6.

The formation of antidrug antibodies (ADA) was analyzed for determining immunogenicity. Blood samples for immunogenicity were collected at −0.5 h (pre-dose), D8, D15, and D22 after end of infusion in during single-dose phase (cycle 1) (for patients in the Q3W dose group, blood samples were not collected on D22). After entering multiple-dose phase, for Q2W cohort, blood samples were collected on D1 and D15 at 0.5h prior to infusion each treatment cycle from cycle 2 (except for cycle 5). Blood samples were collected at −0.5 h (pre-dose), D8, D15, and D22 after end of infusion in cycle 5. For Q3W cohorts, blood samples were collected on D1 at 0.5 h prior to infusion each treatment cycle from cycle 2 (except for cycle 6). Blood samples were collected at −0.5 h (pre-dose), D8, and D15 after end of infusion in cycle 6.

Statistical analysis

No statistical hypothesis was specified for this study. For the dose-escalation phase, an accelerated titration combined with a 3 + 3 dose-escalation design was used. The 0.3 mg/kg cohort planned to enroll one patient (accelerated titration). On the basis of the 3 + 3 design, three to six patients were planned to be enrolled to other dose cohorts. For the expansion phase, additional patients were enrolled to selected dose cohorts to ensure at least eight patients PK evaluable patients in each dose cohort. A total of 21 to 42 patients were to be enrolled in the expansion phase.

The efficacy analysis based on the full analysis set included all patients who received at least one dose of QL1604. Objective response rate (ORR) was defined as the proportion of patients with CR or partial response (PR), assessed by the investigator per RECIST v1.1. Disease control rate (DCR) was defined as the proportion of patients with CR, PR, or stable disease (SD), assessed by the investigator per RECIST v1.1. The safety analysis population included all patients who received at least one dose of QL1604 and had a safety record after treatment.

ORR and DCR point estimates were accompanied by 95% CIs using the Clopper–Pearson exact method. Summary statistics were provided for AEs. PK parameters for QL1604 were calculated using non-compartmental model by WinNonlin 6.4 (Certara, Inc.). All statistical analyses were performed using SAS (version 9.4) (SAS Institute Inc., Cary, NC).

Results

Patient characteristics and disposition

Between 29 May 2019 and 24 July 2020, 40 patients were screened and 35 eligible patients were enrolled and treated with QL1604 (Figure 1) (one in 0.3 mg/kg Q2W, three in 1 mg/kg Q2W, nine in 3 mg/kg Q2W, three in 10 mg/kg Q2W, nine in 3 mg/kg Q3W, and 10 in 200 mg Q3W). Patient demographics and baseline characteristics are listed in Table 1. Patients were predominantly male (62.9%) with a median age of 57 years (range, 35–69 years), and 32 (91.4%) had an ECOG performance status of 1. The majority (n = 33, 94.3%) of patients had stage IV disease. The majority of patients had non–small-cell lung carcinoma (NSCLC) (n = 18, 51.4%). Five patients (14.3%) had brain metastases. All patients received prior anticancer therapy, and 51.4% (n = 18) had ≥3 prior lines of treatment. Across the study, the median time from initial diagnosis to study enrollment was 25.7 months (range, 3.0–155.6).

Safety and tolerability profile

In this study, 13 patients were included for the DLT analysis. DLTs were observed in one (16.7%) of the six patients at the 3 mg/kg Q2W dose level (grade 3 immune-mediated myositis and myasthenia gravis), and maximum tolerated dose (MTD) was not reached.

The majority of patients (33/35, 94.3%) experienced AEs, of which 29 patients (82.9%) had QL1604-related AEs (TRAEs) (Table 2). The most common TRAEs (≥10% in total population) were fatigue (37.1%), anemia (22.9%), increased blood thyroid-stimulating hormone (TSH) (17.1%), increased AST (17.1%), increased ALT (14.3%), decreased WBC count (11.4%), rash (14.3%), and pruritus (14.3%) (Table 2). Grade ≥3 TRAEs occurred in six of the 35 patients (17.1%) at 3 mg/kg (Q2W and Q3W), 10 mg/kg Q2W, and 200 mg Q3W dose levels (Table 3). No grade 5 TRAE occurred.

Serious TRAEs occurred in four (11.4%) patients. TEAEs leading to discontinuation of study drug occurred in three (8.6%) patients, including immune-mediated hepatitis (one patient), myasthenia gravis and immune-mediated myositis (one patient), and sinus bradycardia (one patient).

Immune-related AEs (irAEs) occurred in 17 (48.6%) patients. The most common irAE was increased blood TSH (17.1%). Grade ≥3 irAEs occurred in four (11.4%) patients. Infusion-related reactions occurred in three (8.6%) patients, and all were grade 1 or 2.

Antitumor activity

As of data cutoff (14 July 2022), a PR was observed in seven patients (20.0%): five with NSCLC (one patient had a PR after PD) and two with nasopharyngeal carcinoma (NPC). SD was achieved in five patients (14.3%): two with NSCLC, one with esophageal cancer (EC), one with small-cell lung cancer (SCLC), and one with NPC (Supplementary Figures 2, 3). The ORR of was 20.0% (95% CI,

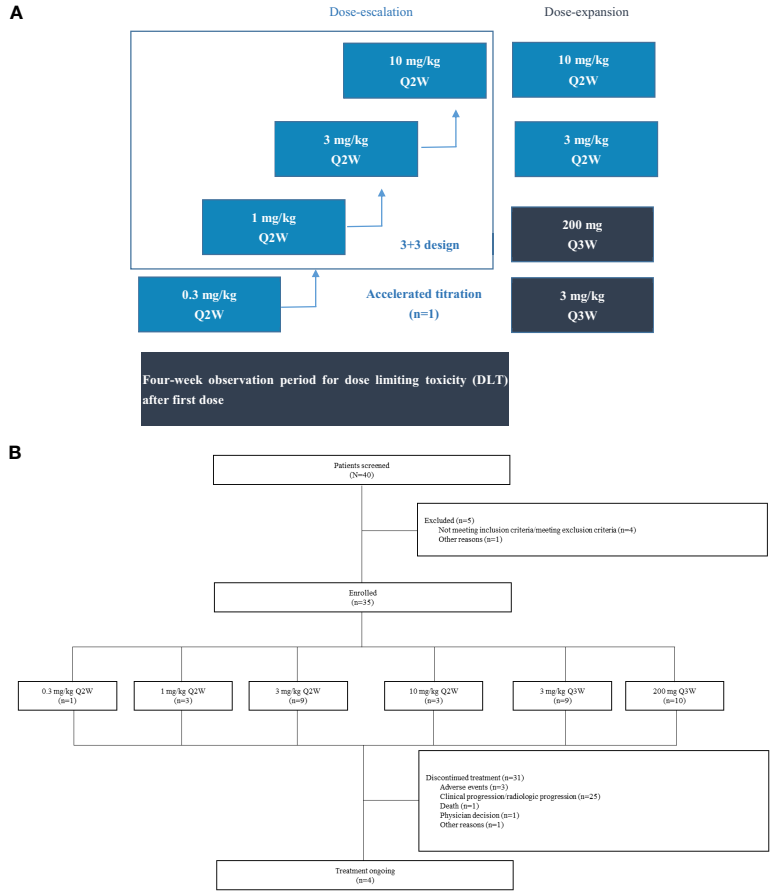

FIGURE 1
Study design and patient disposition. **(A)** Study design. This open-label, phase I study (NCT05649761) consisted of dose escalation and expansion phases in patients with advanced solid tumors. For dose escalation, an accelerated titration combined with a 3+3 dose-escalation design was used. The 0.3 mg/kg cohort planned to enroll one patient, and 3+3 dose escalation method was used for other cohorts. **(B)** Patient disposition. 3 mg/kg Q2W, 3 mg/kg Q3W, and 200 mg Q3W cohorts included patients enrolled in dose-escalation and dose-expansion phases.

8.4–36.9) and DCR was 34.3% (95% CI, 19.1–52.2) (Table 4). The median duration of response (DoR) of all responders was 26.64 months (95% CI, 2.79–not evaluable). The median progression-free survival (PFS) of all patients was 1.38 months (95% CI, 1.35–2.63). A waterfall plot of maximum tumor shrinkage assessed by the investigator showed that, of the 30 patients with at least one post-baseline tumor assessment, nine had tumor shrinkage compared with baseline (Supplementary Figure 1).

Pharmacokinetics/pharmacodynamics and immunogenicity

The PK parameters of single-dose QL1604 are presented in Table 5, and concentration–time profiles by dose levels are shown in Figure 2. The mean C_{max} for QL1604 increased with increasing dose of QL1604 from 4.907 μg/mL to 195.3847 μg/mL. The median time to reach C_{max} ranged from 1.08 h to 7.00 h. The mean half-life ($T_{1/2}$) for QL1604 ranged from 80.93 h to 273.447 h. The mean of AUC_{0-t} ranged from 984 h*μg/mL to 50,300 h*μg/mL. PK of QL1604 at a steady state is presented in Supplementary Table 3.

The RO results indicated PD-1 target engagement on D15 and D22 of cycle 1 after one infusion, which was dose-dependent and with a mean RO >80% at 3 mg/kg Q2W, 3 mg/kg Q3W, 10 mg/kg Q2W, and 200 mg of fixed dose Q3W (Figure 3). The RO for 3 mg/kg Q3W and 200 mg of fixed dose Q3W dose levels was similar.

Three of the 35 patients (3/35, 8.6%) were positive for ADA, and neutralizing antibody (Nab) were negative in all 35 patients (100%) at baseline. After treating with QL1604, 15 (42.9%) patients were ADA-positive, and two (5.7%) patients were Nab-positive (Supplementary Tables 1, 2).

Discussion

This first-in-human phase I study of QL1604 showed that QL1604 was safe and well tolerated at doses from 0.3 mg/kg Q2W to 10 mg/kg Q2W, 3 mg/kg Q3W, and 200 mg Q3W. It is well-known that immunotherapy has received extensive attention and explosive development because of their good safety, durable responses, and application in a broad spectrum of cancers. However, immunotherapies are frequently constrained by their

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TABLE 1 Baseline demographics and disease characteristics (full analysis set).

	0.3 mg/kg Q2W	1 mg/kg Q2W	3 mg/kg Q2W	10 mg/kg Q2W	3 mg/kg Q3W	200 mg Q3W	Total
	(n = 1)	(n = 3)	(n = 9)	(n = 3)	(n = 9)	(n = 10)	(N = 35)
Age (years), median (range)	56.0 (56–56)	57.0 (48–63)	59.0 (49–69)	53.0 (51–62)	59.0 (54–69)	57.0 (35–67)	57.0 (35–69)
Sex, n (%)							
Male	1 (100)	2 (66.7)	4 (44.4)	1 (33.3)	8 (88.9)	6 (60.0)	22 (62.9)
Female	0	1 (33.3)	5 (55.6)	2 (66.7)	1 (11.1)	4 (40.0)	13 (37.1)
Tumor diagnosis, n (%)							
NSCLC	0	2 (66.7)	6 (66.7)	2 (66.7)	4 (44.4)	4 (40.0)	18 (51.4)
EC	1 (100)	0	1(11.1)	0	2 (22.2)	1 (10.0)	5 (14.3)
GC/GEJC	0	0	0	0	0	2 (20.0)	2 (5.7)
Others*	0	1 (33.3)	2 (22.2)	1 (33.3)	3 (33.3)	3 (30.0)	10 (28.6)
Time from initial cancer diagnosis to study enrollment, months, median (range)	32.36 (32.36–32.36)	21.72 (21.09–57.69)	31.97 (3.02–133.59)	30.88 (24.64–89.56)	15.21 (6.60–69.65)	22.28 (5.09–155.60)	25.69 (3.02–155.60)
Current clinical staging, n (%)							
III	0	0	0	0	1 (11.1)	1 (10.0)	2 (5.7)
IV	1 (100)	3 (100)	9 (100)	3 (100)	8 (88.9)	9 (90.0)	33 (94.3)
Number of metastatic sites, n (%)							
0	0	0	0	1 (33.3)	0	0	1 (2.9)
1	0	0	0	0	0	7 (70.0)	7 (20.0)
2	1 (100)	3 (100)	4 (44.4)	1 (33.3)	1 (11.1)	2 (20.0)	12 (34.3)
>2	0	0	5 (55.6)	1 (33.3)	8 (88.9)	1 (10.0)	15 (42.9)
ECOG performance status, n (%)							
0	0	0	1 (11.1)	0	0	2 (20.0)	3 (8.6)
1	1 (100)	3 (100)	8 (88.9)	3 (100)	9 (100)	8 (80.0)	32 (91.4)
Lines of previous anticancer therapies, n (%)							
0	0	0	0	0	0	0	0
1	0	0	3 (33.3)	0	1 (11.1)	0	4 (11.4)
2	0	1 (33.3)	2 (22.2)	1 (33.3)	5 (55.6)	4 (40.0)	13 (37.1)
≥3	1 (100)	2 (66.7)	4 (44.4)	2 (66.7)	3 (33.3)	6 (60.0)	18 (51.4)
Previous anticancer therapies, n (%)							
Chemotherapy	1 (100)	3 (100)	9 (100)	3 (100)	9 (100)	10 (100)	35 (100)
Targeted therapy	0	3 (100)	5 (55.6)	3 (100)	2 (22.2)	5 (50.0)	18 (51.4)
Radiotherapy	0	2 (66.7)	4 (44.4)	2 (66.7)	4 (44.4)	6 (60.0)	18 (51.4)
Surgery	1 (100)	2 (66.7)	6 (66.7)	2 (66.7)	4 (44.4)	5 (50.0)	20 (57.1)
Others	0	2 (66.7)	0	1 (33.3)	0	0	3 (8.6)

NSCLC, non–small-cell lung cancer; EC, esophageal cancer; GC, gastric carcinoma; GEJC, gastroesophageal junction carcinoma; ECOG, Eastern Cooperative Oncology Group.
*Including small-cell lung cancer (four patients), nasopharyngeal carcinoma (three patients), thymic carcinoma (one patient), prostate cancer (one patient), and rectal cancer (one patient).

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TRAEs (12). Most AEs related to QL1604 were grade 1 or 2. The reported AEs were consistent with the overall safety profile of other anti–PD-1 mAb agents (13, 14). Grade 3 or 4 TRAEs occurred in six (17.1%) patients, and no grade 5 TRAE occurred. Three (8.6%) patients discontinued QL1604 because of AEs. A meta-analysis showed that 66% of patients treated with PD-1/PD-L1 inhibitors experienced all grades of TRAEs, 14.0% experienced grade 3 or higher TRAE, and 0.45% died from this factor (15). TRAEs result from blockade of these immune checkpoints and involve lung, liver, heart, skin, neurotoxicity, etc., and even some are occasionally fatal. In our study, no DLT was observed at the highest dose level (10 mg/kg), and, thus, the MTD was not determined. Compared with phase I study of pembrolizumab (TRAE, 70%) (16), the incidence of TRAEs and grade ≥3 TRAEs was higher in our study (TRAE, 82.9%;

TABLE 2 Summary of safety results (safety population).

	0.3 mg/kg Q2W	1 mg/kg Q2W	3 mg/kg Q2W	10 mg/kg Q2W	3 mg/kg Q3W	200 mg Q3W	Total
	(n = 1)	(n = 3)	(n = 9)	(n = 3)	(n = 9)	(n = 10)	(N = 35)
Treatment-related AEs, n (%)	0	3 (100)	6 (66.7)	3 (100)	8 (88.9)	9 (90.0)	29 (82.9)
Grade ≥3 treatment-related AEs, n (%)	0	0	2 (22.2)	1 (33.3)	2 (22.2)	1 (10.0)	6 (17.1)
Immune-related AEs, n (%)	0	1 (33.3)	4 (44.4)	2 (66.7)	3 (33.3)	7 (70.0)	17 (48.6)
Grade ≥3 immune-related AEs, n (%)	0	0	1 (11.1)	0	2 (22.2)	1 (10.0)	4 (11.4)
Treated-related SAEs, n (%)	0	0	2 (22.2)	0	0	2 (20.0)	4 (11.4)
AEs leading to discontinuation of study treatment, n (%)	0	0	1 (11.1)	0	1 (11.1)	1 (10.0)	3 (8.6)
Treatment-related AEs leading to death, n (%)	0	0	0	0	0	0	0
TRAEs in >5% of total population, n (%)							
Fatigue	0	1 (33.3)	3 (33.3)	2 (66.7)	5 (55.6)	2 (20.0)	13 (37.1)
Anemia	0	3 (100)	2 (22.2)	1 (33.3)	2 (22.2)	0	8 (22.9)
Increased AST	0	1 (33.3)	1 (11.1)	0	4 (44.4)	0	6 (17.1)
Increased blood TSH	0	1 (33.3)	1 (11.1)	1 (33.3)	1 (11.1)	2 (20.0)	6 (17.1)
Increased ALT	0	0	1 (11.1)	1 (33.3)	2 (22.2)	1 (10.0)	5 (14.3)
Rash	0	0	1 (11.1)	1 (33.3)	1 (11.1)	2 (20.0)	5 (14.3)
Pruritus	0	0	1 (11.1)	1 (33.3)	1 (11.1)	2 (20.0)	5 (14.3)
Decreased WBC count	0	0	1 (11.1)	0	0	3 (30.0)	4 (11.4)
Increased blood creatinine	0	1 (33.3)	1 (11.1)	0	0	1 (10.0)	3 (8.6)
Increased blood creatinine phosphokinase	0	0	0	1 (33.3)	1 (11.1)	1 (10.0)	3 (8.6)
Nausea	0	0	1 (11.1)	1 (33.3)	0	1 (10.0)	3 (8.6)
Proteinuria	0	1 (33.3)	1 (11.1)	1 (33.3)	0	0	3 (8.6)
Hypothyroidism	0	1 (33.3)	0	1 (33.3)	1 (11.1)	0	3 (8.6)
Hyperthyroidism	0	0	1 (11.1)	1 (33.3)	0	1 (10.0)	3 (8.6)
Weight loss	0	0	0	2 (66.7)	0	0	2 (5.7)
Weight gain	0	0	1 (11.1)	0	1 (11.1)	0	2 (5.7)
Decreased platelet count	0	0	0	0	0	2 (20.0)	2 (5.7)
Prolonged electrocardiogram QT	0	0	0	1 (33.3)	0	1 (10.0)	2 (5.7)
Decreased neutrophil count	0	0	0	0	0	2 (20.0)	2 (5.7)
Pyrexia	0	0	1 (11.1)	0	0	1 (10.0)	2 (5.7)
Decreased appetite	0	0	0	1 (33.3)	0	1 (10.0)	2 (5.7)

(Continued)

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TABLE 2 Continued

	0.3 mg/kg Q2W	1 mg/kg Q2W	3 mg/kg Q2W	10 mg/kg Q2W	3 mg/kg Q3W	200 mg Q3W	Total
	(n = 1)	(n = 3)	(n = 9)	(n = 3)	(n = 9)	(n = 10)	(N = 35)
Hypokalemia	0	0	0	1 (33.3)	0	1 (10.0)	2 (5.7)
Renal impairment	0	1 (33.3)	1 (11.1)	0	0	0	2 (5.7)
Arthralgia	0	0	0	1 (33.3)	0	1 (10.0)	2 (5.7)
γ-GT increased	0	0	0	0	2 (22.2)	0	2 (5.7)
irAEs in >1 patient in total population, n (%)							
Blood TSH increased	0	1 (33.3)	1 (11.1)	1 (33.3)	1 (11.1)	2 (20.0)	6 (17.1)
Rash	0	0	1 (11.1)	0	1 (11.1)	2 (20.0)	4 (11.4)
Hypothyroidism	0	1 (33.3)	0	1 (33.3)	1 (11.1)	0	3 (8.6)
Hyperthyroidism	0	0	1 (11.1)	1 (33.3)	0	1 (10.0)	3 (8.6)
Pruritus	0	0	0	0	1 (11.1)	1 (10.0)	2 (5.7)

TRAE, treatment-related adverse event; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TSH, thyroid-stimulating hormone; WBC, white blood cell; GT, glutamyl transpeptidase; irAE, immune-related adverse event.

grade ≥3, 17.1%). One of the reasons may be that a higher proportion of patients in our study had a bad performance status (ECOG performance status of 1, 91.4%). In addition, the patients in our study were heavily pre-treated, with 37.1% patients received two lines of prior therapy and 51.4% patients received three or more lines of prior therapy. The incidence of skin toxicity with QL1604 (rash, 14.3%; and pruritus, 14.3%) was similar to that reported for pembrolizumab (pruritus, 17%). Compared with pembrolizumab, elevation of liver enzymes was more frequently with QL1604 (increased AST, 17.1%; and increased ALT, 14.3%). but all was grade 1 or 2. Overall, the safety profile of QL1604 is manageable, and the proportion of patients who discontinued study treatment because of AEs (8.6%) was comparable to that reported in the phase I study of pembrolizumab (10%) (16). No QL1604-related death occurred in our study.

QL1604 demonstrated signal of antitumor activity in NSCLC and NPC. In patients who had metastatic NSCLC and had progressed on or after standard therapy, five of the 18 patients had a PR (ORR, 27.8%). In KEYNOTE-001, pembrolizumab resulted in an ORR of 19.4% (96/495) in patients with locally advanced or metastatic NSCLC (17). PR was also observed in two of the three patients with NPC. In KEYNOTE-122, pembrolizumab resulted in an ORR of 21.4% (25/117) in patients with platinum-pretreated, recurrent, or metastatic NPC (18). SD was observed in NSCLC, EC, SCLC, and NPC. One patient with NSCLC had a PR as best response, and response was still ongoing as of data cutoff (120

TABLE 3 Grade ≥3 treatment-related adverse events (safety population).

Variable, n (%)	0.3 mg/kg Q2W	1 mg/kg Q2W	3 mg/kg Q2W	10 mg/kg Q2W	3 mg/kg Q3W	200 mg Q3W	Total
	(n = 1)	(n = 3)	(n = 9)	(n = 3)	(n = 9)	(n = 10)	(N = 35)
Grade ≥3 TRAEs	0	0	2 (22.2)	1 (33.3)	2 (22.2)	1 (10.0)	6 (17.1)
Hypokalemia	0	0	0	1 (33.3)	0	0	1 (2.9)
Hyperglycemia	0	0	0	0	0	1 (10.0)	1 (2.9)
Weight gain	0	0	1 (11.1)	0	0	0	1 (2.9)
Hypertriglyceridemia	0	0	0	1 (33.3)	0	0	1 (2.9)
Immune-mediated hepatitis	0	0	0	0	1 (11.1)	0	1 (2.9)
Immune-mediated myopathy	0	0	1 (11.1)	0	0	0	1 (2.9)
Increased blood creatinine phosphokinase	0	0	0	0	1 (11.1)	0	1 (2.9)
Myasthenia gravis	0	0	1 (11.1)	0	0	0	1 (2.9)
Increased γ-GT	0	0	0	0	1 (11.1)	0	1 (2.9)

TRAE, treatment-related adverse event; GT, glutamyl transpeptidase.

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TABLE 4 Efficacy of QL1604 in patients with advanced solid tumors (full analysis set).

Variable	0.3 mg/kg Q2W (n = 1)	1 mg/kg Q2W (n = 3)	3 mg/kg Q2W (n = 9)	3 mg/kg Q2W (n = 3)	3 mg/kg Q3W (n = 9)	3 mg/kg Q3W (n = 10)	Total (N = 35)
Best overall response, n (%)							
CR	0	0	0	0	0	0	0
PR	0	1 (33.3)	1 (11.1)	1 (33.3)	2 (22.2)	2 (20.0)	7 (20.0)
SD	0	0	3 (33.3)	1 (33.3)	1 (11.1)	0	5 (14.3)
PD	1 (100)	2 (66.7)	4 (44.4)	1 (33.3)	5 (55.6)	6 (60.0)	19 (54.3)
NE	0	0	1 (11.1)	0	1 (11.1)	2 (20.0)	4 (11.4)
ORR (95% CI)[a, c]	0 (0–97.5)	33.3% (0.8%–90.6%)	11.1% (0.3%–48.2%)	33.3 % (0.8%–90.6%)	22.2% (2.8%–60.0%)	20.0% (2.5%–55.6%)	20.0% (8.4%–36.9%)
DCR (95% CI)[b, c]	0 (0–97.5)	33.3% (0.8%–90.6%)	44.4% (13.7%–78.8%)	66.7% (9.4%–99.2%)	33.3% (7.5%–70.1%)	20.0% (2.5%–55.6%)	34.3% (19.1%–52.2%)

CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable; ORR, objective response rate; CI, confidence interval; DCR, disease control rate.
a. ORR was defined as the proportion of patients who had a CR or PR as best response per RECIST version 1.1 by investigator.
b. DCR was defined as the proportion of patients who had a CR, PR, or SD as best response per RECIST version 1.1 by investigator.
c. The 95% CI was calculated by using the Clopper–Pearson method.

TABLE 5 Pharmacokinetics of single dose of QL1604 (pharmacokinetics population).

Variable	0.3 mg/kg (n = 1)	1 mg/kg (n = 3)	3 mg/kg Q2W (n = 9)	10 mg/kg (n = 3)	3 mg/kg Q3W (n = 9)	200 mg (n = 10)
AUC_{0-t} (h*μg/mL), geometric mean (CV%)	984 (NE)	4460 (19.2)	14900 (27.9)	50,300 (5.4)	11,400 (32.2)	12,800 (26.0)
$AUC_{0-\infty}$ (h*μg/mL), geometric mean (CV %)	1010 (NE)	3620 (NE)[a]	14000 (23.4)[b]		11,200 (74.7)[b]	
C_{max} (μg/mL), geometric mean (CV%)	4.907 (NE)	19.865 (4.9)	68.1874 (24.6)	195.3847 (11.6)	55.6076 (22.7)	64.6668 (16.8)
T_{max} (h), median (range)	7.0 (NE)	1.08 (1.08–3.00)	1.08 (1.08–3.03)	1.25 (1.08–3.00)	3.00 (1.08–7.00)	1.08 (0.83–6.78)
$T_{1/2}$ (h), geometric mean (CV%)	136.3 (NE)	80.93 (NE)[a]	98.591 (65.3)[b]		273.447 (86.9)[b]	

AUC_{0-t}, area under the curve from zero up to a definite time t; $AUC_{0-\infty}$, area under the curve from 0 extrapolated to infinite time; C_{max}, maximum concentration; T_{max}, time to C_{max}; $T_{1/2}$, half-life; CV, coefficient of variation.
Drug concentration data below the limit of quantification (BLQ) between two measurable drug concentration data were analyzed as missing values. Other BLQ drug concentration data were imputed with "0" if before T_{max} or analyzed as missing values if after T_{max}.
[a]n = 1.
[b]n = 2.

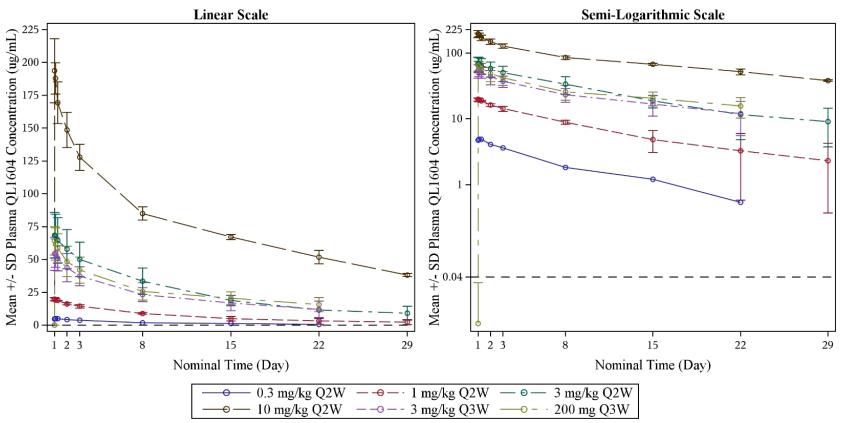

FIGURE 2
Mean blood drug concentration (± standard deviation)–time curve by dose levels (single dose) (PK analysis set). LLOQ = 0.040 μg/mL; PK, pharmacokinetics.

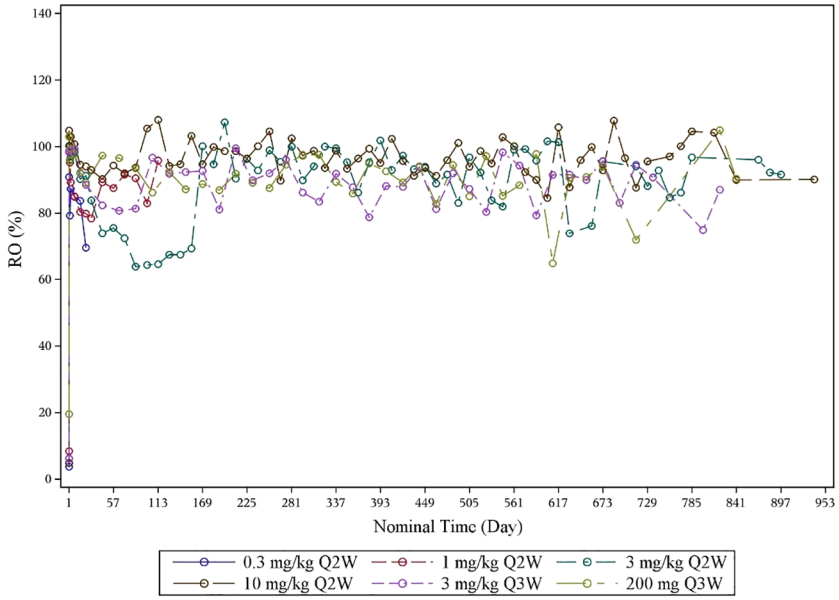

FIGURE 3
Mean RO–time curve by dose levels (RO analysis set). PD-1 receptor occupancy was detected by flow cytometry. RO, receptor occupancy.

weeks after first dose of QL1604). Together, preliminary efficacy results from this study support further clinical studies of QL1604 in multiple tumor types.

Compared with pembrolizumab ($t_{1/2}$, 14 to 22 days), the half-life of QL1604 was shorter ($t_{1/2}$ of QL1604, 3 to 11 days). Serum exposure to QL1604 increased in a dose-proportional manner in the dose range of 0.3 mg/kg to 10 mg/kg in single-dose phase. At a steady state, serum exposure to QL1604 increased approximately in a dose-dependent manner, but the dose proportionality was not observed. Analyses of the PK parameters at a steady state showed accumulation of QL1604 after Q2W or Q3W administration. Similar to pembrolizumab, the PD-1 target engagement by QL1604 was durable for at least one treatment cycle (mean change from baseline in RO on cycle 1 day 22, 81.294%). No difference was observed for 3 mg/kg Q3W and 200 mg Q3W.

The efficacy, safety, and PK/PD data supported dosing of QL1604 every 2 or 3 weeks at doses of 3 mg/kg or 200 mg. No DLT was observed at the planned highest dose level 10 mg/kg. Thus, MTD was not determined yet. In addition, PRs were observed at all dose levels except 0.3 mg/kg Q2W. All doses were well tolerated. Tumor response and incidence of AEs were not dose dependent. Single dose of QL1604 exhibited a PK profile that is typical of mAbs with a dose-dependent increase in the PK exposure ranging from 0.3 mg/kg to 10 mg/kg. RO assessment by flow cytometry is a key PD biomarker, which reflects the relative binding of a therapeutic mAb to its cell-surface target (19). Mean RO for QL1604 at the dose of 3 mg/kg Q2W, 3 mg/kg Q3W, 10 mg/kg Q2W, and 200 mg of fixed dose Q3W was greater than 80% during cycle 1 after one infusion, and no difference was observed for 3 mg/kg Q3W and 200 mg Q3W. The RO results were also comparable to that reported in the phase I study of nivolumab, in which PD-1 occupancy also appeared to be dose-independent, with a mean peak occupancy of 85% (range, 70% to 97%) at 4 h to 24 h after one infusion (20).

Conclusions

In summary, QL1604 monotherapy showed favorable safety, PK, and signal of antitumor activity in patients with advanced or metastatic solid tumors, and the results supported further clinical studies of QL1604. On the basis of the safety, PK, and RO data, the recommended dosage for further clinical trials is 3 mg/kg or a fixed dose of 200 mg given every 3 weeks.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Zhejiang Cancer Hospital Ethics Committee. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

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YX: Data curation, Methodology, Writing – review & editing. WH: Data curation, Methodology, Writing – review & editing. LG: Data curation, Methodology, Writing – review & editing. KC: Data curation, Methodology, Writing – review & editing. JQ: Data curation, Methodology, Writing – review & editing. FX: Data curation, Methodology, Writing – review & editing. FW: Data curation, Methodology, Writing – review & editing. XT: Data curation, Methodology, Writing – review & editing. XM: Data curation, Methodology, Writing – review & editing. WF: Methodology, Project administration, Writing – review & editing. LL: Methodology, Project administration, Writing – review & editing. BZ: Formal Analysis, Methodology, Writing – review & editing. XK: Methodology, Project administration, Writing – review & editing. YF: Conceptualization, Investigation, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

Authors WF, LL, BZ, and XK are full time employees of the company Qilu Pharmaceutical Co., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2023.1258573/full#supplementary-material

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A case report and literature review on reactive cutaneous capillary endothelial proliferation induced by camrelizumab in a nasopharyngeal carcinoma patient

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Camrelizumab, a monoclonal antibody, blocks programmed cell death protein-1 from binding to T cells and programmed cell death ligand 1 on tumor cells, thereby ensuring sustained T cell activation and blocking immune escape of various types of cancer, including nasopharyngeal carcinoma. Reactive cutaneous capillary endothelial hyperplasia (RCCEP) is the most common immune-related adverse event in patients treated with camrelizumab. We report a case nasopharyngeal carcinoma in a patient with camrelizumab-induced RCCEP. A 68-year-old man diagnosed with nasopharyngeal carcinoma developed RCCEP at multiple locations after 3 months of camrelizumab treatment. RCCEP of the right lower eyelid affected closure of the right eye. In this report, we also reviewed previous literature on camrelizumab-induced RCCEP. In summary, the mechanism underlying camrelizumab-induced RCCEP remains unclear. RCCEP typically gradually subsides after discontinuing camrelizumab treatment. Larger nodules can be treated with lasers, ligation, or surgery. Although surgical excision is effective, RCCEP may recur in patients undergoing camrelizumab treatment. RCCEP management may not be required in the absence of adverse effects on the patient's daily life.

KEYWORDS

reactive cutaneous capillary endothelial proliferation, camrelizumab, nasopharyngeal carcinoma, case report, literature review

Introduction

In recent years, immune checkpoint blockade therapy has demonstrated remarkable efficacy in the treatment of various malignant tumors (1, 2). Previous studies have shown that programmed cell death ligand 1 is highly expressed in patients with nasopharyngeal carcinoma (NPC) (3–5). Therefore, the combination of programmed cell death protein 1

and programmed cell death ligand 1, which are expressed by T cells and tumor cells, respectively, can block signal transduction and enhance immune system activity, thereby destroying cancer cells (6, 7). Camrelizumab is a monoclonal antibody against programmed cell death protein 1 that was developed by Jiangsu Hengrui Medicine Co (8).

Immune checkpoint inhibitors are associated with a range of immune-related adverse events (irAEs) (9, 10), which are often associated with immune system overactivation. Reactive cutaneous capillary endothelial hyperplasia (RCCEP) is the most common adverse event associated with camrelizumab use and usually occurs in the skin of the head, face, and trunk (11). However, the mechanism underlying camrelizumab-induced RCCEP remains unclear. We herein present a case in which a patient with NPC developed RCCEP, a "tumor-like" nodule, on the right lower eyelid after undergoing camrelizumab and chemotherapy. RCCEP uncommonly occurs in this location, and the nodule interfered with the patient's ability to close the right eye owing to the thinness of the skin in this area. The nodule was surgically removed, and the patient's prognosis was good.

Case report

In October 12, 2021, a 68-year-old man was diagnosed with T3N2M1 NPC, based on the American Joint Committee on Cancer's Cancer Staging Manual, Eight Edition (12). The patient received chemotherapy (capecitabine, 625 mg/m2 twice daily, orally) and immunotherapy (camrelizumab injection, 200 mg every 21 days). On February 17, 2022, the patient underwent the seventh cycle of camrelizumab injection therapy. The timeline of the patient's entire treatment progress is shown in Figure 1. Approximately 6 days later, the patient developed scattered bright red spots on the head, face, and trunk. Some spots gradually developed into pea-sized nodules (Figure 2). Two months later, the nodule on the right lower eyelid had grown to the size of a peanut (Figure 2). Head computed tomography revealed a nodule that protruded outward and squeezed the normal eye tissue inward (Figure 3). In addition, the nodule pulled the lower eyelid downwards, which affected eyelid closure owing to the thin skin and soft tissue of the lower eyelid (Figures 2A, C). After considering the patient's condition and preferences, we resected the right lower eyelid nodule. The patient recovered well postoperatively and was able to close the right eye without difficulty (Figures 2B, D).

The removed nodule is shown in (Figure 4A). Histopathological examination revealed that the lesions comprised proliferated capillaries, which were distributed in nodular and lobulated forms. Large vessels were surrounded by small vessels; lumens varied in size and contained red blood cells (Figure 4B). Vascular endothelial cells were densely arranged. The nuclei were oval or short and spindle-shaped; mitotic figures were easily observed (Figure 4C). These pathological results supported a diagnosis of RCCEP. During the 6-month follow-up period, the surgical area of the right lower eyelid had recovered well, without RCCEP recurrence (Figure 2E). Because the patient continued to receive camrelizumab postoperatively, dark red nodules remained on other parts of the body (Figure 2E); however, these lesions resolved spontaneously after the patient completed 1 year of immunotherapy. According to Naranjo's adverse drug reaction probability scale (Table 1), RCCEP was most likely caused by camrelzumab.

Discussion

RCCEP occurrence after camrelizumab administration

Skin reactions in various organs are the most frequent irAEs, which are triggered by immune checkpoint inhibitors (13). The most frequent side effect of camrelizumab is RCCEP (8, 11). RCCEP appears primarily in the skin of the head, face, and torso (11). RCCEP in these regions is not typically fatal, although may affect function and coordination in the affected regions. The present case involved a patient who received camrelizumab therapy and subsequently developed right lower eyelid RCCEP that affected the patient's ability to close the eye. According to current RCCEP grading criteria, this patient was classified as having a grade 2 lesion (single or multiple nodules, with the greatest nodule diameter being >10 mm, with or without rupture and bleeding) (14). Surgery was performed to restore the patient's ability to close the right eye. Postoperative histopathological examination confirmed

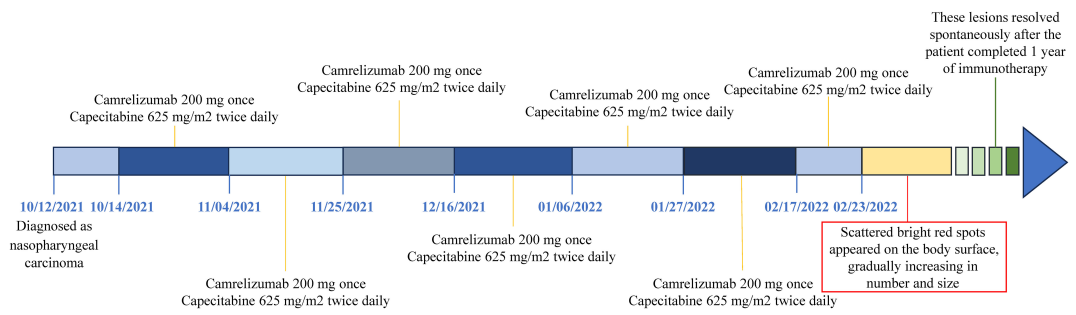

FIGURE 1
The timeline of patient progress throughout treatment.

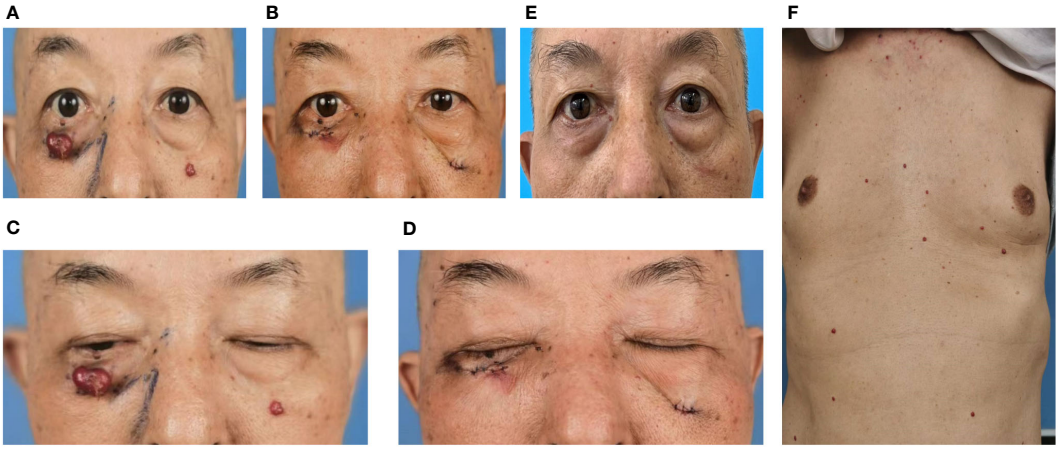

FIGURE 2
Dark red nodules were distributed over the patient's head, face, and trunk. **(A, C)** Before surgery. **(B, D)** After surgery. **(C)** Before surgery, the nodule pulled on the right lower eyelid, resulting in incomplete closure of the right eye. **(D)** After surgery, the patient could close the right eye normally. **(E)** Six months after the operation, no recurrence of the right lower eyelid nodule was observed, although new nodules were noted in other parts of the face. **(F)** Dark red nodules distributed over the patient's trunk before surgery.

the RCCEP diagnosis. Since treatment with camrelizumab and capecitabine was effective, this regimen was continued postoperatively. Although the unresected nodules persisted and new nodules appeared, the patient's daily life was unaffected. The RCCEP spontaneously resolved after the patient completed 1 year of treatment.

Potential mechanism of camrelizumab-induced RCCEP and comparison with other capillary hemangiomas

The mechanism by which camrelizumab triggers RCCEP is currently unclear. The predominant theory is that skin capillary endothelial cells exhibit overly active immune responses. RCCEP is histopathologically characterized by enhanced capillary endothelial cell proliferation and numerous mitotic figures. The molecular mechanism of camrelizumab may be that it activates CD4+ T lymphocytes, thereby increasing interleukin-4 levels in T helper 2 cytokines. This subsequently stimulates CD163+ M2 macrophage differentiation and promotes capillary endothelial cell proliferation by releasing vascular endothelial growth factor (VEGF) A (11, 15). Camrelizumab may also induce RCCEP by causing VEGF receptor-2-induced activation of vascular endothelial cell proliferation (16). These proposed mechanisms offer several potential targets for RCCEP prevention.

RCCEP can be classified as a cherry hemangioma (CH), both grossly and histopathologically (17, 18). CHs are the most prevalent form of acquired cutaneous vascular hyperplasia and are more common in older adults; the most frequently affected sites are the trunk and upper extremities (19, 20). CH etiology is attributed to gene mutations, chemical exposure, and viral infection (19). Gene mutation studies have focused on GNAQ, GNA11, and GNA14 (21, 22). Moreover, evidence suggests that VEGFR2 mutations can cause CHs, although the specific mechanism has not been elucidated (23, 24). CH has distinct clinical and histopathological features, although it is not included in the most recent edition of the International Society for the Study of Vascular Anomalies classification of vascular anomalies (25). The early stage of a CH is usually characterized by a flat red spot that gradually enlarges and becomes a red, blue, or purple papule. Histopathological investigations have revealed that CHs consist of

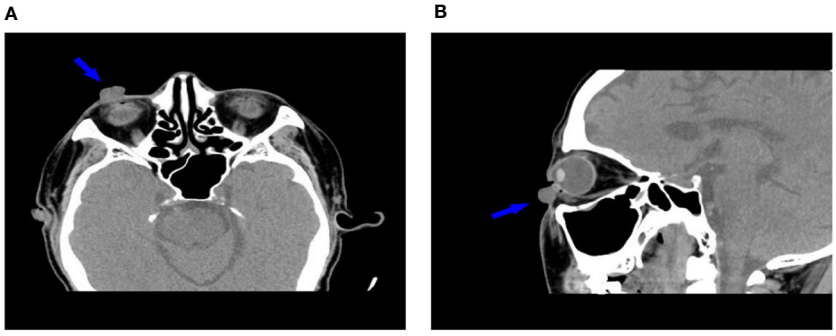

FIGURE 3
Head computed tomography (**A**: transverse plane, **B**: sagittal plane) shows the relationship between the nodule and surrounding soft tissues (arrows).

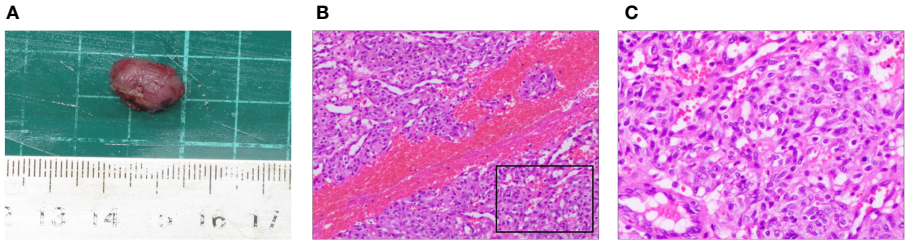

FIGURE 4
Pathological characteristics of the right lower eyelid nodule. **(A)** The size of the surgically resected nodule was about 1.5 cm × 1.0 cm × 0.9 cm. **(B, (C)** Hematoxylin and eosin staining showed extensive capillary proliferation. **(B)** × 200; **(C)** × 400.

lobulated, small-to-mildly dilated, thin-walled vessels with various sized lumens lined with a single layer of endothelial cells (21). These lesions are typically asymptomatic and do not require specific management. Effective treatment methods for CHs can also be used to treat RCCEP.

Infantile hemangioma (IH), also known as infantile capillary hemangioma or strawberry hemangioma, is a benign lesion commonly found on the head, neck, trunk, and extremities. Most of these lesions resolve spontaneously (26). IH growth can be divided into three stages: rapid vascular endothelial cell proliferation, decreased vascular endothelial cell proliferation, and replacement of vascular tissue with fibrofatty tissue (27). Oral propranolol administration, laser therapy, and surgery are the most common clinical treatment options for IH (28). However, the pathogenesis of IH is unclear. The current mainstream view is that pluripotent stem cells respond abnormally to stimuli, such as hypoxia and the renin-angiotensin system (27). As with CH, GNAQ, GNA11, and GNA14 mutations may also cause IH (29–31). Furthermore, gene mutations may interfere with the VEGF A signaling pathway (32, 33). VEGF receptor-2 is the receptor for VEGF A, and some patients with IH have VEGFR2 mutations (34, 35).

Pyogenic granuloma (PG), which is more accurately termed lobular capillary hemangioma, is an acquired benign lesion that occurs in tissues such as the skin and mucous membranes (36, 37). Chronic mild irritation, hormonal imbalances, and drug influences are considered the main PG etiologies (38–40). Cutaneous PG manifests as painless, red, and fleshy nodules that closely resemble RCCEP. Histologically, PG consists of clusters of proliferating capillaries arranged in a lobular structure (41, 42). Current evidence attributes its pathogenesis to effects on the upstream mediator gene, BRAF, on the mitogen-activated protein kinase pathway (43, 44). Although some PGs resolve spontaneously, most require treatment. Treatments include surgical resection, cryotherapy, laser therapy, and imiquimod cream. Among these treatments, surgical resection is the most effective and results in the lowest recurrence rate (37, 45).

Prevention and treatment of RCCEP caused by camrelizumab

Apatinib has successfully lowered the incidence of RCCEP (46–49). Apatinib is a tyrosine kinase inhibitor that selectively inhibits VEGF receptor-2 (50, 51) and inhibits VEGF-induced endothelial cell migration and proliferation, thereby preventing new blood vessel formation in the tumor tissue. Therefore, the combination of apatinib and camrelizumab may prevent RCCEP development by inhibiting capillary endothelial cell proliferation.

Many studies have shown that patients receiving camrelizumab combined with chemotherapy have better progression-free and overall survival rates than those of patients receiving chemotherapy alone (52–57). Camrelizumab and chemotherapy combined can achieve greater clinical benefits in patients with advanced NPC (58, 59). Camrelizumab combined with chemotherapy can also reduce the risk of RCCEP. Fang et al. reported that camrelizumab administration alone in patients with NPC resulted in a RCCEP incidence of 88% (82/93), compared with only 22% (5/23) when camrelizumab was administered in combination with gemcitabine and cisplatin (58).

TABLE 1 Naranjo's adverse drug reaction probability scale.

Related issues	results	score
1. Are there previous conclusive reports of this reaction?	yes	+1
2. Did adverse event appear after the suspected drug was given?	yes	+2
3. Did the adverse reaction improve when the drug was discontinued or a specific antagonist was given?	yes	+1
4. Is the ADR repeated after the use of the suspected drug again?	not known	0
5. Are there alternative causes that could have caused the reaction?	no	+2
6. Did the reaction reappear when a placebo was given?	not known	0
7. Was the drug detected in any body fluid in toxic concentrations?	not known	0
8. Was the reaction more severe when the dose was increased, or less severe when the dose was decreased?	not known	0
9. Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	no	0
10. Was the adverse event confirmed by any objective evidence?	no	0
Total score		6

Naranjo's score ≥ 9 points: definite, 5-8 points: probable, 1-4 points: possible, ≤ 0 points: doubtful.

By preserving immune activity, anti-programmed cell death protein 1 therapy suppresses tumors over the long term. An overactivated immune system may cause irAEs. Improvements in illness prognosis and the emergence of irAEs represent two sides of the same coin. The clinician is responsible for adjusting the medication regimen according to the clinical situation and intervening if adverse reactions occur. As previously stated, most patients with RCCEP do not require special treatment. RCCEP may gradually resolve if camrelizumab is ineffective and subsequently discontinued. If rupture and bleeding occur, the wound surface should be promptly disinfected, and antibacterial drugs should be administered externally if necessary. Therapeutic measures can be taken when RCCEP adversely affects the patients' daily life. Traditional treatment methods include cryotherapy, electrosurgery, ligation, and surgical resection (19, 60). With the recent development of light therapy, safer and more effective options have become available for the treatment of capillary hemangiomas; nevertheless, these treatments are expensive (61–63). Several types of lasers can be used to treat capillary hemangiomas, including pulsed dye, alexandrite, neodymium-doped yttrium aluminum garnet, copper bromide, krypton, 532-nm diode, and potassium-titanyl-phosphate lasers (63–67). Intense pulsed light therapy can also be used to treat capillary hemangiomas (68). In cases of grade 3 or higher RCCEP, drug therapy should be immediately discontinued to reduce the mortality risk.

Conclusion

The development of immunotherapeutic drugs has increased the possibilities for cancer treatment. Meanwhile, cancer diagnosis and treatment require ever-increasing levels of cooperation among multiple disciplines, and increased focus on safe and rational drug administration is required by clinicians. Herein, we described a patient with camrelizumab-induced RCCEP in the right lower eyelid. Although this lesion affected the patient's ability to close the right eye, their prognosis was good after surgery. This case illustrates the importance of considering the therapeutic efficacy versus the risk to maximize the benefit during cancer treatment. In addition, the data obtained in our clinical practice and provided herein can be used as a reference for improved medication regimen guidance.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of the Second Affiliated Hospital of Shantou University Medical College. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YL: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. YXL: Data curation, Writing – original draft. XZ: Methodology, Supervision, Writing – review & editing. QC: Supervision, Writing – review & editing. ST: Methodology, Supervision, Writing – review & editing. JC: Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Revolutionizing cancer immunotherapy: unleashing the potential of bispecific antibodies for targeted treatment

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Recent progressions in immunotherapy have transformed cancer treatment, providing a promising strategy that activates the immune system of the patient to find and eliminate cancerous cells. Bispecific antibodies, which engage two separate antigens or one antigen with two distinct epitopes, are of tremendous concern in immunotherapy. The bi-targeting idea enabled by bispecific antibodies (BsAbs) is especially attractive from a medical standpoint since most diseases are complex, involving several receptors, ligands, and signaling pathways. Several research look into the processes in which BsAbs identify different cancer targets such angiogenesis, reproduction, metastasis, and immune regulation. By rerouting cells or altering other pathways, the bispecific proteins perform effector activities in addition to those of natural antibodies. This opens up a wide range of clinical applications and helps patients with resistant tumors respond better to medication. Yet, further study is necessary to identify the best conditions where to use these medications for treating tumor, their appropriate combination partners, and methods to reduce toxicity. In this review, we provide insights into the BsAb format classification based on their composition and symmetry, as well as the delivery mode, focus on the action mechanism of the molecule, and discuss the challenges and future perspectives in BsAb development.

KEYWORDS

bispecific antibodies, immunotherapy, targeted therapy, tumor microenvironment, cancer

1 Introduction

Currently emerging cancer immunotherapies include cancer vaccines, T cell receptor T cells (TCR-T) or chimeric antigen receptor T cells (CAR-T), cytokine therapies, immune checkpoint blockades (ICBs), and tumor-targeted antibodies (1). Monoclonal antibodies (mAbs), in particular, are powerful tumor-targeted antibodies that have been licensed for use in cancer in the US and Europe for the first time in 2022 (2). However, the complicated

pathophysiology of tumors limits the therapeutic efficacy of mAbs (3, 4), while the combination of two or more mAbs may be subject to safety and efficacy issues (5). Bispecific antibodies (BsAbs) have been developed to bind two specific epitopes or target proteins at the same time. These antibodies have improved specificity, increased targeting ability, and reduced off-target toxicity. Moreover, BsAbs have the potential to effectively lower the cost of treatment, revitalizing the field of cancer immunotherapy (6).

The first BsAb was created in the early 1960s and was based on mild reoxidation of binding fragments from two different polyclonal sera (7). Later, based on enzymatic digestion of hybridoma peptides, hybridoma technology allowed the chemical coupling of mAbs or fragment antigen-binding (Fab) fragments (8). With the rapid development of genetic engineering technology, the multifunctional BsAb formats received great attention in clinical application. Mechanically, BsAbs inhibit tumor progression directly or indirectly mainly by redirecting immune effector cells into tumors, delivering radioactive or drug payloads to cancer cells, targeting multiple signaling pathways, and so on. For example, BsAb drug catumaxomab, which contains anti-epithelial cell adhesion molecule (EpCAM) and anti-cluster of differentiation 3 (CD3) molecule, destroys tumors via T cell-driven lysis, cytotoxicity triggered by antibodies, and phagocytosis via helper cells with Fcγ receptors (FcγRs) (9, 10). Four BsAb medications, including tebentafusp, faricimab, mosunetuzumab, and teclistamab, were approved for marketing in 2022 alone, suggesting that BsAbs are promising approaches to develop antitumor therapies (Figure 1).

In the review, we will systematically cover the antitumor principle and clinical applications of BsAbs in multiple formats. We will also introduce the preparation technology and delivery method of BsAb, and discuss their challenges and prospects in the treatment of solid tumors.

2 Format of BsAbs

The power of BsAbs lies in their capacity to create new activities that demand the union of two binding specificities in a single molecule (28). Their functionality can be greatly impacted by domain composition or "shifting" linker length and unique arrangements of (non-)chemical bonds. The design of BsAbs' forms can also affect other factors including diffusion and pharmacokinetic activity (29, 30). In addition to expression platform's stability and output, the presence or absence of undesirable side products is another factor that must be taken into account. The wide range of BsAb formats produced by the numerous designing methods can be categorized by their design elements or functional characteristics (28).

2.1 Classification of BsAbs based on composition

Based on structural components, BsAbs could be roughly classified into BsAbs with Fragment crystallizable (Fc) regions and BsAbs without Fc regions. BsAbs with Fc regions can help activate the immune system via Fc domain's interaction with cell surface receptors, as well as endow the BsAb molecules with longer half-lives on account of their larger sizes and the neonatal Fc receptors (FcRn)-mediated recycling pathway (31). However, Fc region's engagement with FcγRs can lead to serious cytotoxicity events, which may be a merit of BsAbs without Fc region (32). The Fragment variable (Fv)-only molecules are also easier to produce. While BsAbs without Fc domains lack interactions with CH (constant heavy chain)1/CL (constant light chain) regions, more techniques must be applied to stabilize the Fab regions.

2.1.1 BsAbs with Fc regions

BsAbs that contain the Fc region include Immunoglobulin G (IgG) constructs such as Duobody (controlled Fab-arm exchange technology) (33, 34), Fabs-In-Tandem Immunoglobulin (FIT-Ig) (35), Cross-Mab (36), scFv-Fc constructs (single chain variable fragment), VHH-Fc constructs, and dual-affinity retargeting (DART)-Fc constructs (37).

Fc region offers BsAbs a number of advantages. The engagement of Fc region with membrane Fc receptors (FcRs) and certain complement system proteins help to activate the immune response. The Fc-directed receptor downregulation and malignant cells apoptosis through monocyte/macrophage trogocytosis is required for the antitumor efficacy of amivantamab (38, 39). These BsAbs have longer half-lives because of their big size and recycling pathways controlled by FcRn (37). An entire IgG antibody has a molecular mass of 150 kDa and is removed by the liver, whereas molecules with a molecular weight less than 60 kDa are filtered by the renal system (40). The combination of homologous variable heavy chain (VH) and variable light chain (VL) domains is further driven by the fusion of a heterodimerizing Fc region, making purification with affinity resins like protein G feasible (41).

However, off-target cytotoxicity and reduced treatment efficiency are associated with Fc-mediated downstream actions. When Fc region of medicinal antibodies interact with FcγRs, serious adverse effects may occur (42). Except for safety concerns, CD3-directing BsAbs with an active core demonstrated less effective *in vivo* (28, 43). To reduce the aforementioned negative effects, presently available BsAbs targeting CD3 either omit the Fc region or have modified Fc domains to minimize FcγR interaction (28).

2.1.2 BsAbs without Fc regions

BsAbs without Fc region lack the Fc-mediated effector actions mentioned above, but they aid in eliminating the chain-association problem. Moreover, the formats can be produced economically and high-yieldingly by expressing 1–2 peptides strands in simple eukaryotic and prokaryotic protein synthesis platforms (28, 44, 45).

BsAbs without Fc region mainly consist of tandem single-chain variable fragments (scFv2, taFv), bispecific one-domain antibody hybrid proteins, diabodies, and fragment antigen-binding (Fab fusion protein) (30). The taFv, which stands for the minimum BsAb, can be created by joining two scFvs together with a linker and normally ranges 50-60 kDa in size (30). However, these Fv-only moieties are short of the native-like connections with CH1/CL regions which is required for the stability and solubility of Fab

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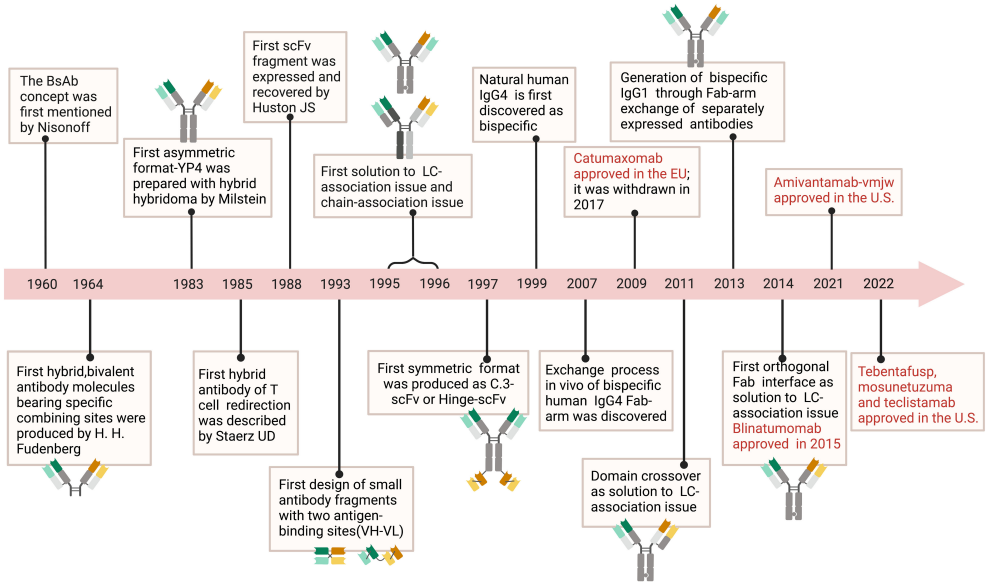

FIGURE 1
Timeline. The timeline showcases the technical innovations and clinical research in tumor of BsAb. In 1960, the concept of BsAb was proposed (11). In 1964, researchers created molecules with two different binding sites (12). BsAb with asymmetric structure was produced using hybridoma technology in 1983 (13). In 1985, the idea of BsAb that can redirect T cells was proposed (14). Diabody, a small molecule BsAb fragment, was designed in 1993 (15). In 1988, researchers developed scFv fragments (16). From 1995 to 1996, the problem of protein subunit pairing was first solved (17, 18). In 1997, BsAb with symmetric structure was manufactured (19). It was discovered in 1999 that natural human IgG4 molecules were bispecific (20). In 2007, the process of Fab fragment exchange in human IgG4 was explained (21). Catumaxomab was approved by the EU in 2009 but later withdrawn in 2007 (22). In 2011, the problem of light chain pairing was solved through domain swapping strategy (23). Bispecific IgG1 was produced using Fab fragment exchange in 2013 (11). In 2014, the problem of light chain pairing was solved through orthogonal Fab fragments (24). In 2015, Blinatumomab was approved (24). Amivantamab-vmjw was approved in the U.S. in 2021, and in 2022, Tebentafusp, Mosunetuzuma, and Teclistamab were also approved in the U.S (25–27).

regions (46). By creating a disulfide connection between the VH and VL domains, the stability of tandem scFv can be enhanced (30, 47). Bispecific single-domain antibody hybrid molecules can be made by one-domain antibodies, such as VH or VL domains, VHH, variable new antigen receptor (VNAR) and nanobodies (Nbs) (30). Compared to human programmed cell death-ligand 1 (PD-L1)-targeting mAb or vascular endothelial growth factor receptor type 1 domain 2 (VEGFR1D2) fusion protein alone, the HB0025 that combines the VEGFR1D2 and anti-PD-L1 mAb was more effective at preventing the growth of tumor (48). Diabody is a noncovalent heterodimer comprising the VH and VL portions of the scFv fragment linked by a short peptide. Since only some of the potential arrangements and orientations preserved binding potential for both antigens, it is crucial to choose the ideal VH/VL organization and alignment (30, 49, 50). In addition to domain order, the diabody-Ig platform utilize the dimerization domains CH1/CL, heterodimerizing EH Domain Containing 2 (hetEHD2), EH Domain Containing 2 (EHD2), and IgM heavy chain domain 2 (MHD2) to stabilize the diabody (51, 52). Furthermore, the domain connection was modified to promote heterodimerization (30). Fabs can serve as the foundation to which other binding elements are attached (30). A scFv may be attached to the C-terminal of either the light strand or the VH-CH1 (Fd) chains (e.g., bibody Fab-L-scFv, Fab-H-scFv), or to both strands (e.g., tribody, Fab(scFv)2) when Fabs are connected by hinge-regions (30, 45, 53–59).

The antigen-combining abilities of heavy-chain antibodies are entirely preserved in Nbs created from variable heavy-chain segments (VHH) in camelid heavy-chain antibodies (60). The molecule weight of the Nbs is 12–15 kDa, which is considerably less than the molecule weight of typical antibodies (150 kDa) (61, 62). Nbs with hydrophilic interfaces prevent the discrepancies in the heavy and light chain pairing of traditional antibodies, are not bound to light chains, which are vulnerable to polymerization, and are distinguished by tiny molecular mass, excellent solubility, and persistence (62). Nbs exhibit lower immunogenicity and simpler for humanizing and application in the clinic than traditional antibodies (62, 63). BsAbs can be created by modifying two Nbs which hit separate tumour antigens in order to enhance the selectivity of anticancer antibodies and render them optimal antibodies. In the detection and management of infection, cancer, and immunity, BsNbs are a scientific focus due to the improvement of BsNbs binding capacity, lengthening of plasma half-life, decrease in drug resistance, and severe side effects (62, 64). Liu et al. created the anti-CD20/CD3 BsNb by merging the anti-CD20 VHH gene with a thoroughly validated anti-human CD3 VHH built on the acquired anti-CD20 Nbs. After being incubated with human sera at 37°C for 48 hours, the anti-CD20/CD3 BsNb was still able to retain 80% of its binding efficacy. The findings demonstrated the potent anticancer activity of the developed anti-CD20/CD3 BsNb (62). Employing a BsNb which could concurrently target epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2) on cancer cells, Hong et al. created a dual-directed non-IgG form of BsAbs. The absence of Fc effector functional capabilities was restored by site-selective alteration of the EGFR-HER2-targeted

BsNb employing the rhamnose (Rha) hapten through sortase A-regulated binding. The adjusted BsNb-Rha combination demonstrated significantly better pharmacokinetics and effective inhibiting actions against *in vivo* development of xenograft tumors (65). With the application of in silico methods, an improved bispecific design was created that can engage the therapeutically important antigens TNF-α and TNF-α-23 concurrently and, thanks to its increased avidity, efficiently block the death of TNF-α-sensitive L929 cells (66).

2.2 Classification of BsAbs based on symmetry

Through the lens of symmetry, BsAbs can be categorized into the asymmetric ones and the symmetric ones (Figure 2). Asymmetric BsAbs are initially created by combining two antibody-producing cell lines; with the advancement of genetic engineering, the technology is employed to produce BsAb, greatly assisting with the "chain" problems (it will be clarified in the following text) (Figure 3). Another approach to get around the "chain" issues and make the construction simpler is to design symmetric BsAbs, which could be generated by fusing or modifying IgG proteins.

2.2.1 BsAbs of an asymmetric architecture

Every bispecific IgG molecule (antibody that is similar to natural immunoglobulins in constitute) is bivalent and has an asymmetric architecture because it contains at least distinct Fv regions (30).

Asymmetric BsAbs can be generated by merging two antibody-generating cell lines, e.g., producing a hybrid-hybridoma by fusing YTH12 and the MGICD19 cell lines (73). However, nine unwanted products will be generated by simply fusing two cell lines together (30). Genetic engineering is an alternative to remedy the issue. Through genetic methods, it is possible to create cell lines that produce two separate heavy and light chains and enable their proper integration (30). The heavy chain issue can be handled by BsAbs featuring an asymmetric Fc domain. Several methods created over the past 20 years leverage specific interchain disulfides, as well as steric or electrostatic steering effects to create a complimentary interface, benefiting heterodimerization against homodimerization (30). The knobs-into-holes (KIHs) strategy is a promising way to create BsAbs by inducing heterodimerization with mutations in the CH3 domain of each half antibody (74, 75). It was found that there was no significant change in the conception kinetics of BsAb produced by the KIHs technique, and the stability was similar to that of the wild-type antibody structure (74). Epcoritamab which recognises CD3 and CD20 and was created via cFAE of a humanized CD3 mAb and the human CD20 mAb7D8 (68). In extremely resistant patients with large B-cell lymphoma, notably those who had previously been exposed to CAR T cells, subcutaneous Epcoritamab produced profound reactions as well as reasonable safety (76). Flexible linker peptides may also be employed to fuse Fabs at their C-termini to a highly hereodimerizing Fab-like molecule (30). For instance, TriFabs are BsAbs with an IgG structure made of two conventional Fab connected by elastic linker protein to a single asymmetrical third Fab-sized interaction unit. The third module is S354C-Y349C disulfides connect CH3 knob-hole heterodimers, which replaced the original Fc region (70).

To maintain the related functions and favorable qualities, the major methods to create the forms in the category aim to preserve the structure of natural antibodies precisely. Nevertheless, in certain formats, the complex architecture to address the chain-association problem may negate these benefits (28). Compared to formats that permit multivalent target binding, asymmetric forms' lower avidity may influence their strength (28, 77).

2.2.2 BsAbs of a symmetric architecture

Incorporating two particularities into single heavy & light combination or peptide strand will result in symmetrical BsAbs, and can solve the chain-association problem whilst preserving the Fc domain (28). Also, the symmetric form is easier to construct (58).

One strategy is to produce IgG fusion proteins, to be more specific, by fusing scFv, domain antibodies and scaffold proteins, Fab arms, or additional VH and VL domains. For instance, the T cell-stimulating BsAb CLN-049, which binds to CD3 and FLT3, was created as an IgG heavy chain/scFv hybrid (78). Another strategy is to modify IgG molecules. Either the VH and VL domains' original antigen-binding sites was altered or an extra binding site was transplanted to the Fc fragment's bottom portion (30). With two unique, regionally separated interaction sites inside the human antibody CDR loops, dual targeting Fab (DutaFab) molecules was developed (79).

While almost mimicking natural antibodies, symmetric forms bear differences in size and organization. These variations may adversely alternate antibodies' features (eg, consistency and solubility), which could disrupt their physicochemical and/or pharmacokinetic qualities (28, 80, 81). Most clinical test formats feature tetravalent 2+2 configurations due to the symmetric character and thus anticipated to have enhanced affinity against both malignant cells and T cells. However, this was just of secondary significance in terms of therapeutic efficacy. Despite sharing identical neoplasm binding and having improved T cell interaction compared to 2+2, 2+2B (Bispecific T cell engager (BiTE)-Fc) and 2+2HC (IgG-[H]-scFv) both failed to exhibit anticancer efficacy *in vivo* (28, 58).

3 Preparation technology of BsAbs

Methods of preparation of BsAbs are classified as chemical coupling, hybridoma binding and gene cloning methods (82, 83). Chemical cross-linking is the process of forming disulfide bonds between antibody molecules of different specificities or F(ab') by using a specific chemical cross-linking agent, thus creating a heterodimer. This can be in the form of coupling between whole antibody molecules, or between F(ab') and F(ab')2 (84). BsAbs

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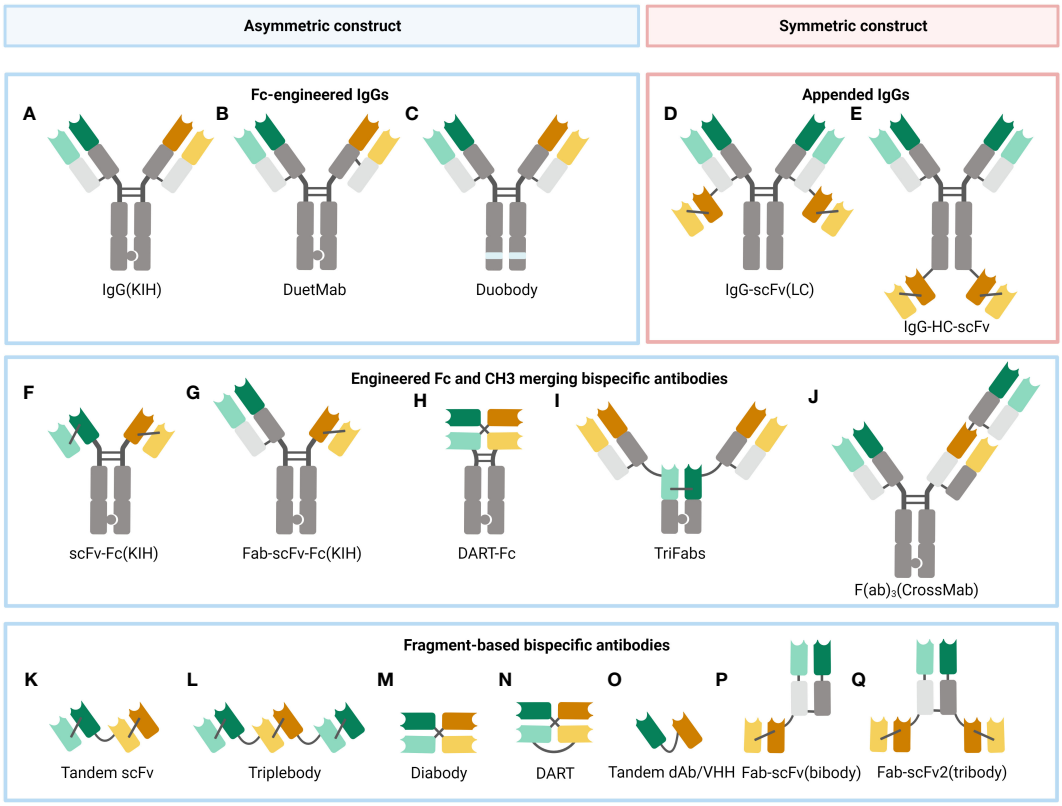

FIGURE 2
A selection of some common BsAb formats. **(A)** Fc-modified IgG format, built with the KIH technology to heterodimerize two different heavy chains. **(B)** DuetMab, improving the efficacy of homologous heavy and light chain coupling by designing a new disulfide bond to substitute the natural one in one of the CH1-CL interfaces (67). **(C)** Duobody, its Fc region was suppressed by inserting mutations, which circumvents the Fc-mediated cytotoxicity (68). **(D)** Appended IgG format, IgG- single-chain variable fragment (scFv) (light chain, LC). **(E)** Appended IgG format, IgG-heavy chain (HC)-scFv. **(F)** scFv-Fc format, constructed with the KIH approach. **(G)** Fab-scFv-Fc format, built with the KIH method. **(H)** DART-Fc construct, a DART protein unites two separate antigen-binding regions in a stable, diabody-like architecture (69). **(I)** TriFabs, IgG-based BsAbs made up of two normal Fab arms connected by flexible linker peptides to a third Fab-sized interaction unit (70). **(J)** CrossMab, antibody domain crossover enables the proper connection of generic light chains (71). **(K)** Tandem scFv (taFv), the minimum BsAb. **(L)** Triplebody, a construct similar to taFv. **(M)** Diabody (db), a short protein linker joins the heavy chain variable (VH) and light chain variable (VL) domains of a scFv segment to form a noncovalent heterodimer. **(N)** DART, made up of two Fv segments that heterodimerize to generate two distinct antigen-binding sites. **(O)** Tandem single-domain antibody (dAb)/VHH, made up of the antigen-engaging portion of heavy chain-only antibodies (72). **(P)** Fab-scFv (bibody), a scFv segment is fused to the C-terminus of the Fab framework to produce the bibody. **(Q)** Fab-scFv (tribody), a format similar to the bibody.

prepared by this method can directly utilise existing antibodies and has a high yield, but its activity may be affected by damage to the antigen-binding site (85). Hybridoma technology is based on monoclonal antibody technology, in which hybridoma cells secreting two antibodies are fused to produce hybridomas that stably secrete BsAb. Co-expression of the respective immunoglobulin (Ig) genes produces two types of H and L chains, which combine to form a BsAbs with the characteristics of the parental Ig (86). BsAb prepared using the hybridoma method is more random and relatively inefficient, but it has better biological activity and a more stable antibody structure (87). Genetic engineering techniques have opened up new avenues for the preparation of BsAbs, either by cloning the gene encoding the parental antibody and transfecting it into host cells for direct expression of BsAbs, or by gene shearing and constructing a scFv for the preparation of modified BsAbs (30).

A quality technology platform is key to the success of BsAbs development, and several technology platforms are in progress (88). Among the BsAbs technology platforms with Fc are Crossmab/KIH, DVD-Ig (dual variable domain-Ig), IgG-scFv, FIT-Ig, mAb-Trap, duobody ect (48, 89–92). Dual antibody technology platforms without Fc include BiTE, DART, TandAb, ImmTAC, BriKE, etc (93, 94). Developing BsAbs with the aforementioned functions requires careful adjustment of a number of variables in order to attain the ideal practical result. A blend of complementary binders and other elements in formats which allow the required functionality is necessary for the creation of BsAbs (95). Here, we will introduce several promising preparation methods.

3.1 Knob-into-hole technology

BsAbs possess two distinct paratopes on their variable regions that recognize two separate antigens, in contrast to normal mAbs that contain two same antigen-binding or Fab components. Due to this special characteristic, BsAbs can take on more intricate forms, such as homodimers made of two distinct arms or light chain mismatches, among others. The KIH configuration was employed

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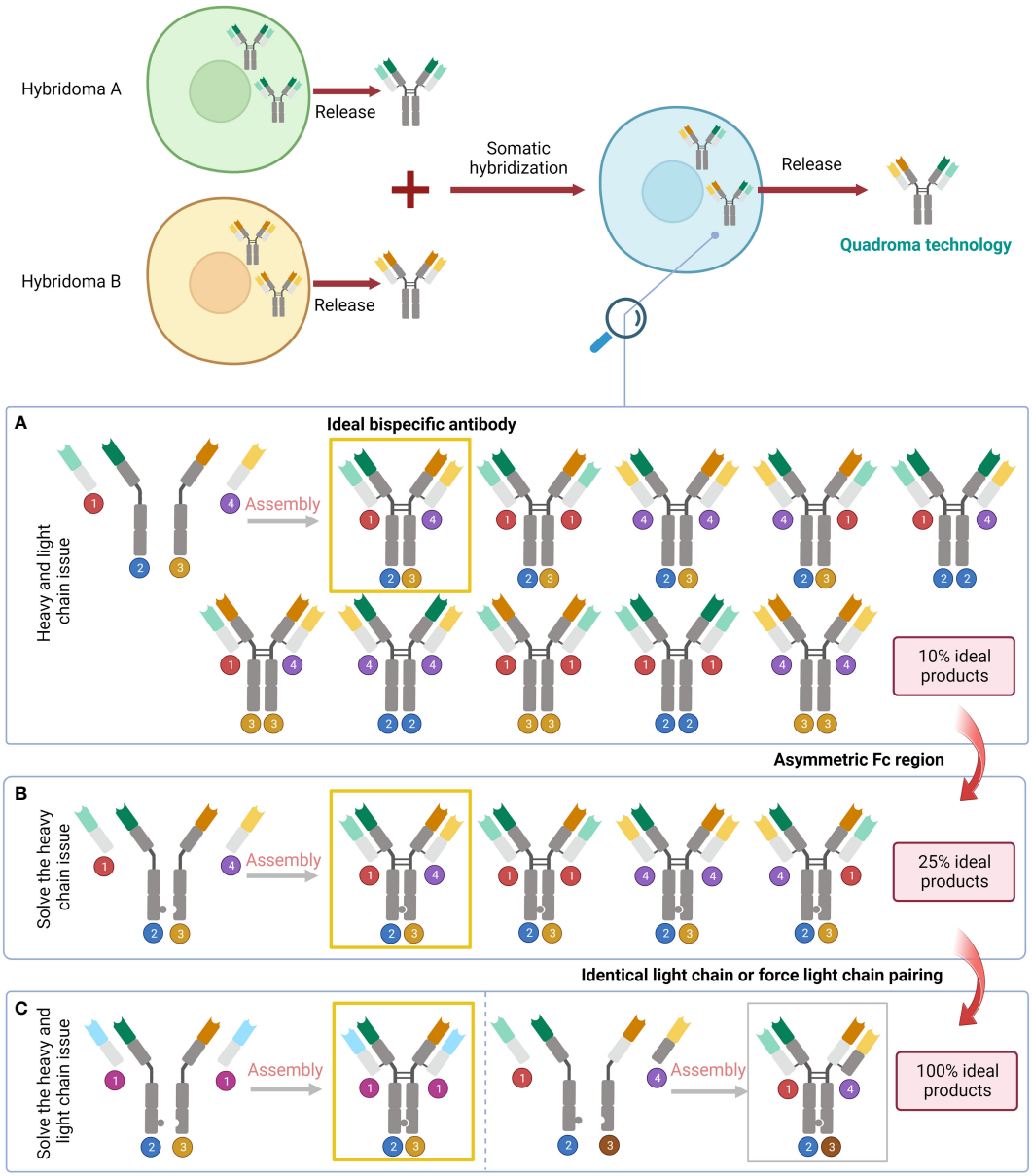

FIGURE 3
Problems raised by the variety of pairings and the corresponding solutions. **(A)** The result of simply fusing two different cell lines. **(B)** Solve the heavy chain problem by designing an asymmetric Fc region. **(C)** Address the issue with light chains by subsequent design of the identical light chain or force light chain pairing.

to create BsAbs to foster heavy-chain heterodimer pairing of the two hemi-antibodies (17, 96). Knob and hole mutations shouldn't affect antigen recognition or Fc activity since they are in the CH3 domain interaction surface. There are multiple ways to prevent light chain mismatching over assembly (97). One method involves expressing two half-antibodies in two separate host cells during an *in vitro* production process. Following two distinct Protein A specificity grab procedures, the two hemi-antibodies are combined for *in vitro* synthesis by reduction and oxidation, which is proceeded by subsequent BsAb purifying (98, 99). It was possible to detect chemical alteration sites and evaluate the steadiness and wholeness along with the operation of a BsAb by applying a variety of stressful situations together with dimension isolation chromatography, ion switch chromatography, LC-MS/MS peptides mapping, and practical examination by cell-based tests. Grunert et al. observed that IgG1 KIH CrossMab-engineered BsAbs were significantly more stable than commercially available antibodies (100). Furthermore, Liu et al. discovered that the KIH architecture did not significantly modify the organization or conformation motion, and the structural security is comparable to that of wild-type (WT) IgG4 (apart from a minor change in the CH3 domain) generated in *E. coli* (74).

The KIH structure and *in vitro* construction may effectively promote the heterodimerization of the heavy chains; nevertheless, throughout hemi-antibody isolation and BsAb installation, some homodimers (such as knob-knob and hole-hole dimerization)

remain detectable (96). In the Fc region of an IgG1 BsAb, Elliott et al. uncovered the molecular specifics for KIH and homodimer engagements (101). The knob-knob and hole-hole Fc component homodimers' X-ray crystal structures have been resolved, revealing a juxtaposed Fc configuration. Bispecific variations have been identified and quantified via intact mass evaluation (99, 102–104).

3.2 CrossMab technology

Combined with techniques that allow for accurate heavy-chain connection with already-existing pairs of antibodies, CrossMab technology, in conjunction with KIH technology, permits an appropriate antibody light-chain interaction with its corresponding heavy chain in BsAbs (105). The BsAbs are made up of two arms: one altered, and the other is not. Adjustments may be restricted to the VL-VH domain, the whole Fab region, or the CL-CH1 area (23). Due to the modifications, the intended chain interaction is enacted because the unaltered heavy chain could no longer interact with the altered light chain. In terms of structure and purity, the CL-CH1 CrossMAb displayed the best results among the three potential changes (106). Clinical trials are now being conducted to assess a number of BsAbs developed by CrossMAb innovation (71). The BsAbs that have currently been produced using CrossMAb include RO6958688 (CD3, CEA) for carcinoembryonic antigen $(CEA)^+$ solid tumors, RG7221 (Ang2, VEGF), RO7121661 (PD-1, TIM3), and RO5520985 (Ang2, VEGF) (28, 107).

3.3 FORCE technology

Being a high-throughput method, Format Chain Exchange (FORCE) provides effective combined production of BsAbs in various arrangements for screening in ultimate form. The technique is based on the formation of BsAb from monospecific educts carrying various binders in various forms. Input agents for the production of BsAbs are monospecific entities with matching CH3-interface-regulated and imitation chains with affinity tags, analogous to KIH hemi-antibodies. These comprise mutations which result in minor interaction repulsions without affecting the production or biological characteristics of educts. Instability at the CH3-educt interfaces resolves to complete compatibility upon mild reduction of pairings of educts, initiating unprompted chain interchange events. This results in the formation of sizable BsAb matrices including various binders in various forms. Processing automation is made possible by benign biological characteristics, excellent production outputs of educts, and ease of purification. The monospecific input components comprise designed Fc-mimic chains that induce heavy-chain interchange processes that lead to formation. Production automation is made possible by efficiency, sturdiness, and simplicity (including assembly and one-step output purifying), allowing for thorough screens of BsAb binder-format layout spaces (95).

3.4 SEEDBodies technology

By creating strand-exchange engineered domain (SEED) CH3 heterodimers, Davis et al. built a heterodimeric Fc system which facilitates the construction of bispecific and asymmetric hybrid proteins. Human SEED CH3 heterodimers, which are made up of alternate parts of human IgA and IgG CH3 patterns, are produced by the variants of human IgG and IgA CH3 regions. When produced in mammal cells, the resultant pair of SEED CH3 regions selectively interacts to generate heterodimers. SEEDbody (Sb) fused proteins are made up of [IgG1 hinge]-CH2-[SEED CH3], and one or more fused couples could be genetically related to them. Mammal cells producing SEEDbody (Sb) fusing proteins result with large outputs of Sb heterodimers which can be easily separated to get rid of the modest byproducts. To simplify examination of heterodimer production in the current study, fusion companions are usually introduced to the N- or the C-terminal of one Sb chain. The lengthy plasma half-life prolongation characteristic of analogous fusion involving Fc segments and IgG1 standards were visible in the Sb pharmacokinetics after being delivered intravenously to rodents (108).

3.5 Duobody® technology

DuoBody® innovation was created by Engelberts et al. to produce complete IgG1 BsAb employing cFAE. Here, under carefully monitored fabrication circumstances, two original IgG1 mAb with paired single spot mutations in the IgG Fc region rearrange into full-length bispecific IgG1s (33, 109). At both the laboratorial and industrial scales, it was demonstrated that the cFAE technique is a simple and reliable way to produce durable BsAb with a greater output contrasted with other BsAb technologies (110). Additionally, the approach offers the chance to create and screen sizable and different arrays of BsAb, allowing for the identification of BsAb with the best functionality (68). In patients with progressed solid cancers, DuoBody-CD40-4-1BB has the potential to improve anticancer immunity by altering DC and T-cell activities (111). A transformed CD3 mAb and the human CD20 mAb 7D8 were combined to create DuoBody-CD3xCD20 (GEN3013), a BsAb recognizing CD3 and CD20 (76, 112). The subcutaneous injection route might offer a way to lower patients' peak cytokine levels, as well as a way to ease their medical strain and make more efficient use of the facility's resources (68).

4 Delivery of BsAbs

Currently, there are two ways to deliver BsAbs to the tumor sites. The first is to administer BsAbs after they have been produced *in vitro*, a process that is generally costly, time-consuming, and ineffective. The second is to enable the *in vivo* synthesis of BsAbs, which can counteract the quick kidney elimination of Fc-free forms, rendering a prolonged potent antibody level and can bypass issues

with recombinant antibody assembly and long-term preservation (113, 114) (Figure 4).

4.1 Delivery of *in vitro*-produced BsAbs

The majority of antibody-based treatments are administered following *in vitro* synthesis. In terms of the scope of action, these deliveries could be classified into circulatory delivery (e.g., intravenous (IV), intracutaneous (IC), subcutaneous (SC), or intramuscular (IM) delivery) and local delivery. Delivery to cancer is regulated by an intricate interaction of factors: the spot of infusion (e.g., IV, IC, or intratumor), carry via the plasma and lymphatics, permeation across the endothelium and basal lamina into the interstitium, hydrodynamic tension in the blood *vs.* the carcinoma tissues, and removal of biologics from the scheme (e.g., by renal filtration, hepatic evacuation (115)).

Although the IV method provides 100% bioavailability, physiological obstacles and circulatory dispersion significantly lower the real BsAb level in the target sites (116). Additionally, IV infusion is inconvenient and takes time, as purified BsAbs that require higher concentrations must be supplied by gradual IV in order to prevent injection responses (114). Considering the maximal amount of infusion is limited, accordingly, low BsAb solubility at high densities is the most typical barrier for SC or IM. Clinical-grade BsAbs are very costly and have production difficulties, such as low durability over long-range preservation and a propensity to congregate (117, 118). The BBB is a tough hurdle that prevents drug transport to the brain. The employment of intrinsic brain endothelial delivery channels is a viable strategy to bypassing the biological hurdle via receptor-mediated transcytosis (RMT). BsAbs are the perfect choice for the purpose since treatments engineered demand at least two capabilities, one that aids delivery and the other to give clinical effect (119). The most typical RMT (TfR, InsR, and LRP1 receptor) have been effectively exploited to cross the BBB (120, 121). Since the BBB is disrupted in brain malignancies, it is necessary to particularly look into the production of novel candidate receptors facilitating RMT particularly for the blood–brain tumor barrier (122). Besides getting to the pathways, innovations to effectuate BsAb distribution into the brain should also be taken into account (119).

Regional administration can improve potency and lessen overall contact for various ailments. In certain malignant situations, intratumoral or intraperitoneal injection of BiTEs may confine effects to malignancies (123–125). An efficient and feasible approach is to use solid implants, granules, or injected storage made of biodegradable and biocompatible polymers to entangle BsAbs and unleash them as the polymer breaks down. PEG-PLA copolymers, a depot-injectable polymeric platform created *in situ*, were utilized to transport BiJ591 (a BsAb targeting $CD3^+$ T cell and prostate-specific membrane antigen (PSMA) on prostate cancer cell) against prostate tumor. The transport method could regulate BiJ591's discharge while preserving its durability and activity, and the treatment efficiency was higher than IV delivery (126, 127).

Transport techniques utilizing nanoparticles also showed impressive performance. Contrasted to XA-1 protein alone, lipid nanoparticle-encased mRNA-expressed XA-1 displayed greater potential anti-cancer effectiveness (128). Administration platforms like liposomes and cell-infiltrating peptides have also been demonstrated to be more efficient than the use of a solitary drug (127).

4.2 Delivery of *in vivo*-produced BsAbs

In vivo gene treatment was created, in an effort to strike a balance between potency and security. The two major schemes are the *in vitro* inoculation of genetically engineered cells and straight gene transport via vectors, mRNA, and plasmid DNA (127).

Both viral and nonviral vectors could be employed to convey the genetic material encoded for BsAb *in vivo*, while utilizing mRNA or plasmid DNA, direct *in vivo* administration of synthesized nucleic

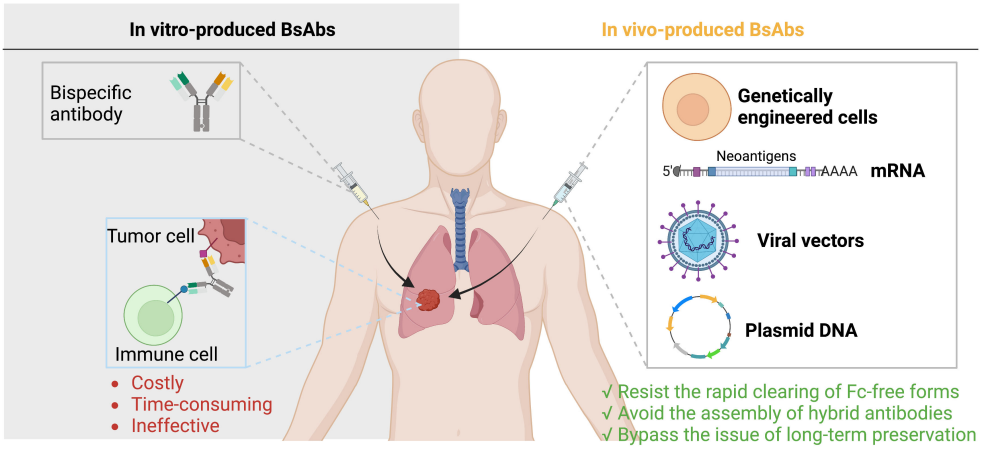

FIGURE 4
Delivery of BsAbs. There are two ways to deliver BsAbs to tumors: administer pre-made BsAbs, which is expensive and inefficient, or allow for *in vivo* synthesis of BsAbs, which can avoid elimination assembly, and preservation issues.

acid-coded BsAbs suggests new methods (114, 117, 129, 130). A CaPO-nanoneedle/minicircle DNA platform generated BsAb (EpCAM/CD3-targeted) resulting in a considerable slowdown of tumor development and a prolongation of animal life-span with minimal toxicity in an intraperitoneal xenograft model with human ovarian carcinoma cell line SKOV3 (131). A synthesized HER2 plasmid DNA-coded BiTE effectively recruited T cells to identify and ruin $HER2^+$ melanoma cells, and it exhibited potent anti-cancer effects *in vivo* (127, 132). Additionally, numerous oncolytic viruses were equipped with expression cassettes generating BiTEs, proving that when straight oncolysis and T cell-regulated destruction are combined, anticancer potency is increased in contrast with the original equivalent in syngenic and xenograft malignancy models (114, 127, 129, 132). The strategy may speed up the clinical progression of new BsAbs as it is quick to produce pharmaceutical-grade mRNA and DNA. Moreover, the temperature tolerance of DNA could make it simpler to carry and administer to larger populations due to its long-term preservation and temperature durability. Also, the *in vivo* synthesis could maintain an efficacious protein level, allaying worries about a quick kidney clearance (129, 130, 133, 134).

It is possible to *in vitro* transmit genetic information into cells obtained from patients, after which the BsAb-releasing cells are infused back to the patient. Compared to straight gene transfer methods, tumor infiltration and general on-target/off-cancer cytotoxicity problems may be addressed by the tumor anchoring of injected cells and ensuing intratumoral release (114, 135). New methods centered on modified cells secreting BiTEs (STAb cells) natively are now being developed (135). Research detailed the creation of anti-CEA and anti-CD3 dAb-releasing human T cells and revealed that the intratumoral delivery of lentivirally transfected STAb-T cells dramatically decreased *in vivo* cancer progression in human HCT-116 colon malignancy xenografts (135).

5 Anti-tumor mechanism of BsAbs

BsAbs could executive its antitumor effect in three major ways, including redirecting immune effector cells to tumors, delivering radioactive or drug payloads to carcinoma cells, targeting multiple signaling pathways to suppress tumor progression directly or indirectly. The classification of BsAb clinical applications based on target antigens is presented in Table 1.

5.1 Reorientating immune effector cells

In the case of cancer treatment, one arm of the BsAb targets a tumor-associated antigen (TAA), while the other arm targets a molecule present on immune cells. Via targeting both the TAA on tumor cells and immune cell molecules, the BsAb brings the immune cell in close proximity to the tumor cell, resulting in the immune cell's stimulation and then destroying the tumor cell (Figure 5).

Under microhomeostatic circumstances, anticancer immunity is one of the essential cancer therapy techniques. However, in order to survive and propagate, tumor cells are able to escape the "cancer-immunity cycle" which describes how the innate and adaptive immune systems collaborate to prevent malignancy genesis through sequential events (149). This is accomplished by mechanisms that suppress anti-tumor immunity, such as increased expression of molecules like PD-L1 that block T-cell action or decreased production of human leucocyte antigen (HLA) class I molecules that hinder antigen presentation (150–153).

Substantial therapy outcomes can be attained by reviving and strengthening the latent immune cells, which has been demonstrated by the development of several mAb immune checkpoint inhibitors (ICIs) that bind PD1, PD-L1, CTLA-4, etc. during the past ten years (154–157). Major improvements in total and advancement-free survival have been obtained in melanoma, lung carcinoma, and urothelial cancer (58, 130).

5.1.1 Reorientating the cells of the adaptative immune system

As of October 2023, the majority of BsAbs bridge cells as their primary action mode and have T cell reorientation as their shared thread. Rerouting effector T cells with cytotoxic activity to destroy malignant cells is a classic paradigm of these BsAbs (28).

BiTEs primarily stimulate T cells via interaction with CD3ϵ in the T-cell receptor (TCR) complex, thus are defined as pan-T-cell engagers (158, 159). The high affinity between BiTEs and TAA/CD3a renders a huge proportion of activation receptors (TCR/CD3 complexes) gather between cells, resulting in effectual T-cell excitation with just one receptor-ligand interplay and the killing of cancer cells through releasing perforin and granzymes (130, 160, 161). Most CD3-targeted pan-T-cell stimulators at the clinical phase are designed to treat blood tumors, such as targeting CD19 and CD20 for non-Hodgkin lymphoma (NHL), targeting B cell maturation antigen (BCMA), GPRC5D and CD38 for multiple myeloma (MM), targeting CD33, CD123, and CLEC12A for acute myeloid leukemia (AML) (162). Although these targets are widely expressed on healthy blood cells as well, their depletion can be handled without eliciting serious negative impacts (114). A relatively small amount of TCRs target MHC-presented TAAs in solid tumors (163). Phase I clinical studies are being conducted with AMV564, a TandAb with two CD3 binding sites and two CD33 binding sites respectively (162).

The TAA selection is crucial for BiTEs to perform properly. The performance of BiTEs is associated with expression ratios of targets, as was the case with BiTEs targeting EpCAM, CD33, and HER2 (164). Three distinct cell lines exhibiting high (EOL-1), medium (MOLM-13), and low (MV4-11) rates of FMS-like tyrosine kinase 3 (FLT3) expression were employed to analyze the influence of receptor density on the potency of FLT3 BsAb *in vivo*. Compared with the MOLM-13 model, the EOL-1 model demonstrated total potency at a lower dose of 7370, which is aligned with EOL-1's higher membrane FLT3 expression (165). BiTEs' activity is also influenced by TAA's characteristics, such as size and mobility on cell membrane (166). Chinese hamster ovary (CHO) cells

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TABLE 1 Clinical-stage BsAbs for cancer indications.

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
Targeting immune effector cells: T cell							
CD3×CD19	AMG562	Fab-scFv (bibody) *P	DLBCL; MCL; FL	Phase 1	Amgen	NCT03571828	
	Blinatumomab	Tandem scFv *K	B-ALL	Phase 2	Amgen Research Munich GmbH	NCT01209286	
	A-319	scFv-Fab *P	DLBCL	Phase 1	EVIVE Biotechnology	NCT04056975	
	TNB-486	IgG4 *A	B-Cell Lymphoma	Phase 1	TeneoTwo Inc.	NCT04594642	
	AFM-11	scFv×scFv (diabodies) *M	Leukemia, B-Cell	Phase 1	Affimed GmbH	NCT02848911	
CD3×CD20	Plamotamab (XmAb13676)	Fab-scFv-Fc (KIH) *G	B-cell NHL; CLL	Phase 1	Xencor, Inc.	NCT02924402	
	Odronextamab (REGN1979)	IgG4 *A	NHL; CLL	Phase 1	Non-Hodgkin Lymphoma Chronic Lymphocytic Leukemia	NCT02290951	
	Epcoritamab (GEN3013)	IgG1 *A	LBCL	Phase 1	Genmab AbbVie	NCT05733650	
	FBTA05	IgG-HC-scFv *E	Leukemia; SCT	Phase 1 Phase 2	Technical University of Munich	NCT01138579	
	Mosunetuzumab (BTCT4465A)	IgG1 *A	BCL	Phase1	Hoffmann-La Roche	NCT04313608	
	GB261	Fab-scFv-Fc (KIH) *G	B-Cell NHL; CLL	Phase 1 Phase 2	Genor Biopharma Co., Ltd.	NCT04923048	
	Obinutuzumab (RO7082859)	Fab3CrossMab *J	DLBCL	Phase 2	Hoffmann-La Roche	NCT00576758	(136)
CD3×CD22	JNJ-75348780	IgG4 *A	Lymphoma, Non-Hodgkin Leukemia, Lymphocytic, Chronic, B-Cell	Phase 1	Janssen Research & Development, LLC	NCT04540796	
CD3×CD28	rM28	Tandem scFv *K	Malignant Melanoma	Phase 1 Phase 2	University Hospital Tuebingen	NCT00204594	
CD3×CD33	AMV564	diabody *M	AML	Phase 1	Amphivena Therapeutics, Inc.	NCT03144245	
	AMG330	Tandem scFv *K	AML, Myelodysplastic Syndrome	Phase 1	Amgen	NCT02520427	
	AMG673	Triplebody *L	Recurrent Squamous Cell Lung Carcinoma	NA	SWOG Cancer Research Network	NCT02154490	
	JNJ-67571244	IgG4 *A	Leukemia, Myeloid, Acute, MDS	Phase 1	Janssen Research & Development, LLC	NCT03915379	(137)
	GEM333	Fab-scFv-scFv *P	AML	Phase 1	AvenCell Europe GmbH GCP-Service International Ltd.	NCT03516760	
CD3×CD38	ISB 1342	Fab-scFv-Fc (KIH) *G	Relapsed/Refractory MM	Phase 1	Ichnos Sciences SA Glenmark Pharmaceuticals S.A.	NCT03309111	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
	Y150	Fab-scFv-IgG1 *A	Relapsed/Refractory MM	Phase 1	Wuhan YZY Biopharma Co., Ltd.	NCT05011097	
	AMG424 (Xmab13551)	Fab-scFv-Fc (KIH) *G	Relapsed/Refractory MM	Phase 1	Amgen	NCT03445663	
CD3×CD123	Vibecotamab (XmAb14045)	Fab-scFv-Fc (KIH) *G	AML MDS	Phase 2	M.D. Anderson Cancer Center	NCT05285813	
	JNJ-63709178	IgG4 *A	Leukemia, Myeloid, Acute	Phase 1	Janssen Research & Development, LLC	NCT02715011	
	Flotetuzumab (MGD006)	DART *N	AML	Phase 1 Phase 2	MacroGenics	NCT02152956	
	APVO436	SCFV-Fc (KIH) *F	AML MDS	Phase 1	Aptevo Research and Development LLC	NCT03647800	
CD3×B7-H3	Orlotamab (MGD009)	DART-FC *H	Mesothelioma, Bladder Cancer, Melanoma	Phase 1	MacroGenics	NCT02628535	
	INCA32459-101	Fc-silenced IgG1 *A	Advanced Malignancies	Phase 1	Incyte Corporation	NCT05577182	
CD3×BCMA	Elranatamab (PF-06863135)	IgG2 *A	MM	NA	Pfizer	NCT05565391	
	Linvoseltamab (REGN5458)	IgG4 *A	MM	Available	Regeneron Pharmaceuticals	NCT05164250	
	REGN5459	IgG4 *A	Relapsed/Refractory MM	Phase 1 Phase 2	Regeneron Pharmaceuticals	NCT04083534	
	Pavurutamab (AMG701)	Fab-scFv (bibody) *P	Relapsed/Refractory MM	Phase 1	Amgen	NCT03287908	
	Pacanalotamab (AMG420)	Tandem scFv *K	MM	Phase 1	Amgen	NCT02514239	(138)
	Teclistamab	IgG4 *A	Relapsed/Refractory MM	Marketed	Janssen Research & Development, LLC	NCT05463939	
	EMB-06	Fab-scFv-Fc (KIH) *G	Relapsed/Refractory MM	Phase 1 Phase 2	Shanghai EpimAb Biotherapeutics Co., Ltd.	NCT04735575	
	TNB-383B (ABBV-3830)	IgG4 *A	MM	Phase 1 Phase 2	TeneoOne Inc. AbbVie	NCT03933735	
	ARB202	IgG-HC-scFv *E	Gastrointestinal Cancer Cholangio-carcinoma Liver Cancer	Phase 1	Arbele Pty Ltd Arbele Limited	NCT05411133	
	CC-93269 (EM801)	Fab3CrossMab*J	MM	Phase 1	Celgene	NCT03486067	
	JNJ-64007957	IgG4 *A	Hematological Malignancies	Phase 1	Janssen Research & Development, LLC	NCT03145181	
CD3×CEA	Cibisatamab (CEA-TCB)	Fab3CrossMab*J	Carcinoma, NSCLC	Phase 1 Phase 2	Hoffmann-La Roche	NCT03337698	
	CEA-TCB (RO6958688)	Fab-scFv-Fc (KIH) *G	Solid Tumors	Phase 1	Hoffmann-La Roche	NCT02324257	(139)
CD3×CLEC12A	Tepoditamab (MCLA-117)	IgG1 *A	AML	Phase 1	Merus N.V.	NCT03038230	
CD3×DDL3	TarlatamabAMG757	Fab-scFv (bibody) *P	SCLC	Phase 1	Amgen	NCT04885998	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
CD3×EGFR	AMG596	Tandem scFv *K	Glioblastoma, Malignant Glioma	Phase 1	Amgen	NCT03296696	
CD3×EpCAM	Catumaxomab	Rat-mouse hybrid IgG	Solid malignancies	Withdrawn from the market	Neovii Biotech	NCT00836654	
	M701	Fab-scFv-Fc (KIH) *G	MPEs, NSCLC Stage IV	Phase 1 Phase 2	Wuhan YZY Biopharma Co., Ltd.	NCT05543330	
	MT110	Tandem scFv *K	Solid Tumors	Phase 1	Amgen Research Munich GmbH	NCT00635596	
	A-337	scFv2-Fab TriFabs *I	NSCLC	Phase 1	Generon Shanghai Corporation	ACTRN12617001181392	
CD3×FLT3	AMG427	Fab-scFv-Fc (KIH) *G	Relapsed/Refractory AML	Phase 1	Amgen	NCT03541369	
CD3×FcRH5 CD307	Cevostamab	Tandem scFv *K	MM	Phase 1	Genentech, Inc.	NCT03275103	
CD3×GPC3	ERY974	IgG4 *A	Solid Tumors	Phase 1	Chugai Pharmaceutical	NCT02748837	
CD3×GD2	Nivatrotamab (Hu3F8-BSAB)	IgG-scFvLC *D	SCLC	Phase 1 Phase 2	Y-mAbs Therapeutics	NCT04750239	
CD3×GPRC5D	Talquetamab (JNJ-64407564)	IgG4 *A	Hematological Malignancies	Phase 1	Janssen Research & Development, LLC	NCT03399799	
CD3×GPA33	MGD007	DART-FC *H	Colorectal Carcinoma	Phase 1	MacroGenics	NCT02248805	
CD3×gp100	Tebentafusp (IMCgp100)	SCFV-TCR	Uveal Melanoma	Phase 1 Phase 2	Immunocore Ltd	NCT02570308	
CD3×HER2	M802	Fab-scFv-Fc (KIH) *G	HER2-Positive Solid Tumors	Phase 1	Wuhan YZY Biopharma Co., Ltd.	NCT04501770	
	ISB 1302 (GBR1302)	Fab-scFv-Fc (KIH) *G	Breast Cancer	Phase 1 Phase 2	Ichnos Sciences SA Glenmark Pharmaceuticals S.A.	NCT03983395	
	Runimotamab (BTRC4017A)	Undisclosed	Solid Tumors	Phase 1	Genentech, Inc.	NCT03448042	
CD3×HLA-G	JNJ-78306358	IgG4 *A	Neoplasms	Phase 1	Janssen Research & Development, LLC	NCT04991740	
CD3×ROR1	EMB07	Fab-scFv-Fc (KIH) *G	Advanced/Metastatic Solid Tumors	Phase 1	EpimAb Biotherapeutics SuzhouCo., Ltd.	NCT05607498	
CD3×PD-L1	ONO-4685	Undisclosed	Relapsed/Refractory T Cell Lymphoma	Phase 1	Ono Pharmaceutical Co. Ltd	NCT05079282	
CD3×PSMA	AMG160	Fab-scFv-Fc (KIH) *G	NSCLC	Phase 1	Amgen	NCT04822298	
	AMG 340	Tandem scFv *K	mCRPC	Phase 1	Amgen	NCT04740034	
	CC-1	IgG4-SC *A	Lung Cancer Squamous Cell	Phase 1 Phase 2	German Cancer Research Center	NCT04496674	
	ES414	IgG-HC-scFv *E	Prostate Cancer	Phase 1	Aptevo Therapeutics	NCT02262910	

(Continued)

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TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
	Pasotuxizumab (BAY20101120)	Tandem scFv *K	Prostatic Neoplasms	Phase 1	Bayer	NCT01723475	
	REGN4336	IgG1 *A	mCRPC	Phase 1 Phase 2	Regeneron Pharmaceuticals	NCT05125016	
CD3×PSCA	GEM3PSCA	Fab-scFv-scFv *P	NSCLC, Prostate Cancer, Renal Cancer, Transitional Cell Carcinoma	Phase 1	AvenCell Europe GmbH GCP-Service International Ltd. & Co. KG	NCT03927573	
CD3×P-cadherin	PF-06671008	DART-FC *H	Neoplasms	Phase 1	Interventional	NCT02659631	(140)
CD3×SSTR2	Tidutamab (XmAb18087)	Fab-scFv-Fc (KIH) *G	Neuroendocrine Tumor, Gastrointestinal Neoplasm	Phase 1	Xencor, Inc.	NCT03411915	
CD27×PD-L1	CDX-527	IgG-HC-scFv *E	NSCLC, Breast Cancer, Gastric Cancer	Phase 1	Celldex Therapeutics	NCT04440943	
CD28×EGFR	REGN7075	IgG1 *A	Advanced Solid Tumors	Phase 1 Phase 2	Regeneron Pharmaceuticals	NCT04626635	(141)
CD39×TGF-β	ES014	IgG-HC-scFv *E	Advanced Solid Tumor	Phase 1	Elpiscience Biopharma, Ltd.	NCT05381935	
MUC2×CD4018	Cemiplimab (REGN4018)	IgG4 *A	Recurrent Ovarian Cancer	Phase 1 Phase 2	Regeneron Pharmaceuticals	NCT03564340	
TCR VB	STAR0602	Fab-scFv-Fc *G	Advanced Solid Tumor	Phase 1 Phase 2	Marengo Therapeutics, Inc.	NCT05592626	
OX40×4-1BB	FS120	IgG1 *A	Advanced Cancer Metastatic Cancer	Phase 1	F-star Therapeutics Limited Merck Sharp & Dohme LLC	NCT04648202	(142)
Targeting immune effector cells: NK cells							
CD30×CD16A	AFM13	Tandem (diabodies) *M	Lymphoma, T-Cell, Cutaneous	Phase 1 Phase 2	Ahmed Sawas Columbia University	NCT03192202	
CD16×CD33	GTB-3550	Tandem scFv *K	HR-MDS AML Systemic Mastocytosis	Phase 1 Phase 2	GT Biopharma, Inc.	NCT03214666	
Targeting immune effector cells: B cells							
CD19×CD64	4G7xH22	IgG-HC-scFv *E	Leukemia Lymphoma	Phase 1	Dartmouth-Hitchcock Medical Center National Cancer Institute NCI	NCT00014560	
CD19×CD22	OXS-1550 (DT2219ARL)	Tandem scFv *K	B-cell lymphoma leukaemia	Phase 1 Phase 2	GT Biopharma	NCT02370160	
CD19×CD47	TG-1801	IgG1 *A	B-Cell Lymphoma	Phase 1	TG Therapeutics, Inc.	NCT03804996	
Targeting multiple checkpoints							
PD-1×CD47	HX009	IgG-HC-scFv *E	Relapsed, Refractory Lymphoma	Phase 1 Phase 2	Waterstone Hanxbio Pty Ltd	NCT05189093	
PD1×CTLA4	Cadonilimab (AK104)	IgG-HC-scFv *E	Advanced Solid Tumors Melanoma	Phase 1 Phase 2	Akeso Pharmaceuticals, Inc.	NCT04172454	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
	XmAb20717	Fab-scFv-Fc (KIH) *G	Solid malignancies	Phase 1	Macrogenics	NCT03517488	
	MEDI5752	IgG1 *A	Solid malignancies	Phase 1	AstraZeneca	NCT03530397	
PD-1×ICOS	XmAb23104	Fab-scFv-Fc (KIH) *G	Solid malignancies	Phase 1	Xencor	NCT03752398	
PD-1×LAG3	EMB-02	Fabs-In-Tandem Ig	Advanced Solid Tumors	Phase 1 Phase 2	Shanghai EpimAb Biotherapeutics Co., Ltd.	NCT04618393	
	MGD013	SCFV-Fc(KIH) *F	Advanced Solid Tumors Hematologic Neoplasms Ovarian Cancer	Phase 1	MacroGenics	NCT03219268	
	AK129	IgG1 *A	Advanced Malignant Tumors Stage IA-IB	Phase 1	Akeso	NCT05645276	
PD-1×TIM3	AZD7789	undisclosed	Carcinoma, NSCL	Phase 1 Phase 2	AstraZeneca	NCT04931654	
	Lomvastomig (RO7121661)	IgG1 *A	NSCLC, SCLC, ESCC	Phase 1	Hoffmann-La Roche	NCT03708328	
	LB1410	undisclosed	Solid Tumor Lymphoma	Phase 1	L & L biopharma Co., Ltd., Shanghai China	NCT05357651	
PD1×HER2	IBI315	IgG1 *A	Advanced Solid Tumor	Phase 1	Innovent Biologics Suzhou Co. Ltd.	NCT04162327	(143)
PD-1×VEGF	Ivonescimab (AK112)	IgG-HC-scFv *E	Solid Tumor, Adult	Phase 1 Phase 2	Akeso	NCT04597541	
PD-1×4-1BB	PRS-344/S095012	IgG-HC-scFv *E	Solid Tumor	Phase 1 Phase 2	Pieris Pharmaceuticals, Inc.	NCT05159388	(144)
PD-1×PD-L1	LY3434172	IgG1 *A	Advanced Cancer	Phase 1	Eli Lilly and Company	NCT03936959	
	IBI318	IgG1 *A	Advanced Malignancy	Phase 1	Innovent Biologics Suzhou Co. Ltd.	NCT03875157	
PDL1×4-1BB	MCLA-145	IgG1 *A	Advanced Cancer Solid Tumor, Adult B-cell Lymphoma, Adult	Phase 1	Merus N.V. Incyte Corporation	NCT03922204	
	INBRX-105	SCFV-Fc (KIH) *F	lymphoma, solid tumours	Phase 1	Inhibrx	NCT03809624	
	GEN1046	hetero Fab assembly IgG1 *A	Non-SCLC Metastatic	Phase 2	Genmab BioNTech SE	NCT05117242	
	PM1003	IgG-HC-scFv *E	Advanced Solid Tumors	Phase 1 Phase 2	Biotheus Inc.	NCT05862831	
	ATG101	IgG-HC-scFv *E	Advanced Solid Tumor Metastatic Solid Tumor B-NHLs	Phase 1	Antengene Hangzhou Biologics Co., Ltd.	NCT05490043	
	QLF31907	IgG-HC-scFv *E	Melanoma Urothelial Carcinoma	Phase 2	Qilu Pharmaceutical Co., Ltd.	NCT05823246	
	FS222	IgG1 *A	Advanced Cancer, Metastatic Cancer	Phase 1	F-star Therapeutics Limited	NCT04740424	(145)
	ABL503	IgG-HC-scFv *E	Advanced Solid Tumor	Phase 1	ABL Bio, Inc.	NCT04762641	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
PDL1×CTLA4	KN046	IgG1 *A	ESCC, Triple-negative Breast Cancer, Advanced Solid Tumors Lymphoma	Phase 2	Jiangsu Alphamab Biopharmaceuticals Co., Ltd	NCT03925870	
	SI-B003	undisclosed	Solid Tumor	Phase 1	Sichuan Baili Pharmaceutical Co., Ltd. SystImmune Inc.	NCT04606472	
PDL1×LAG3	FS118	IgG1 *A	Advanced Cancer Metastatic Cancer HNSCC	Phase 1 Phase 2	F-star Therapeutics Limited	NCT03440437	
	ABL501	IgG-HC-scFv *E	Advanced Solid Tumor	Phase 1	ABL Bio, Inc.	NCT05101109	
	RO7247669	Fc silenced IgG1 *A	Solid Tumors Metastatic Melanoma NSCLC,ESCC	Phase 1	Hoffmann-La Roche	NCT04140500	
PD-L1×TGF-β	Y101D	IgG-scFvLC *D	Metastatic or Locally Advanced Solid Tumors	Phase 1	Wuhan YZY Biopharma Co., Ltd.	NCT05028556	
	QLS31901	IgG-HC-scFv *E	Advanced Malignant Tumor	Phase 1	Qilu Pharmaceutical Co., Ltd.	NCT04954456	
PDL1×TIGI1	HLX301	IgG-LC-scFv *D	Locally Advanced or Metastatic Solid Tumors NSCLC	Phase 1 Phase 2	Shanghai Henlius Biotech	NCT05102214	
PD-L1×TIM-3	LY3415244	Undisclose	Solid Tumor	Phase 1	Eli Lilly and Company	NCT03752177	(146)
PDL1×VEGF	B1962	IgG1 *A	Neoplasms Malignant	Phase 1	Tasly Biopharmaceuticals Co., Ltd.	NCT05650385	
PD-L1×OX-40	EMB-09	undisclosed	Advanced Solid Tumor	Phase 1	Shanghai EpimAb	NCT05263180	
CTLA-4×LAG3	Bavunalimab (XmAb22841)	IgG-HC-scFv *E	Metastatic Melanoma	Phase 1 Phase 2	University of California, San Francisco	NCT05695898	
CLDN18.2×4-1BB	ABL111TJ0033721	IgG-HC-scFv *E	Solid Tumor	Phase 1	I-Mab Biopharma Co. Ltd.	NCT04900818	
Targeting Growth factors and their recepters							
EGFR×FcγRI	MDX447	IgG-HC-scFv *E	Brain and Central Nervous System Tumors	Phase 1	Dartmouth-Hitchcock Medical Center National Cancer Institute NCI	NCT00005813	
EGFR×MET	JNJ-61186372	IgG1 *A	NSCLC	Phase 1	Janssen R&D	NCT02609776	
	LY3164530	IgG4 *A	Neoplasms	Phase 1	Eli Lilly and Company	NCT02221882	(147)
EGFR×c-Met	MCLA-129	IgG1 *A	NSCLC Metastatic Gastric Cancer ESCC HNSCC	Phase 1 Phase 2	Merus N.V.	NCT04868877	
	EMB-01	Fabs-In-Tandem Ig	Neoplasms Neoplasm Metastasis	Phase 1 Phase 2	Shanghai EpimAb Biotherapeutics Co., Ltd.	NCT05176665	
	Amivantamab	IgG1 *A	NSCLC	Phase 1	Janssen Research & Development, LLC	NCT02609776	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
	MCLA-129	IgG1 *A	Solid Tumor NSCLC HNSCC	Phase 1 Phase 2	Betta Pharmaceuticals Co., Ltd.	NCT04930432	
EGFR×4-1BB	HLX35	IgG-HC-scFv *E	Solid Tumors Squamous-cell NSCLC	Phase 1	Shanghai Henlius Biotech	NCT05360381	
EGFR×HER3	SI-B001	IgG-HC-scFv *E	Epithelial Tumor	Phase 1	Sichuan Baili Pharmaceutical Co., Ltd. SystImmune Inc.	NCT04603287	
	Duligotuzumab (MEHD7945A)	IgG1 *A	Neoplasms	Phase 1	Genentech, Inc.	NCT01986166	
EGFR-LGR5	Petosemtamab (MCLA-158)	IgG1 *A	Solid Tumors NSCLC HNSCC	Phase 1 Phase 2	Merus N.V.	NCT03526835	
HER2×HER2	Alphamab (KN026)	IgG1 *A	breast and gastric cancer	Phase 1	Jiangsu Alphamab Biopharmaceuticals Co., Ltd	NCT03619681	
	MBS301	IgG1 *A	Solid malignancies	Phase 1	Beijing Mabworks Biotech Co., Ltd.	NCT03842085	
	ZW49	Fab-scFv-Fc (KIH) *G	HER2-expressing Cancers	Phase 1	Zymeworks Inc.	NCT03821233	
ECD2×ECD4HER2	ZW25	Fab-scFv-Fc (KIH) *G	HER2-Positive Advanced BTC	Available	Jazz Pharmaceuticals	NCT04578444	
HER2×HER3	MM-111	IgG-scFv *	Her2 Amplified Solid Tumors Metastatic Breast Cancer	Phase 1	Merrimack Pharmaceuticals	NCT00911898	
	Zenocutuzumab (MCLA-128)	IgG1 *A	Tumours Harboring NRG1 Fusion	Phase 2	Merus N.V.	NCT02912949	(148)
	Zenocutuzumab (MCLA-128, PB4188)	IgG1 *A	breast cancer	Phase 2	Merus	NCT03321981	
HER2×4-1BB	YH32367 (ABL105)	IgG-HC-scFv *E	HER2-Positive Solid Tumor	Phase 1 Phase 2	Yuhan Corporation	NCT05523947	
IGF1R×HER3	Istiratumab (MM-141)	IgG1 *A -scFv *E	Colorectal Cancer NSCLC HNSCC	Phase 1	Merrimack Pharmaceuticals	NCT02538627	
VEGF×Ang2	BI 836880	Tandem VHH *O	NSCLC	Phase 1	Ablynx/ Boehringer Ingelheim	NCT02689505	
	Vanucizumab, (RO5520985)	IgG1 *A	Solid malignancies	Phase 1	Roche	NCT02715531	
VEGF×DLL4	Dilpacimab (ABT-165)	Tandem Fv-IgG1 *A	CRC	Phase 1	AbbVie	NCT03368859	
	Navicixizumab	IgG2 *A	ovarian, peritoneal fallopian tube cancers	Phase 1	Celgene/Oncomed	NCT03030287	
	NOV1501 (ABL001)	IgG-HC-scFv *E	Advanced Solid Tumors	Phase 1	ABL Bio, Inc. National OncoVenture	NCT03292783	
Targeting other points							
CD73×TGFβ-Trap	Dalutrafusp alfa (AGEN1423)	IgG1 *A	PDAC	Phase 2	Bruno Bockorny, MD Agenus Inc.	NCT05632328	

(Continued)

TABLE 1 Continued

Target	Interventions	Format	Conditions	Phase	Sponsors	NCT Number	Ref
CD40×MSLN	ABBV-428	SCFV-Fc (KIH) *F	Solid malignancies	Phase 1	AbbVie	NCT02955251	
CEA×HSG	CrossMabTF2	Tandem scFv *K	SCLC CEA-expressing NSCLC	Phase 1 Phase 2	Centre René Gauducheau	NCT01221675	

Data available as of 1 August 2023. Molecules are ordered on the basis of the antigens in the first column. The capital letters after * in the third column represent the type of BsAbs in Figure 2. Fab, antigen-binding fragment; ScFv, single-chain variable fragment; DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; B-ALL, B cell acute lymphoblastic leukaemia; IgG, immunoglobulin G; Fc, fragment crystallizable region; VHH, variable heavy-chain only fragment antibodie; BCL, B-cell lymphoma; FL, follicular lymphoma; NHL, non-hodgkins lymphoma; CLL, chronic lymphocytic leukemia; LBCL, large B-cell lymphoma; SCT, stem cell transplantation; AML, acute myeloid leukemia; LUSC, lung squamous cell carcinoma; MDS, myelodysplastic syndromes; MM, multiple myeloma; 7-H3, B7 homologue 3; BCMA, B cell maturation antigen; CEA, carcinoembryonic antigen; CLEC12A, C-type lectin domain family 12 member A; DLL3, delta-like ligand 3; EpCAM, epithelial cell adhesion molecule; FLT3, Fms-like tyrosine kinase 3; FcRH5, Fc receptor homologue 5; GPC3, glypican 3; GPRC5D, G protein-coupled receptor family C group 5 member D; GPA33, Glycoprotein A33; gp100, glycoprotein 100; HER2, human epidermal growth factor receptor 2; HLA-G, human leucocyte antigen-G; ROR1, receptor tyrosine kinase-like orphan receptor 1; PD-1, programmed cell death 1; PD-L1, programmed cell death 1 ligand 1; SSTR2, somatostatin receptor 2; EGFR, epidermal growth factor receptor; TGF-β, transforming growth factor-β; MUC2, recombinant mucin 2; TCR, VBT-Cell receptor; OX40, tumor necrosis factor receptor superfamily member 4; 4-1BB, tumor necrosis factor receptor superfamily member 9; CTLA4, cytotoxic T lymphocyte antigen 4; PSMA, prostate-specific membrane antigen; ICOS, inducible T cell co-stimulator; LAG3, lymphocyte-activation gene 3; TIM3, T cell immunoglobulin mucin 3; VEGF, vascular endothelial growth factor; CLDN18.2, Claudin18.2; FcγRI, receptor I for the Fc region of immunoglobulin G; MET, mesenchymalepithelial transition factor; c-MET, cellular-mesenchymalepithelial transition factor; HER3, human epidermal growth factor receptor 3; LGR5, leucine-rich repeat-containing G protein-coupled receptor 5; IGF1R, insulin-like growth factor 1; Ang2, Angiopoietins2; DLL4, delta-like ligand 4; MSLN, mesothelin; HSG, hysterosalpingography; NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; MPEs, malignant pleural effusions; mCRPC, metastatic castration-resistant prostate cancer; ESCC, esophageal squamous cell carcinoma; B-NHLs, mature B-cell non-hodgkin lymphoma; HNSCC, head and neck squamous cell carcinoma; HR-MDS, high-risk myelodysplastic syndromes; BTC, biliary tract cancer; PDAC, pancreatic ductal adenocarcinoma; NK, natural killer.

presenting small surface target antigens were typically more effectively lysed than those with bigger antigens (167). The antigen's affinity to candidates is also an important determinant for the strength of BiTE. High affinity for HER2 was essential for the HER2/CD3-targeted BiTEs' ability to destroy cancer cells. Nevertheless, a worse safety profile, such as cytokine release and impairment to HER2-expressing tissues, was also linked to increased HER2 affinity. Adopting a dose-fractionation method could enhance the HER2/CD3-targeted tolerance (168).

Architecture of CD3-binding part impacts the biodistribution of BiTEs. Despite BiTEs with high CD3 affinity demonstrated better efficacy in co-culture tests *in vitro*, a reduced affinity of the CD3-binding domain is preferred to enable effective tumor diffusion *in vivo* without triggering rapid CD3-regulated plasma elimination or antibody entrapment in organs which store T cells (169–173). Many BiTEs only have one CD3-binding domain, whereas some clinical-stage BsAbs possess two CD3-targeting sites. However, whether such formats could functionally connect CD3 bivalently is unclear, which is essential for antigenic regulation and tolerance evoked by CD3-mAbs (28, 174). A monovalent CD3 interplay is favored because bivalent CD3 binding may crosslink the TCR/CD3 complex even if it is not simultaneously bound to TAA-expressing cells, resulting in systemic T-cell stimulation and cytokine release syndrome (CRS) (114, 175).

There are several FDA-authorized BiTEs for tumor therapy. In 2009, catumaxomab was granted clinical approval as the first BiTE. This antibody has two distinct antigen-targeting sites—one for the CD3 antigen on T-cells and another for the EpCAM on tumor cells—and also binds to accessory cell FcγR via its preserved Fc region (9). However, the IV injection of catumaxomab was linked to serious harmful effects that were ascribed to the active Fc site's off-target adhesion to other immune cells expressing FcγRs, causing CRS and T cell-mediated hepatic damage (114, 176, 177). In 2014, Blinatumomab, a BiTE created by Amgen Inc. for the treating blood malignancies derived from B-cell lines (178), was authorized by the FDA for the therapy of acute lymphoblastic leukemia (ALL). Blinatumomab is a tiny BsAb with a molecular weight of around 55 kDa and a brief plasma half-life of 1.25±0.63 hours *in vivo* (28, 179–181). In this regard, the switch from sporadic IV infusion to constant IV infusion was a crucial choice in the clinical development of blinatumomab, which not only elevated security but also permitted more sustained T cell activity by preserving effective drug serum rates for the duration of a treatment cycle (182). Motivated by the promising clinical data of binatumomab, a variety of BiTEs with multivalent TAA affinity and monovalent CD3 binding, as well as the DART format have been developed to improve tumor selectivity and reduce off-target side effects (28, 114). In 2021, zenocutuzumab, an innovative IgG1 class HER2/HER3 BsAb utilizing the "dock-and-block" strategy, was granted the Fast Track Designation for $NRG1^+$ metastatic neoplasms. Owing to its selectivity for HER2's domain 1, zenocutuzumab can suppress HER2/HER3 signals regardless of the presence of HER2. Furthermore, it has no synergistic toxicity on cardiac myocytes conducted by HER2/HER4, thanks to its selectivity in blocking HER2/HER3 dimerization (183, 184).

Despite their potential, certain investigations revealed that T cells activated by BiTEs become less potent over time since they deplete more quickly (130, 185). As is the case with blinatumomab and catumaxomab, the administration of BiTEs is linked to CRS, which is indicated by abrupt elevations in the serum amounts of inflammatory cytokines such interleukins-6 (IL-6), tumour necrosis factor (TNF), and interferons (IFNs) (186–188). In an intriguing study applying an anti-PSMA T-BsAb, the scFv-Fc-scFv T-BsAb design allowed for the generation of powerful T-cell-dependent cellular cytotoxicity (TDCC) *in vitro* while limiting cytokine release, indicating that carcinoma cytotoxicity and cytokine storm might be distinct events or that an ideal balance between efficacy and toxicity can be realized by altering BiTE layout (189). Another significant drawback of CD3-targeted BiTEs is a significant fraction of the T-cell population is awakened. Therefore, compared to existing CD3-targeted pan-T-cell activators, BiTEs specifically activating distinct T-cell subtypes would be advantageous (114).

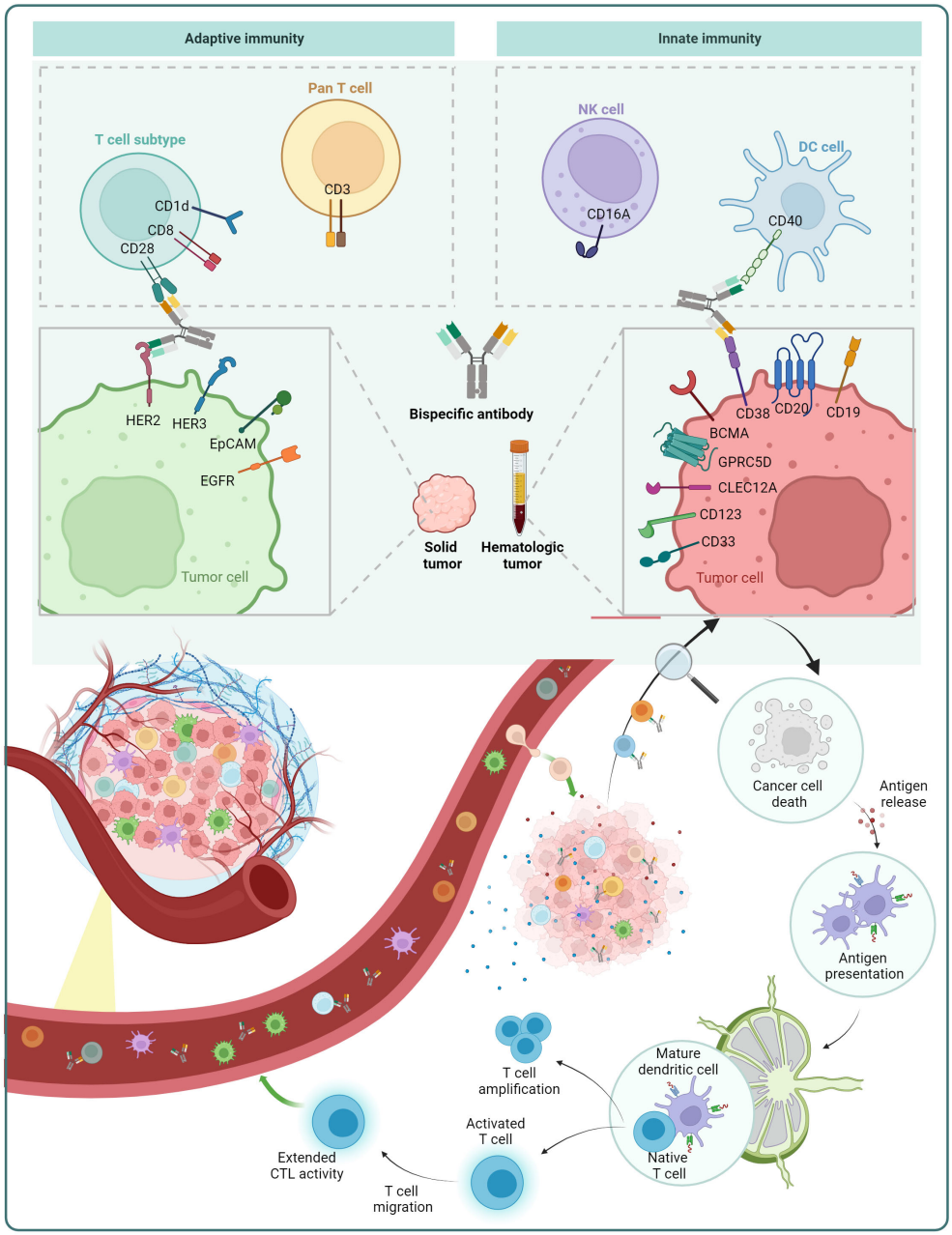

FIGURE 5
Mechanism of action of BsAb: redirection of immune cells. BsAb can redirect pan T cells and T cell subsets from the adaptive immune system, as well as natural killer (NK) cells and dendritic cells (DCs) in the innate immune system. The figure also displays some of the immune cell surface antigens and tumor cell surface antigens that are already under investigation.

Targeting particular T cell subgroups render BsAb more effective in the destruction of tumor cells (28). Blinatumomab could stimulate Tregs *in vitro*, which inhibited effector T cells' cytotoxicity (190). Additionally, in 42 patients with B cell ALL, the number of Tregs in the peripheral blood before blinatumomab administration negatively predicted response (190). Therefore, one of the objectives to construct a $CD8^+$ T cell and prostate stem cell antigen binding tandem scFv was to avoid the induction of Tregs (40, 191). Vγ9Vδ2-T cells are a small and conserved T-cell fraction with a powerful inherent immunotherapeutic prospective. Vγ9Vδ2-T cell concentration and powerful CD1d-reliant tumor lysis are made possible by a bispecific Vγ9Vδ2-T cell engager (192).

Obstructing inhibitory checkpoints can revive weary neoplasm-permeating T cells (114). Inhibitory receptors such as PD1, mucindomain containing3 (TIM3), and lymphocyteactivation gene 3 (LAG3) are abundantly expressed when T cells are in a worn-out condition, which results in defective effector outcomes (193–195). Immune checkpoint-targeting BsAbs are arising following the therapeutic efficacy of anti-CTLA4 (cytotoxic T lymphocyte antigen 4), anti-programmed cell death protein 1

(PD-1), and anti-PD-L1 antibodies, while the enhanced therapeutic effect seen in coupled research using mAbs that engage the checkpoints serves as justification for concurrently engaging two immune checkpoints (28, 196). The majority of the BsAbs inhibit the PD-1/PD-L1 pathway with one arm while blocking CTLA-4, LAG3, or TIM3 with the other (28, 114). Fc-silenced BsAbs were developed to block the PD-1 cascade via high-affinity PD-1 interaction whilst obstructing CTLA4 with a low-affinity binding domain in order to enhance the safety aspect of simultaneous engagement of PD-1 and CTLA4 (28).

5.1.2 Reorientate cells of the innate immune system

BsAbs are also evolving as a substitute therapy strategy geared at the induction of intrinsic immune effector cell toxicity versus cancers with prospects for therapeutic potency and reduced therapeutic toxicity (197, 198). The bulk of BsAbs regarding the innate immune system focuses on dendritic cell (DCs), natural killer (NK) cells, and phagocytes (114, 199).

DCs are professional antigen-presenting cells (APCs). BsAbs with intact Fc domain can be employed to boost the chances of DCs and T cells coming into contact (130). In this regard, a BsAb which concurrently and agonistically activated CD28 on naïve T cells and CD40 on AML-DC was developed. In addition to improving CD28-mediated messaging, it was proposed that the ensuing cellular cross-linking would strengthen and prolong T cell/AML-DC contacts, thus boosting T cells' sensitivity to AML antigens (200).

NKs may identify and destroy stressed cells, triggering an immune response much more quickly without antibodies or MHC. Tandem scFv, also known as "bispecific killer cell engager" (BiKE) or "trispecific killer cell engager" (TriKE), is a technique for directing NK cells toward cancer cells (201, 202). The innate cell engager (ICE®) AFM13 is a tetravalent BsAb that targets CD16A, the main FcR on NK cells, and CD30, which is prevalent in blood malignancies. In individuals with recurrent or resistant Hodgkin lymphoma, it has exhibited early clinical efficacy without significant therapeutical toxicity (197, 203–207). NK cells can also be recruited to cancer cells based on a scFv-Fc-scFv format. RO7297089, a bispecific BCMA/CD16A-targeted ICE® intended to cause $BCMA^+$ MM cell lysis via strong affinity interaction of CD16A and redirection of NK cell toxicity and macrophage phagocytosis, promotes antibody-dependent cell-mediated cytotoxicity (ADCC) and ADCP against myeloma cells effectively, as well as pharmacodynamic efficacy in cynomolgus monkeys (197).

The modification of *in vitro* activated or expanded immune cells with BsAbs represents a new therapy for cancer treatment. The first clinical report of this approach emerged in 1990. Nitta et al. applied the method to malignant gliomas by using anti-CD3/glioma BsAb-modified lymphokine-activated killer (LAK) cells, which exhibited a favorable anti-tumour effect (208). Following the emergence of cytokine-induced killer (CIK) technology, modified CIK cells with BsAbs have been introduced into clinical studies (209). In nude mice, the use of BsAb-CIK cells resulted in a significant ($p<0.05$) reduction in CD133 (high) tumour growth (210). Golay et al. utilized CIK cells from cryopreserved cord blood units along with blinatumomab for the treatment of CD19 malignancies, which showed meaningful therapeutic effects with no evidence of toxicity or graft-versus-host disease (210). BsAbs *ex vivo* armed T cells (EAT) could potentially overcome certain limitations of chimeric antigen receptor-modified T cells (CAR-T), including cytokine release syndrome and neurotoxicity (211). Park et al. developed EAT utilizing the IgG-[L]-scFv platform BsAb. This resulted in a more efficient infiltration of tumors and a lower concentration of TNF-α released compared to the use of BsAb or T-cell injections alone (212).

5.2 Delivery of medicines

The distribution of payloads such as medicines, radiolabels, and nanoparticles is an intriguing deployment of BsAbs (97). A payload comprising an isotope or a medication is directly attached to a BsAb in this method (40).

5.2.1 Radioactive payload

Inferior therapeutic indices (TI) cause the majority of radioligand treatments to have unforeseen dose-limiting toxicities to crucial organs, which leads to many patients receiving subtherapeutic dosage of treatment. BsAbs can be applied to enhance radioactive payloads in neoplasm areas, which can greatly increase the tumor/blood percentage and serum retention duration (97, 213–215) (Figure 6A).

A BsAb with specificity for both the TAA and the load can be cultured with the load before infusion (40). Optionally, pre-targeting strategies can be applied to avoid sustained exposure of normal tissue to the payload, reducing toxicity and side effects, which involve first injecting the BsAb, followed by administering the payload to deliver radioactive loads to a malignancy (40, 217). Such BsAbs with a radioactive payload could be utilized for radioimmunotherapy as well as cancer imaging. Pilot research revealed that pretargeted immunological positron emission tomography (immuno-PET) employing a CEA/IMP288-targeted BsAb and a [^{68}Ga] Ga-labelled hapten was secure and practical with encouraging diagnostic accuracy (218). A creative cancer-targeted DOTA-hapten pre-targeted radioimmunotherapy platform, Pr, is composed of a vacant DOTA-chelate for ^{225}Ac, which is connected to a lutetium-composited DOTA for picomolar DOTA-bound chelate scFv adhering through a short polyethylene glycol linker. In three solid patient tumor xenograft models for neuroblastoma (GD2), colorectal cancer (GPA33), and breast cancer (HER2), extended general survival, complete responses, and histologic healing were noted. Also, [^{225}Ac]Pr has a significantly higher security profile in contrast with RIT with tumor-targeted IgG antibodies (219).

5.2.2 Drug payload

By combining targeted treatment with a strong cytotoxic payload to antibodies, antibody-drug conjugate (ADC) medicines destroy malignant cells through a pharmaceutical Trojan horse approach (37) (Figure 6A). The monoclonal antibody-based ADCs,

including the anti-CD33 drug gemtuzumab ozogamicin, the first ADC authorized by the FDA, and the anti-HER2 drug adotrastuzumab emtansine to treat advanced breast carcinoma, have achieved a significant progress (220).

However, the potency of therapy may be restricted gradually since tumors may evolve defences against drug effects. Downregulation of the antigen on the cell membrane, which makes the ADC comparatively less likely to perform the cytotoxic activity, may be one cause of resistance (221, 222). Bispecific or biparatopic mAbs, which bind to non-overlapping epitopes on a single target antigen or two distinct antigens simultaneously, are novel strategies to circumvent antigen-specific rejection mechanisms (223). M1231 is an experimental ADC combining a BsAb which concurrently targets MUC1 and EGFR with a payload linked to hemiasterlin. Patients with progressive solid tumors, such as esophageal cancer, and non-small-cell carcinoma (NSCLC) are currently being studied using the BsAb as a monotherapy in phase I trials (224).

It is vital to internalize proteins effectively and direct them to lysosomes where proteolysis can occur. Nevertheless, the amplitude of these activities for many cell membrane proteins and carbohydrate compounds on tumor cells is inadequate to support an efficient ADC strategy. One approach is to incorporate BsAbs into ADCs, where one binding region would offer malignant affinity while the other binding site would enable localization to the lysosome (224). A bispecific ADC that targets both HER2 and the lysosome membrane protein CD63 appears to increase lysosomal aggregation as well as cargo delivery (225, 226).

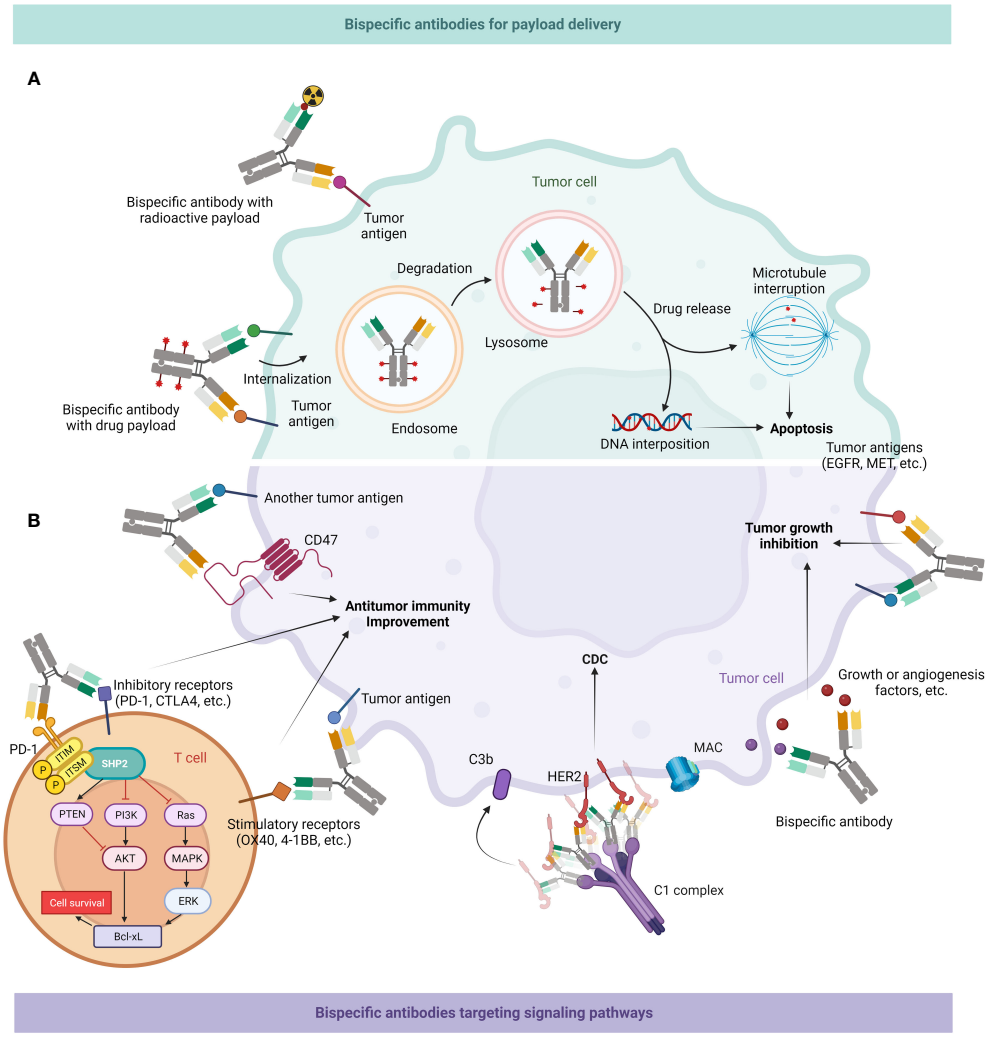

FIGURE 6
Mechanism of action of BsAb: delivery of payloads & targeting tumor-associated signaling pathways. **(A)** BsAb can enrich radioactive substances around tumor cells; they might also load drugs and then enter cancer cells through endocytosis, first into endosomes and then into lysosomes, and then release drugs to interfere with microtubule formation or DNA replication, ultimately leading to apoptosis of tumor cells. **(B)** BsAb can directly inhibit tumor growth. It can interfere with multiple signaling pathways related to neoplastic growth, as well as inhibit growth factors or angiogenic factors in the tumor microenvironment (TME). BsAb may also facilitate antitumor immune activity. It can bind CD47 on the surface of tumor cells to promote macrophages-mediated phagocytosis of them, or target inhibitory or stimulatory receptors on T cell surfaces to enhance the effector function of T cells. BsAb can also form hexamers by binding to receptors like HER2, recruiting the C1 complex, and subsequently mediating tumor cell death through the CDC effect (216).

5.2.3 Targeted delivery of nanoparticles

Delivery of nanoparticles with BsAbs can overcome the inefficiency of the enhanced permeability and retention (EPR) effect of nanomaterials and improve the active targeting and delivery efficiency of the drug itself. At the same time, BsAbs can enhance the anti-tumour activity of nanoparticles and reduce the amount of drug used, thus reducing off-target cytotoxicity.

Active targeting of nanomaterials can be achieved through coupling BsAbs to the surface of the nanomaterials. BsAbs and nanoparticles are linked via antibody-antigen interactions, which are more specific than random coupling methods (227). The vast majority of connections between BsAbs and nanoparticles are non-covalent, and upon cellular endocytosis of drugs, these bonds are readily broken, thus increasing drug release and improving delivery efficiency (228). Furthermore, the selectivity of BsAbs allows for the specific targeting of tumour tissues through nanomedicines, holding immense significance in the treatment of solid tumors. EGFR-targeted EDV nanocells loaded with paclitaxel are currently part of clinical studies and can be safely administered to patients with advanced solid tumors at a maximum tolerated dose (MTD) of 1×10^{10} microcells per dosage (229). Pre-targeted therapies are another method of delivering nanomedicines based on BsAbs (230). A CD20 Ab-mPEG scFv was able to target CD20-expressing Raji cells while carrying simultaneously grasping polyethylene glycolated liposomal DiD with a 24% increase in internalization capacity (60 h). It showed a nine-fold increase in tumour cytotoxicity (LC50: 3.45 nM) and improved anti-liquid tumour efficacy (p=0.005) compared to CD20 Ab-mPEG scFv and PLD alone (231). Another study showed that BsAbs pre-targeted delivery of D-Dox-PGA reduced off-target toxicity in human breast cancer xenografts and had antitumor efficacy comparable to Dox therapy (232).

5.3 Signaling pathway targeting

Some cancer cells can develop resistance to single-target therapies by activating alternative signaling pathways. BsAbs can co-target several tumor-related receptors on the cell membrane (Figure 6B), providing powerful anticancer strategy and reversing resistance, eliciting a more potent and effective response than single-target antibodies.

5.3.1 Bridge receptors

Resistance is a main downside of RTKs, such as EGFR and HER2. Resistance typically entails the activation of parallel signal transduction by upregulating other RTKs that avoid targeted receptor blockage. For example, the MET oncogene is amplified to induce HER3 and the PI3K-AKT cell survival pathway, enabling NSCLC to escape the suppression of EGFR-TKIs (226, 233). Several BsAbs co-targeting several RTKs were developed and are currently undergoing clinical trials (233).

The members of the ErbB family, EGFR, HER2, and HER3, are typical targets for BsAbs that interrupt two signals because of their interaction (40, 234–236). Zenocutuzumab targets the HER2 and HER3 cytoplasmic regions. By blocking Neuregulin 1 (NRG1) binding to HER3 and preventing HER3 from going through the conformational alteration necessary for heterodimerization with HER2 and possibly with EGFR, zenocutuzumab inhibit downstream oncogenic signals and the phosphorylation of HER3's cytoplasmic region (148, 183). Clinical success was verified in patients with pancreatic and lung malignancies induced by NRG1 rearrangements (148). Other oncogenes such as MET can also become targets. Amivantamab (JNJ-372) is a first-in-class, completely humanized, IgG1 BsAb that targets both EGFR and MET synchronically. It hinders the stimulation of ligand-binding receptors, encourages their internalization and destruction to impede downstream signaling cascades. It can involve Fc-mediated attachment to macrophages and then trigger a potent antibody-reliant ADCC which is vital for the blockage of both EGFR and MET pathways (38, 39, 237). Additional targets under research include lysosomal internalization-related receptors like CD63 and death receptors like death receptor 5 (DR5) (40). Shivange et al. develop antibodies that limit DR5-mediated apoptotic activity toward FOLR1-expressing ovarian tumor cells yet minimizing the need for ADCC to trigger cell destruction. It turned out that these antibodies operated better than DR5 agonist antibodies reported from clinical testing (238).

Besides, the activation of the complement-dependent cytotoxicity (CDC) system is regarded as a method for enhancing the clinical efficiency of anticancer Abs (239). It comprises one of the identified modes of action (MOA) for B-cell specific monoclonal Abs such as rituximab and atumumab (240–242). The capacity of the antigen-Ab combination to adopt a shape which permits effective C1q interaction is influenced by an array of variables, such as antigen diameter and density, which regulate activation of the classical complement system (243). Hexameric arrangement of Ab Fc regions in the Ab-antigen complexes is necessary for optimum CDC action (244). In preclinical investigations, techniques that improve CDC, such as Ab hexamerization and Fc alterations, have showed potential for improving anticancer efficacy. For instance, hexamerization was employed to create a biparatopic anti-CD37 B-cell specific Ab with increased *in vitro* CDC efficacy (216, 245). Utilizing BsAbs which antagonize the C-mediators CD55 and CD59 to promote C-regulated activities, Macor et al. devised an innovative method to improve CDC (246). Although trastuzumab, pertuzumab, and tras +pert exert anticancer action via a variety of MOAs, they are unable to induce CDC in HER2-exhibiting cells when human serum is present (247–249). According to Weisser et al.'s hypothesis, a biparatopic Ab designed to increase receptor crosslinking and gathering might offer the receptor-Ab complex needed to connect C1q and activate the effective anti-cancer mechanism, as well as preserve and/or improve all other identifiable anti-cancer MOA assigned to authorized anti-HER2 Ab therapies (216).

5.3.2 Biparatopic BsAbs

BsAbs might be constructed to concurrently attach to two non-overlapping epitopes on the same target rather than two distinct antigens. Biparatopic targeting mimics the actions of antibody

cocktails and polyclonal antibodies by enhancing binding efficiency via antigen crosslinking and accumulation (28). Earlier research has demonstrated that it is crucial for biparatopic BsAb to structurally identify two epitopes or to have a strong specificity for the antigen (250). Therefore, the membrane protein, which has extensive extracellular domains and has been the subject of a few mAbs, is a viable target for BsAbs (251). ZW25 is a BsAb that adheres to two HER2 epitopes concurrently, one is the pertuzumab (Perjeta; Genentech) binding site ECD2, and one is the trastuzumab binding site ECD4. According to preclinical studies, ZW25 are able to mute HER2 signaling more efficiently than trastuzumab or pertuzumab and exhibit high anticancer efficacy at a variety of HER2 expression rates. Currently, the drug is being evaluated in a phase I basket test for HER2-positive tumors (11).

5.3.3 BsAb targeting redundant ligand

Another area of concern for BsAbs is tackling redundancy for numerous growth or angiogenesis factors in the tumor microenvironment (TME). By reducing angiogenin-2 (Ang-2) and vascular endothelial growth factor-A (VEGF-A) in the TME, the CrossMab construction vanucizumab prevents angiogenesis, while VEGF and delta-like ligand 4 are the objectives of the BsAb OMP-305B83. In these architectures, both BsAbs carry Fc as a lengthy half-life is essential for efficient factor elimination (40). Moreover, ZVEGFR2 Bp2, which has anti-angiogenic impacts equivalent to the clinically authorized Ramucirumab, was regarded as the best candidate for further assessment because of the slow dissociation level and the benefits of a shorter linker in a study to test the potential of four biparatopic constructs (252).

6 Challenges and prospects in solid tumor

The application of BsAbs in cancer has also encountered numerous bottlenecks, particularly in solid tumors, which are mainly attributed to the more complex microenvironment of solid tumors. In this section, we will discuss the challenges of identifying suitable targets within solid tumors, penetrating the tumor, as well as the difficulties related to tolerance, side effects, and large-scale production of BsAb therapy.

6.1 Identifying the promising targets in solid tumor

For solid malignancies, the first step to effective immunotherapy is identifying the right targets, which are probably TSAs uniformly expressed on cancer cells and are crucial in the growth of tumors (253). Although both immune cell-redirecting and antigen-bridging BsAbs are being investigated, over three-quarters of these investigations focus on the second one. Bridged antigen pairings can be single targets like HER2/HER2 (biparatopic BsAb) or associations of two separate antigens like PD-1/CTLA4, PD-1/PD-L1, VEGF/Ang-2, IGF-1/IGF-2, etc. These BsAbs primarily target double signaling transductions to produce reinforced inhibited or activated impacts in lymphocytes or tumor cells. Immune cell-redirecting, CD3-targeted/TAA-targeted BsAbs have used TAAs such as EGFR, HER2, and c-Met, whereas TSAs like EGFRvIII (254), p95HER2 (255), and RAS G12V are more desirable targets to prevent on-target, off-tumor impacts caused by target expression on healthy cells. In fact, hemopoietic differentiation antigens like BCMA, CD123, and CD20 satisfy these requirements, while there are few TSAs accessible for solid tumors and, if exists, antigen heterogeneity would be an issue (256).

6.2 Penetration of the immune cells

Only a modest percentage (less than 1%) of migrated T cells were ultimately able to penetrate solid neoplasm tissues, according to preclinical research (257). Therefore, in order to maximize the efficacy of immunotherapies in cancers, T cell penetration must be improved (258). Macromolecules like BiTEs force out via transvascular holes with diameters ranging from 200 nm to 1.2 μm inside the solid tumor vasculature and permeate into the neoplasm (259). TILs are present in a variety of solid malignancies, and their number is closely correlated with cancer reaction to ICIs and prognosis (260–262). In malignancies lacking TILs (263), it is depended on the BiTEs' capacity to draw T cells from the blood. The TME presents a variety of difficulties for BiTE medication, as it does for other macromolecule treatments for solid malignancies. A solid tumor's physical hurdle prevents trafficking and penetration, greatly lowering the quantity of both BiTEs and T cells involved (264). An alluring remedy is the gene editing of an oncolytic virus to generate a BsAb following its invasion of a tumor cell. After selectively infecting tumor cells, the virus releases its offspring into the surroundings. In the vicinity of the tumour, the virus releases BiTEs that trigger a T cell activation during the viral cycle. A study reveals the viability and efficiency of using an erythropoietinproducing human hepatocellular carcinoma A2 receptor (EpHA2)-targeted BiTE in combination with oncolytic adenoviruses and adenovirus-delivered gene treatment; this design can provide a robust tumor-specific cytotoxic response that brings long-term management of pediatric high-grade gliomas (265). Adenoviruses and other oncolytic viruses can also destroy cells immediately via oncolysis, which might cause the liberation of neoantigens from the ruptured cells. The APCs' further exposure of them may serve as an *in situ* vaccine, boosting the precise immune reaction (266). Comparable methods are reported by Gardell et al., who administered a retrovirally edited macrophage that could release a BiTE selective for EGFRvIII to mouse models of glioma. The macrophages would stay in the solid malignancy and continue to secrete IL-12 and BiTE, which would strengthen the T cell activity (267, 268). Given that the effectiveness of BsAbs in the therapy of solid tumors appears to be constrained by low tumor infiltration and on-target, off-tumor activity ending in dose limiting toxicity (DLTs), BiTE-armed oncolytic viruses represent an attractive new strategy for enhanced performance (187, 269–272).

6.3 Resistance to BsAb therapy

Another concern is the emergence of resistance to BsAb treatment. One typical tactic of resistance is the downregulation or removal of BsAb-specific TAAs on carcinoma cells, which results in cancer immune evasion. ICI resistance is linked to cancer-intrinsic deficiencies in antigen presentation, such as lack of β2 microglobulin needed for MHCI protein production on the cell membrane. Immune checkpoint overexpression, a key component of the immunosuppressive TME, could help with BsAb resistance. Although ICIs have transformed lung carcinoma therapy and are now the standard of care for patients with advanced solid cancers, but the general reaction is still poor, which reveals significant unsatisfied therapeutic needs (37, 273–275). A monovalent IgG1-based BsAb named MEDI5752 was developed to specifically inhibit the PD-1 cascade and CTLA-4 on PD-1$^+$ stimulated T cells. Notably, in contrast to the individual PD-1-targeted or CTLA-4-targeted mAbs, MEDI5752 was discovered to promote swift internalization and breakdown of PD-1 and to concentrate selectively in malignancies, generating stronger anti-cancer efficacy (276). OX40/CTLA-4-specific BsAbs are in the preliminary stages of research. Agonist mAbs have been developed to stimulate the immunological costimulatory protein OX40. A number of BsAbs that can selectively attach to OX40 and CTLA-4 to target Tregs in the TME have been invented. These BsAbs are reported to have anticancer impact on bladder, pancreatic, and colon cancers and to work in conjunction with anti-PD-1 antibodies to control colorectal tumor (277, 278).

6.4 Side toxicity of BsAbs

The two major issues associated with the cytotoxicity of BsAbs are CRS and neurotoxicity. Exhaustion, headache, and fever are common signs of CRS, as well as more serious symptoms like hypotension, tachycardia, acute respiratory distress syndrome, and circulatory failure. Sequential dosage, premedication with dexamethasone, and SC dosing might reduce the frequency and intensity of CRS (37, 68, 163). The symptoms of neurotoxicity comprise headache, intention tremor, speech difficulties, cognitive problems, and seizures (187). Step dosage and prophylactic corticosteroid usage are among the methods for managing neurotoxicity (37, 279), but there are also ideas to utilize anti-adhesive medicines attempting to avoid neurotoxicities. According to Klinger et al., T-cell adherence to endothelium is a required but inadequate precursor to the emergence of blinatumomab-related neurological negative effects, and inhibiting adherence is a potential mitigating strategy (279).

6.5 Large-scale manufacturing of BsAbs

For a number of factors, producing BsAbs is difficult. For instance, challenges with protein production, stability, or the adoption of atypical production techniques may lead to low yields or unsuitable facility design (110). Since the essential methods for regulated Fab-arm interchange are consistent with typical procedures for commercial synthesis of conventional human IgG1, this technology seems to be well suited for large-scale manufacture. The adaptability was validated by the large-scale application of the bench-scale technique, which did not require extensive tuning or result in a decline of output quality (33). CHO cells were used to produce two original antibodies, each of which had a single matching point alteration in the CH3 domain. These antibodies were then produced at a volume of 1000 L utilizing a system fed-batch and purifying procedure created for conventional antibody manufacturing. By combining the two mother molecules in carefully monitored reduction circumstances, the BsAb was produced, featuring an effective Fab-arm interchange of >95% at kilogram scale. By using diafiltration to eliminate the reductant, the disulfide between chains spontaneously reoxidized. In addition to the molecule's bispecificity, in-depth evaluation showed that the IgG1 structural coherence was preserved, involving functionality and durability (110).

7 Conclusion and future perspectives

Recent advances in cancer immunotherapy have highlighted the enormous potential of BsAbs as innovative targeted therapy tools. Over the past two decades, the development of BsAbs has accelerated significantly, benefiting from groundbreaking innovations in antibody formatting. Notably, strategies such as modifying the Fc region have proven effective in mitigating adverse effects (68, 280), while antibody tandem techniques and dimerization enhanced the stability of BsAbs without involving the Fc region (173, 281, 282). However, the paradox between stability and adverse effects associated with the presence or absence of the Fc fragment remains a complex issue requiring further exploration through deliberate design strategies (173). Emerging gene therapies such as genetically engineered cells or nucleic acid drugs hold promise for improving the efficacy and safety paradox.

The landscape of BsAb therapies continues to expand with some preclinical and clinical studies suggesting macrophages and neutrophils as potential therapeutic targets (283). These innate immune cells possess unique cytotoxic properties that can be leveraged to eliminate cancer cells (284). Notably, tumor-associated neutrophils (TANs), especially tumor-associated macrophages (TAMs), are already abundantly present in the TME, precluding the need for external recruitment (285–288). By stimulating these immune effectors *in situ*, BsAb therapies offer the potential for highly efficient tumor cell clearance (289). The strategy of combining BsAbs with other antitumor approaches such as radiotherapy, chemotherapy, mAb, Small-molecule inhibitors or other BsAbs, can overcome the limitations of monotherapy. ATIM-05 (anti-CD3 and anti-EGFR) used prior to radiation/temozolomide, can overcome the highly immunosuppressive glioblastoma (GBM) microenvironment (290). A clinical study (NCT02892123) has shown that ZW25 is well tolerated in combination with chemotherapeutic agents (paclitaxel, capecitabine, or vinorelbine). This approach has excellent and long-lasting antitumor activity in patients with HER2$^+$ BC (291). The clinical study (NCT04626635) has demonstrated that RENG7075 used in tandem with cemiplimab boosts the anti-

tumour effectiveness of cemiplimab immunotherapy by targeting CD28 with BsAbs (292). Combining BsAb treatment with immune checkpoint blockade provides another avenue for enhanced anti-tumor responses (293). Such combinations take advantage of BsAbs preferential targeting of specific cell types like CD47 and PD-L1 expressing cells to ensure selective cancer cell elimination while protecting normal cells (294, 295). V-aCD3Mu in combination with ICIs can eliminate solid tumors in different cancer models such as B16-F10, 4T1 and CT26 Models (296). The combination of two BsAbs, such as AK117 and AK112 was used in the humanized PD-1 mice models, showing good anti-tumour effects (297). These combined approaches present a promising therapeutic option that provides a wider outlook for BsAbs.

Despite significant progress, BsAb development in oncology remains unfinished. While several BsAbs have gained marketing approval, the rapid emergence of trispecific and multispecific antibodies highlights their dynamic potential (298–301). Robust validation through larger phase II-III clinical trials across cancer types and individualized treatment regimens will be key to fully harnessing the advantages of BsAbs. However, in the realm of solid tumor treatment, BsAbs still face many challenges including but not limited to off-target effects, immune cell penetration, toxicity, resistance mechanisms, manufacturing complexity, and cost (188, 302, 303).

In summary, BsAbs represent a promising direction for designing and developing novel anti-cancer drugs, and their continued R&D will be an integral part of biotherapeutic oncology. Meanwhile, optimizing BsAb pharmacokinetics, manufacturability, deeply understanding factors impacting their penetration and activity in the TME, developing rational combination therapies to improve response rates, and evaluating their engagement with other immune cells like NK cells and CAR-T cells, while elucidating resistance mechanisms, will be critical to advancing BsAbs clinical translation and realizing their anti-tumor potential.

Author contributions

GS: Conceptualization, Writing – original draft, Writing – review & editing. XG: Writing – original draft, Writing – review & editing. YW: Writing – original draft, Writing – review & editing. YX: Writing – original draft. NX: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Glossary

BsAbs	bispecific antibodies
BsNbs	bispecific nanobodies
TCR-T	T cell receptor T cells
CAR-T	chimeric antigen receptor T cells
ICBs	immune checkpoint blockades
mAbs	monoclonal antibodies
Fab	fragment antigen-binding
EpCAM	epithelial cell adhesion molecule
FcγRs	Fcγ
Fc	fragment crystallisable
IgG	Immunoglobulin G
FcRn	feonatal Fc receptors
Fv	fragment variable
CH	constant heavy chain
CL	constant light chain
DART	dual-affinity retargeting
VH	variable heavy chain
VL	variable light chain
SCFV	single chain variable fragment
VHH	variable heavy-chain segments
VNAR	variable new antigen receptor
hetEHD2	heterodimerizing EH Domain Containing 2
MHD2	IgM heavy chain domain 2
Nbs	nanobodies
EGFR	epidermal growth factor receptor
HER2	human epidermal growth factor receptor 2
Rha	rhamnose
TNF	tumour necrosis factor
IL	Interleukin
PD-L1	programmed cell death-ligand 1
VEGFR1D2	vascular endothelial growth factor receptor type 1 domain 2
KIHs	knobs-into-holes
DutaFab	dual targeting Fab
DVD-Ig	dual variable domain-Ig
FIT-Ig	Fabs-In-Tandem Immunoglobulin
Duobody	controlled Fab-arm exchange technology
TandAb	tandem diabodies
BiTE	bispecific T cell engager

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ImmTAC	immune mobilizing monoclonal TCRs against cancer
BriKE	bi-specific killer cell engagers
CD3	cluster of differentiation 3
CEA	carcinoembryonic antigen
Ang2	angiopoietin-2
VEGF	vascular endothelial growth factor
TIM3	T cell immunoglobulin domain and mucin domain-3
FORCE	format chain exchange
SEED	strand-exchange engineered domain
cFAE	controlled Fab-arm exchange
IV	intravenous
IC	intracutaneous
SC	subcutaneous
IM	intramuscular
RMT	receptor-mediated transcytosis
PEG-PLA	polyethylene glycol-lactic-co-glycolic acid
P-gP	P-glycoprotein
PD-1	programmed cell death protein 1
TAA	tumor-associated antigen
HLA	human leucocyte antigen
ICIs	immune checkpoint inhibitors
TCR	T-cell receptor
NHL	non-hodgkin lymphoma
MM	multiple myeloma
AML	acute myeloid leukemia
CHO	Chinese hamster ovary
TDCC	T-cell-dependent cellular cytotoxicity
LAG3	lymphocyteactivation gene 3
DCs	dendritic cells
NK	natural killer
APCs	antigen-presenting cells
LAK	lymphokine-activated killer
CIK	cytokine-induced killer
EAT	*ex vivo* armed T cells
TI	therapeutic indices
ADC	antibody-drug conjugate
NSCLC	non-small-cell carcinoma
EPR	enhanced permeability and retention
MTD	maximum tolerated dose

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NRG1	neuregulin 1
ADCC	antibody-dependent cell-mediated cytotoxicity
CDC	complement-dependent cytotoxicity
TME	tumor microenvironment
DLTs	dose limiting toxicity
TAMs	tumor-associated macrophages

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Manifestation of subacute cutaneous lupus erythematosus during treatment with anti-PD-1 antibody cemiplimab – a case report

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Introduction: The anti-programmed cell death protein 1 (PD-1) antibody cemiplimab has shown promising results in the treatment of unresectable or metastatic squamous cell carcinoma, however, frequently leads to immune-related adverse events limiting therapy efficacy. Although cutaneous side effects are common, only very few cases of cutaneous lupus erythematosus have been reported under anti-PD-1 immunotherapy. So far, no case of cutaneous lupus has been described under treatment with cemiplimab.

Case report: For the first time, we report the case of a patient with advanced squamous cell carcinoma, who developed clinical and histological findings in sun-exposed skin that were consistent with anti-SS-A/Ro antibody-positive subacute cutaneous lupus erythematosus (SCLE) under treatment with cemiplimab. Additionally, laboratory chemical analyses revealed a severe immune-related hepatitis without clinical symptoms. Both, the SCLE and the hepatitis, resolved after the administration of topical and systemic steroids and the discontinuation of anti-PD-1 therapy.

Conclusion: Treatment with cemiplimab can be associated with the appearance of cutaneous lupus erythematosus in sun-exposed areas. Application of topical and systemic glucocorticoids can lead to a rapid resolution of the skin eruptions. Moreover, our case illustrates the possibility of simultaneously occurring severe immune-related adverse events. This highlights the importance of additional diagnostics to avoid overlooking additional immune-related adverse events.

KEYWORDS

case report, cutaneous squamous cell carcinoma, immunotherapy, anti-PD-1 antibody, cutaneous lupus erythematosus

Introduction

The anti-programmed cell death protein 1 (PD-1) antibody cemiplimab has shown remarkable efficacy in the treatment of unresectable or metastatic cutaneous squamous cell carcinoma (CSCC) (1). However, immune-related adverse events frequently occur during anti-PD-1 treatment and can cause discontinuation of therapy (1). Subacute cutaneous lupus erythematosus (SCLE) is a rare but known adverse event during therapy with other anti-PD-1 antibodies, such as pembrolizumab or nivolumab (2). SCLE presents with erythematous, circular, scaly skin lesions on sun-exposed skin and is triggered by various drugs and UV light (3, 4). Diagnosis is based on skin lesion morphology, laboratory findings (e.g., anti-Ro (SS-A) antibodies) and histopathological findings (3, 4). Therapy includes sun protection and anti-inflammatory topical or systemic drugs, such as corticosteroids or hydroxychloroquine. Moreover, it should be considered to discontinue or pause the potentially triggering drug (3, 4). Cutaneous adverse events are very frequently in up to 50% of patients treated with anti-PD-1 therapy (1, 5–8). Among these, the appearance of SCLE was described for pembrolizumab and nivolumab in less than 0.01% of patients (9). In contrast to the vast majority of cutaneous adverse events, the appearance of SCLE frequently leads to discontinuation of therapy and thus constitutes a relevant potential adverse event (10–12). So far, no case of SCLE has been reported for treatment with cemiplimab (5). For the first time, we describe the occurrence of histopathologically and serologically confirmed SCLE in a patient undergoing treatment with cemiplimab (Figure 1).

Case description

A 64-year- old woman with lymphogenic metastasized CSCC of the left limb presented in our dermatological department with a three-week history of severely pruritic, coalescent, erythematous, and scaly macules and papules in sun-exposed areas of the skin (V-area of the upper trunk and neck, forearms, dorsal hands, lower legs, and dorsal feet, Figure 2A) five weeks after the administration of the anti-PD-1 antibody cemiplimab. The patient's medical history included several benign and malignant light-induced skin eruptions (actinic keratoses, basal cell carcinoma) of the left limb, type 2 diabetes mellitus, and cardiovascular diseases, but no known autoimmune diseases. Despite the successful surgical removal of the primary tumor, the patient developed lymph node metastases of the left axilla (Figure 1). Because of disease progression that could not be managed surgically, we decided to initiate anti-PD-1 antibody therapy as first-line treatment in accordance with the current interdisciplinary guideline on invasive CSCC (13). Five weeks after the occurrence of lymph node metastases the patient received a single dose of cemiplimab 350 mg. Due to an infection of the upper airways three weeks after the first application, which resolved completely after treatment with Amoxicillin for seven days, cemiplimab treatment was halted. Additionally, three weeks after treatment initiation with cemiplimab, the patient underwent fractionated irradiation of symptomatic lymphogenic CSCC metastases of the left axilla for three weeks with a total dose of 40 Gray. The skin eruptions occurred two weeks after administration of cemiplimab and progressed over three weeks.

A skin biopsy of the chest showed an interface dermatitis with colloid bodies, perifollicular mucin, and mixed immune cells (Figure 3A). A second skin biopsy of the right forearm displayed an acanthosis, parakeratosis, and intraepithelial neutrophil granulocytes, consistent with a psoriasiform inflammation (Figure 3B). Immunoblotting revealed highly positive anti-Ro-52 (SS-A) antibodies (299 U/ml using ELISA), whereas direct immunofluorescence did not show precipitations of IgA, IgG, IgM, C3, or fibrin. Routine blood testing revealed highly elevated liver enzymes and cholestasis parameters (peak values: AST 401 U/l, ALT 267 U/l; γ-GT 1947 U/l, alkaline phosphatase 640 U/l, total bilirubin 2.56 mg/dl; Figure 1) without any pathologies in the clinical examination and computed tomography imaging of the liver. Hepatitis virus serology as well as autoimmune hepatitis panel were negative. The patient was diagnosed with drug-induced, immune-related SCLE, Common Terminology Criteria for Adverse Events version 5.0 (CTCAE v5.0) grade 2 and immune-related hepatitis, CTCAE v5.0 grade 3 (14). Treatment with prednisolone for the severe hepatitis and topical steroids (mometasonfuroate 0.1% ointment) were initiated and tapered over eight weeks. The cutaneous eruptions

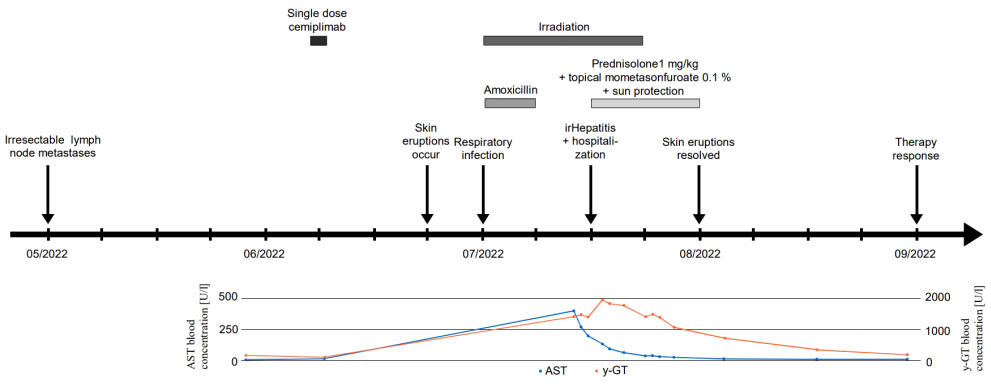

FIGURE 1
Timeline. Timeline of therapeutic regimens, patient's symptoms, and laboratory studies.

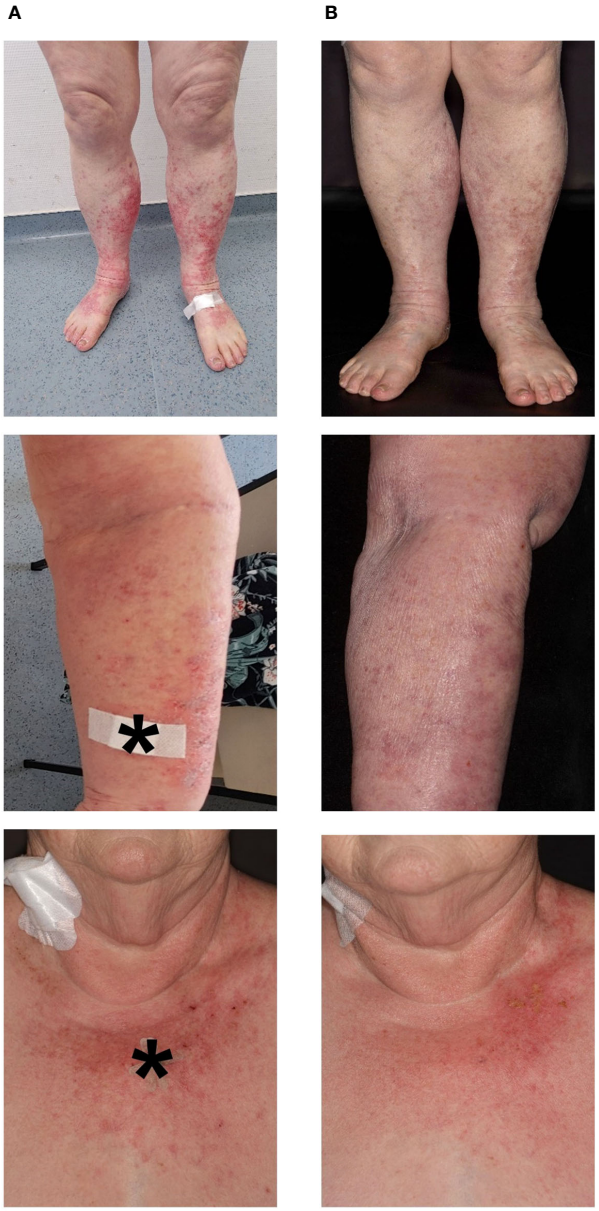

FIGURE 2
Clinical images **(A)** on initial contact and **(B)** 14 days after initiation of steroid treatment. Severely pruritic, scaly, coalescent, and erythematous macules and papules on sun-exposed skin (V-area of the upper trunk and neck, forearms, dorsal hands, lower legs, and dorsal feet). * indicates biopsy site.

gradually resolved (Figure 2B) as well as the elevated liver enzymes and cholestasis parameters (Figure 1). Due to the severe CTCAE v5.0 grade 3 hepatitis we decided not to re-initiate treatment with cemiplimab according to the current ASCO guideline (15). Imaging after three months showed therapy response and the patient remained progression-free for eight months.

Discussion

For the first time, we present the rare occurrence of SCLE related to cemiplimab therapy complicated by immune-related hepatitis. As described by Marzano et al. drug-induced SCLE usually goes along with elevated anti-Ro (SS-A)-antibodies and is chronologically related to the triggering drug (3). Because of the typical clinical appearance and distribution of the skin eruptions and the high concentration of anti-Ro (SS-A)-antibodies, we diagnosed SCLE despite the lack of lesional immune precipitates. The skin biopsies taken from the chest and the right forearm revealed an interface dermatitis and a psoriasiform inflammation, respectively. Marzano et al. described two different predominant phenotypic variants of drug-induced SCLE: 'papulosquamous' and 'annular polycyclic' (3). SCLE mostly goes along with histological interface dermatitis (3). However, biopsies taken from 'papulosquamous' SCLE can show psoriasiform changes as well (4, 5). In accordance with the clinical phenotype both biopsies contribute to a 'papulosquamous' SCLE in the case of our patient.

It remains unclear whether a topical application of glucocorticoids would have been sufficient for the recovery of the skin lesions, as systemic steroid treatment was necessary to treat the concomitant immune-related hepatitis. In patients treated with the anti-PD-1 antibodies pembrolizumab or nivolumab moderate SCLE eruptions could be treated successfully by discontinuation of immunotherapy and were kept under control after re-initiation of systemic treatment by the use of topical steroids (16, 17). In addition to treatment discontinuation and topical steroids the use of systemic hydroxychloroquine and/or glucocorticoids alone or in combination was described to resolve severe SCLE skin eruptions (10–12, 18–23). By this means, immunotherapy could be continued successfully in some cases without or with only a mild relapse of SCLE (20–22). Although the patient only underwent irradiation of the left axilla, radiotherapy is a known trigger of cutaneous lupus erythematosus and was temporally related to the occurrence of the presented skin eruptions in our case (24, 25). Therefore, irradiation could have acted as a trigger for SCLE in the described case. Moreover, the simultaneous irradiation of the metastatic site could have masked response to cemiplimab. In our case, cemiplimab treatment was discontinued because of the severe hepatitis. Monitoring of SCLE and evaluation of possible therapy options under cemiplimab treatment might be of interest for future patients.

In conclusion, our case demonstrates that patients receiving cemiplimab can develop cutaneous lupus erythematosus. The concomitant immune-related hepatitis underlines the need to screen for additional adverse events other than skin conditions. Diagnosis and treatment of patients with adverse events on cemiplimab treatment will be a challenge for dermatologists in the future.

Patient perspective

For the patient, an improvement of the sever pruritus accompanying the cutaneous lesions was of primary importance. As she did not develop any clinical symptoms from the hepatitis, it was challenging for the patient to stay motivated to continue on

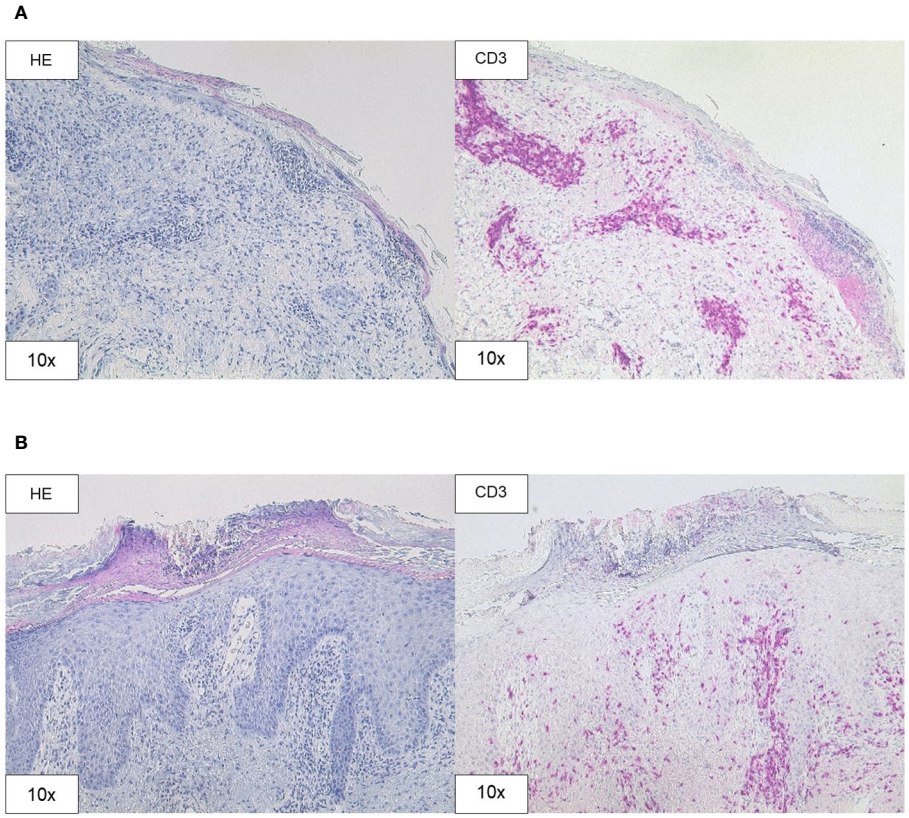

FIGURE 3
Histopathology. **(A)** H&E and CD3 staining (original magnification 10x) of the skin biopsy of the chest demonstrating interface dermatitis with colloid bodies, perifollicular mucin, and mixed immune cells, containing lymphocytes, histiocytes, and neutrophil granulocytes. **(B)** H&E and CD3 staining (original magnification 10x) of the skin biopsy of the right forearm showing acanthosis, parakeratosis, intraepithelial neutrophil granulocytes, and CD3 positive T cell infiltration.

systemic steroids after resolution of the skin symptoms. Transparent communication of diagnostic results and therapeutic consequences was important for the patient to reach a high level of compliance.

Data availability statement

The data analyzed in this study is subject to the following licenses/restrictions: a publication of datasets containing original patient-related data could affect the patient's anonymization. Original datasets are available from the corresponding author on reasonable request. Requests to access these datasets should be directed to Simon Fietz, Simon.Fietz@ukbonn.de.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

SF: Conceptualization, Formal Analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing, Data curation, Project administration, Software, Validation. AF: Supervision, Writing – original draft. CM: Conceptualization, Supervision, Writing – original draft. LV: Supervision, Writing – original draft. TF: Data curation, Investigation, Writing – original draft. JL: Supervision, Writing – original draft. FH: Conceptualization, Project administration, Supervision, Writing – original draft. JS: Conceptualization, Project administration, Supervision, Writing – original draft.

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Conflict of interest

JS is a consultant/advisory board member and received speaker's honoraria from Novartis, BMS, MSD, and Roche.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Complete and early response to cemiplimab associated to severe immune toxicity in advanced cervical cancer: a case report

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Cervical cancer (CC) is the second most commonly diagnosed cancer and the third leading cause of cancer death among females. The options of treatment for recurrent/advanced CC are limited and patients experiencing recurrence after first line platinum-based chemotherapy have a poor prognosis. In this context, immune checkpoint inhibitors (ICI)s antagonizing PD-1 and programmed death-ligand 1 (PD-L1) have profoundly changed the treatment scenario and outcomes in CC in the first or subsequent lines both as monotherapies or in combination with chemotherapy or other ICIs. Herein, we report the clinical case of a 74-year-old woman with metastatic CC with negative tumor PD-L1 expression who having disease progression after first-line of systemic treatment with platinum, thus undergoing to anti-PD-1 namely cemiplimab. The patient achieved a surprising, fast and complete metabolic response to cemiplimab immediately discontinued after only two cycles due to the onset of rare and severe immune-related adverse events (irAE)s such cardiovascular toxicity and hypertransaminasemia. Despite this, thirteen months later, the patient remains disease-free despite cemiplimab was withdrawn.

KEYWORDS

cemiplimab, immunotherapy, cervical cancer, immune-related adverse events, cardiotoxicity, hepatotoxicity, spleen immune activity

Introduction

Globally, cervical cancer (CC) is the second most commonly diagnosed cancer and the third leading cause of cancer death among females (1). The most significant cause of CC is persistent papillomavirus infection (2).

Metastatic or recurrent CC is usually a symptomatic and devastating condition for the patient (3, 4). The combination of paclitaxel plus cisplatin showed the highest response rate

(29%), median PFS (5.8 months) and median OS (12.8 months) and was considered the preferred regimen (5). The combination of paclitaxel and carboplatin could be considered a valid alternative (6). The addition of anti-angiogenic drug as bevacizumab to combination chemotherapy in first line showed an improvement of 3.7 months in median overall survival (OS) (7). Despite the improvement in OS conferred by anti-angiogenic therapy, most patients have progression after first-line and have limited treatment options (8).

To this regard, the background for employing immunotherapy in CC is strongly supported be several features such as HPV-driven carcinogenesis, high tumor mutational burden (TMB), T-cell infiltration, and microsatellite instability (MSI). In addition, the integrated genomic and molecular characterization has amply reported the amplifications in PD-L1 and PD-L2 in CC specimens (9).

In October 2021, the Food and Drug Administration (FDA) granted approval to pembrolizumab in combination with platinum-based chemotherapy with or without bevacizumab in patients with persistent, recurrent, or metastatic PD-L1-positive (CPS≥1) CC (10).

Unfortunately, there was no survival benefit with second-line systemic therapy (8). About this, accelerated approval for pembrolizumab monotherapy for the treatment of patients with recurrent or metastatic CC with disease progression on or after chemotherapy whose tumors express PD-L1 (CPS≥1) was granted in June 2018, based on results from the phase II KEYNOTE-158 basket trial (11, 12).

Cemiplimab, another anti-PD-1 monoclonal antibody, showed significant activity results in pre-treated advanced CC population. In the phase III EMPOWER-Cervical 1 trial, cemiplimab led to significantly longer OS and PFS than standard chemotherapy among patients with recurrent CC who had disease progression after first line platinum-chemotherapy regardless of histology and previous bevacizumab exposure (13). Patients were enrolled regardless of PD-L1 expression. Interestingly objective responses were seen also in patients with PD-L1 expression of less than 1%. The good safety profile of cemiplimab was consistent with that previously reported for the other ICIs in other tumor types (14). Importantly, only 8.7% of patients receiving cemiplimab discontinued treatment for any grade of immune-related adverse events (irAE)s.

Therefore, in September 2021, cemiplimab was granted priority review by the FDA for patients with recurrent or metastatic CC who are not candidates for surgery. In November 2022, also the European Medicines Agency (EMA) has approved cemiplimab (Libtayo®) for the treatment of recurrent or metastatic CC that has progressed on or after platinum-based chemotherapy regardless of PD-L1 expression status or tumor histology.

Although comparative data are lacking, immunotherapy with anti-PD-1 such as pembrolizumab, cemiplimab, and nivolumab is generally expected to show similar clinical activity (15).

Herein, we report an advanced CC case pre-treated with platinum-based regimen that responded favorably to cemiplimab after only two cycles. The patient immediately discontinued immunotherapy due to the onset of rare irAE such cardiovascular toxicity.

Notably, in our patient a complete response to cemiplimab was observed notwithstanding the tumor PD-L1 expression of less than 1%.

To our knowledge, this is the first clinical case of a patient with advanced CC successfully treated with cemiplimab obtaining a complete and early metabolic response in association to severe irAEs.

Case presentation

In February 2022, a 74-year-old woman was diagnosed with G3 adenosquamous cell carcinoma of the uterine cervix, International Federation of Gynecology and Obstetrics (FIGO) clinical stage IIIA.

Radiological staging with computed tomography (CT) scan at baseline showed a large cervical mass of 5 cm associated with intense hyperaccumulation of the 18-fluorodeoxyglucose (FDG) at to positron emission tomography (PET) examination.

Her family history was negative. Prior personal history was complicated by several important comorbidities such as coronary vasculopathy without significant stenosis, chronic cerebral vascular disease in a patient with a previous ischemic stroke, Von Willebrand disease, chronic obstructive pulmonary disease in a patient strong smoker, and dyslipidemia. Despite that, the patient was functioning well, as indicated by an Eastern Cooperative Oncology Group performance status of 1.

The patient received from April to May 2022 a definitive external beam radiotherapy (EBRT) with a dose of 45Gy/25 fractions and subsequently brachytherapy with a dose of 28 Gy/4 fractions completed in June 2022. The patient obtained a complete radiological and metabolic response following radical radiotherapy treatment on the site of cervical tumor.

Approximately one month after completion of radiotherapy, the patient showed a clinical disease progression with the appearance of a subcutaneous nodule in the right knee that was radically removed. The histological examination confirmed the diagnosis of CC metastases.

The staging with 18-FDG PET scan showed disease progression with several bilateral lung metastases and also subcutaneous nodulation of the left foot.

Following multi-disciplinary discussion and cardiovascular assessment including coronary angiography without evidence of relevant stenosis, the patient was treated with carboplatin (AUC 5) as first-line systemic therapy for a total of three cycles from July to September 2022. Paclitaxel and bevacizumab therapy were excluded for multiple comorbidities.

In September 2022, a new metabolic assessment revealed disease progression with multiple lung metastases to the anterior segment of the right upper lobe (12x11 mm and 18x17 mm) with maximum standardized uptake value (SUVmax) of 9.1 and 7.7 respectively, to the anterior segment of the left lower lobe (17x14 mm) with SUVmax 7.8, new appearance of hyperaccumulation in two hypodense hepatic lesions in the VII and VI segments (SUVmax 5.7), and a lymphadenopathy to the right hilar region (SUVmax 5.3) (see Figure 1).The combined positive score (CPS) for PD-L1 on tumor tissue from the last surgery was negative. Given the unavailability of clinical trials in our Institute for this patient,

following approval by the Institute's ethics committee, immunotherapy with cemiplimab, a PD-1-blocking antibody, was provided for compassionate use in a managed access program.

In October 2022, the patient started treatment with cemiplimab at the standard dose of 350 mg every 3 weeks until disease progression or unacceptable toxicity. The immunotherapy was performed for a total of two cycles and then withdrawn due to the onset of severe toxicity.

During the course of cemiplimab treatment, after the first cycle, the patient experienced asymptomatic toxicity of grade 1 such as hepatic adverse event in the form of elevation of alanine aminotransferase (ALT) and aspartate transaminase (AST), for which continued immunotherapy with close laboratory monitoring.

Unfortunately, 14 days after the second cemiplimab infusion, the patient was urgently hospitalized for acute anterior ST-elevation myocardial infarction (STEMI) complicated by severe left ventricular dysfunction, and hypertransaminasemia (grade 3).

During hospitalization, the patient was undergoing to coronary angiography and subsequently coronary CT scan without evidence of relevant stenosis or acute plaque rupture, thus excluding an acute coronary syndrome.

Importantly, echocardiography revealed a complete, apical and midventricular akinesia and left ventricular dysfunction (ejection fraction of 38%) associated to elevated cardiac markers such as brain natriuretic peptide and cardiac troponin I. The cranial CT scan showed no signs of ongoing bleeding.

The patient was not subjected to further investigation with cardiac magnetic resonance.

These imaging features were suggestive of Takotsubo cardiomyopathy, however, immunotherapy-related myocarditis could not be excluded.

To confirm the suspected diagnosis of Takotsubo syndrome, a later echocardiography performed at four weeks reported a recovery of the left ventricular systolic dysfunction with resolution of apical akinesia (Lyon).

Therapy for heart disease included antiplatelet drug, beta-blocker, diuretics and norepinephrine injection for acute hypotension. For the management of irAEs, immunosuppressive therapy with oral systemic steroid was initiated resulting in rapid improvement and progressive reduction of transaminases. She was discharged after fourteen days of hospitalization on prednisone 50 mg daily with plan for gradual taper over 6 weeks.

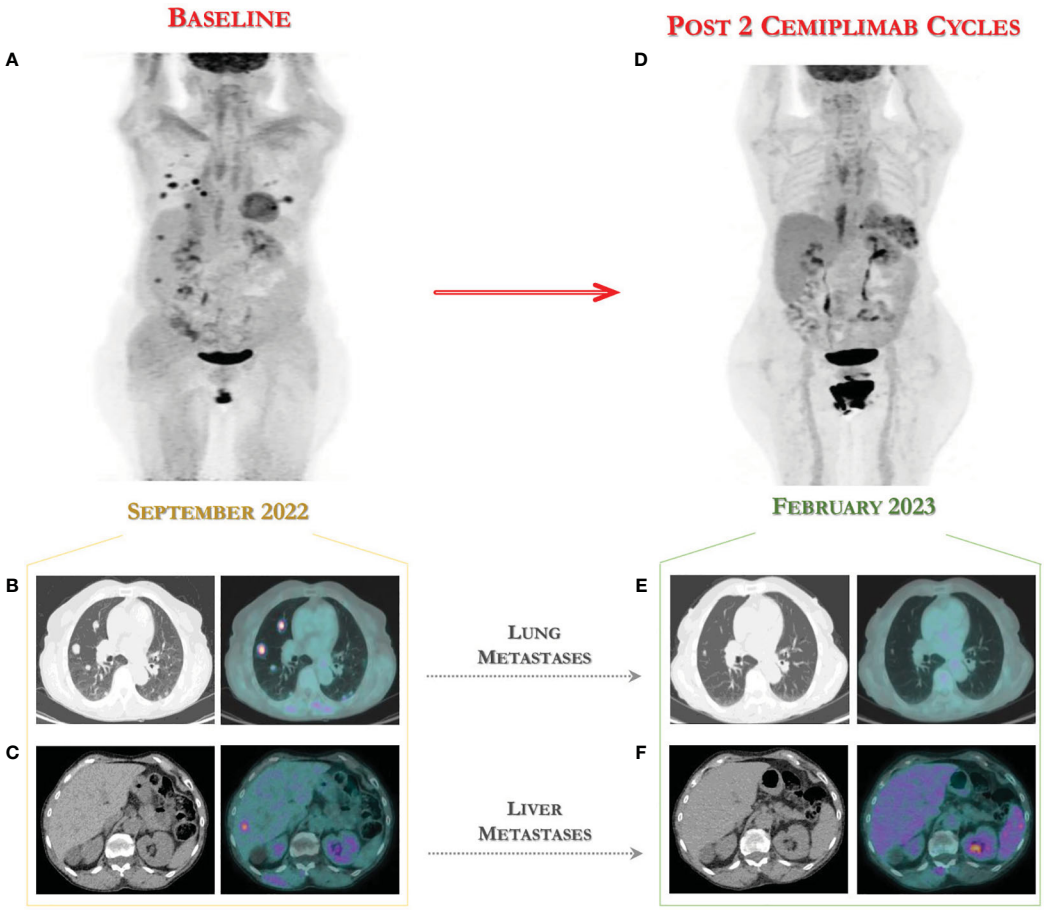

FIGURE 1
18-FDG PET/CT for assessing the fast tumor response to immunotherapy with cemiplimab. **(A)** Baseline 18-FDG PET images in advanced CC patient showing multiple lung **(B)**, liver **(C)** and lymph node metastases. **(D)** Early assessment after two cycles of cemiplimab showed a metabolic complete response to immunotherapy with disappearing of lung metastases **(E)** and of two hepatic nodules **(F)** to 18-FDG PET scan performed in February 2023.

In terms of effectiveness, after only two cycles of immunotherapy, she obtained a clinical benefit with surprising complete metabolic response according to PET Response Criteria in Solid Tumors (PERCIST) (16), with disappearing of two hepatic nodules and of lung metastases to 18-FDG PET scan performed in February 2023, as shown in Figure 1.

Probably as result of an effective immune activation, the 18-FDG PET scan showed an intense increase in uptake to the spleen, a finding not present at the previous pre-immunotherapy examination (see Figure 2). Importantly, the spleen is the largest secondary lymphoid organ, although mechanisms underlying lymphocyte trafficking to the spleen induced by immunotherapy remain unclear.

Therefore, in consideration of the objective and metabolic response, the clinical benefit and the onset of severe and unacceptable toxicities, the immunotherapy was definitively discontinued.

Interestingly, nine and thirteen months after the start of cemiplimab treatment, the patient is off therapy and still disease-free (complete response) as documented by the last 18-FDG PET scan (see Figure 2). In addition, she is currently completely asymptomatic without any systemic symptoms.

Discussion

In advanced CC setting, the strategy based on the use of platinum-based regimens is the most active in first line. Advanced CC patients who progress after fist line therapy have a poor prognosis and the subsequent available options were until recently disappointing.

In this setting, the use of ICIs has changed even the management of gynecological cancers over the last few years (17).

The immunotherapeutic approach with anti-PD-1 ICI cemiplimab led to significantly longer OS than chemotherapy among patients with recurrent CC who had had disease progression after first-line chemotherapy (18).

Here we report the first case of patient with pre-treated advanced CC with PD-L1 expression of less than 1%, effectively treated with cemiplimab as a part of a compassionate use in a managed access program.

Our decision to manage this case with cemiplimab was based on the positive results of the phase III EMPOWER-Cervical 1 trial (13).

This trial showed promising improvement in the OS of patients treated with immunotherapy compared to single-agent chemotherapy in patients regardless of their PD-L1 status.

To date the PD-L1 expression is the only immune-related biomarker investigated in CC patients that could potentially predict benefit to immunotherapy. In the pivotal trial, among the patients with PD-L1 expression of 1% or greater, median OS was 13.9 months with cemiplimab, as compared with 9.3 months with chemotherapy. Instead, among the patients with PD-L1 expression of less than 1% median OS was 7.7 months with cemiplimab and 6.7 months with chemotherapy (13).

In the overall population, an objective response occurred in 16.4% in the cemiplimab group, as compared with 6.3% in the chemotherapy group. An objective response occurred in 18% of the cemiplimab-treated patients with PD-L1 expression ≥1% and in 11% of those with PD-L1 <1% (13).

These results suggest that some patients with PD-L1 negative may still have a benefit to cemiplimab, as we demonstrate in our clinical case. The main best overall tumor response to cemiplimab was the stable

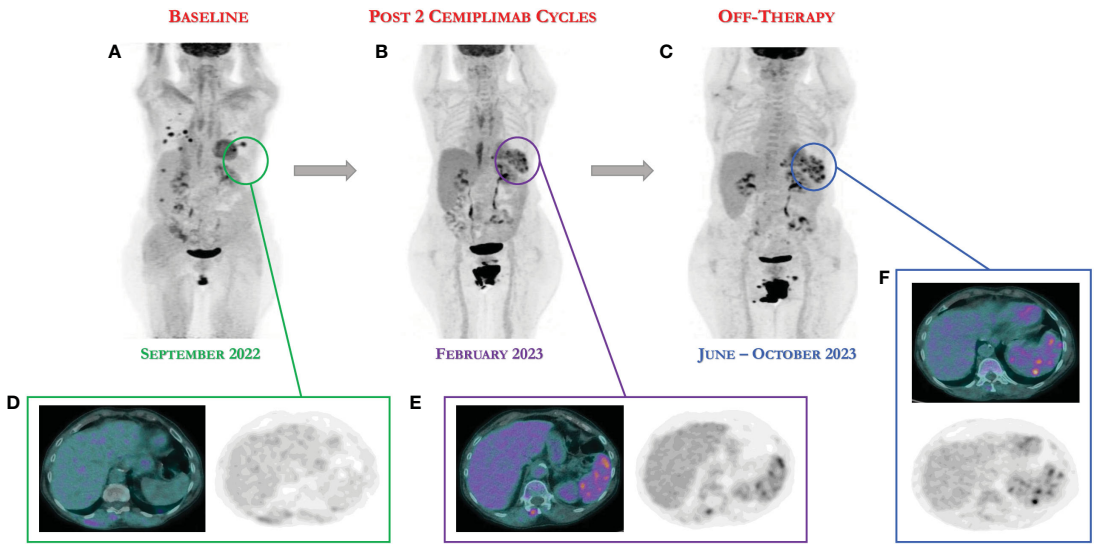

FIGURE 2
18-FDG PET/CT scan demonstrates the persistence of complete response after only two cycles of cemiplimab definitively discontinued for severe toxicities. **(A)** Baseline 18-FDG PET image. **(B)** 18-FGD PET showing complete response after two cycles of immunotherapy. **(C)** 18-FDG showing the persistence of complete response following nine and thirteen months (June and October 2023), despite the discontinuation of immunotherapy. **(D–F)** As result of an effective immune activation, the 18-FDG PET scan showed an intense increase in uptake to the spleen, a finding not present at the previous pre-immunotherapy examination.

disease (41.4%). In our clinical case we report an early and complete response to cemiplimab that occurred in only 3.3% in the pivotal trial.

Unfortunately, the immune system activation may lead to a novel type of toxicities known as irAEs (19). These events may potentially involve any organ system with variable clinical presentation and prognosis (20). Specifically, cardiovascular irAEs are rarely reported with an incidence of 1.3% (21) but are often severe and can be life threatening. Myocarditis represents the most frequent immune-related cardiovascular toxicity (22). Pericarditis, arrhythmias, and pericardial effusion are less frequent. Typically, the incidence is higher with the combined use of different ICIs, although it has been reported to occur with a single ICI.

Regarding the safety profile of cemiplimab, in EMPOWER-Cervical 1 the irAEs of grade 3 or higher occurred in 45% in the cemiplimab group (13). The irAEs that resulted in discontinuation of the trial treatment occurred in 8.7% receiving cemiplimab. The increase in transaminases of any grade were described in only 2.7% of cemiplimab group and 0.7% of severe grade. Interestingly, no cardiovascular irAEs were reported by the investigators (13). Therefore, this is the first clinical case in which an immune-related cardiological toxicity such as Takotsubo syndrome is described. As this is a relatively uncommon ICI-associated cardiotoxicity, to date there are not many data in the literature regarding the incidence and prognosis (23).

Although no data are available regarding the correlation between the occurrence of severe irAEs and the effectiveness of cemiplimab in the setting of advanced CC, we describe for the first time the case of a patient undergoing immunotherapy who showed an exceptional and early response concomitantly with the onset of severe and rare irAEs. Indeed, it has been hypothesized that irAEs, especially in skin, endocrine organ or gastrointestinal tract are related to a significant survival benefit (24). This correlation has been widely described in patients with melanoma and lung cancer treated with ICIs (25, 26).

Another interesting aspect of this case is represented by the persistence of clinical benefit and disease progression-free for thirteen months, despite the early immunotherapy discontinuation (27). There are several reports that anecdotally indicate the durable response for the patients who discontinued immunotherapy due to toxicity, but no data with cemiplimab in CC setting.

Therefore, in the event of immunotherapy discontinuation due to irAEs, one should not immediately switch to a subsequent line of therapy especially in the case of clinical benefit or subsequent evidence of radiological response.

Finally, in our patient perhaps as result of an excessive and effective immune activation, the 18-FDG PET scan showed an intense increase in uptake to the spleen following the onset of severe immune toxicity. Regarding this, it has been reported that the PET scan can potentially monitor the metabolic changes in peripheral lymphoid organs such as lymph nodes and spleen (28). The spleen is the largest secondary lymphoid organ, while the mechanisms underlying lymphocyte trafficking remain unclear. Thus, it has been hypothesized that PET examination can predict the efficacy of immunotherapy identifying patients with effective immune activation.

Anyway, the optimal approach to detect the metabolism of peripheral lymphoid organs through the 18-FDG PET and to predict the therapeutic effect of ICIs is yet to be established.

Conclusion

To our knowledge, we report for the first time a case of advanced CC refractory to standard of care platinum-based with a complete and early metabolic response after only two cycles of immunotherapy with cemiplimab.

Our case provides unequivocal clinical evidence for the immunotherapy effectiveness in treating CC even in patients with no tumor PD-L1 expression.

Importantly, this case highlights that the therapeutic effect of cemiplimab could be maintained for a long period after early discontinuation of its administration, thus suggesting a potential correlation between the activity of immunotherapy and the onset of severe and rare irAEs.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

AP: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. CP: Validation, Visualization, Writing – review & editing. EC: Validation, Visualization, Writing – review & editing. JV: Validation, Visualization, Writing – review & editing. SCC: Validation, Visualization, Writing – review & editing. MDN: Validation, Visualization, Writing – review & editing. LC: Data curation; Visualization, Writing – review & editing. SL: Data curation; Visualization, Writing – review & editing. SP: Conceptualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

SP received honoraria from MSD, Pfizer, AZ, Roche, Clovis, and GSK. CP received honoraria from MSD, AZ, and GSK. MN received honoraria from MSD and BMS.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Adrenal crisis mainly manifested as recurrent syncope secondary to tislelizumab: a case report and literature review

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As an immune checkpoint inhibitor (ICI), tislelizumab is an anti-programmed cell death protein 1 (PD-1) drug. With the extensive application of ICIs, there is an ever-increasing proportion of immune-related adverse events (irAEs) in clinical settings, some of which may even be life-threatening. Herein, we present a patient with tislelizumab-induced adrenal crisis. The main clinical manifestation was recurrent syncope accompanied by high-grade fever. Timely identification and hormone replacement therapy helped the patient overcome the crisis well. Finally, the patient discontinued tislelizumab and switched to antibody–drug conjugate (ADC) therapy. We report this case to improve our understanding of this situation, identify this kind of disease, and prevent adrenal crisis in time. Eventually, limiting toxicities reduces the interruption of immunotherapy. Since irAEs are multisystem damage with more non-specific symptoms, except for oncologists, general practitioners who endorse the need for taking a holistic approach to the patient should play a vital role in the management of cancer treatment.

KEYWORDS

tislelizumab, immune-related adverse events (irAEs), adrenal crisis, immune checkpoint inhibitors (ICIs), recurrent syncope

Introduction

Nowadays, the treatment of multiple malignancies has been revolutionized by immune checkpoint inhibitors (ICIs), which prolong patients' long-term survival and produce durable remissions. ICIs are monoclonal antibodies that target two key signaling pathways related to T-cell activation and exhaustion by binding and inhibiting cytotoxic T lymphocyte antigen (CTLA)-4 or programmed death (PD)-1 and its ligand PD-L1 (1, 2). However, ICIs may also demolish the maintenance of immunological tolerance to self-antigens (3), leading to immune-related adverse events (irAEs) in different organ systems, especially autoimmune-like manifestations targeting endocrine glands (4). These

toxic effects are a major cause of onset, often leading to treatment discontinuation, and can have debilitating long-term consequences (1). Endocrine dysfunction is one of the most commonly reported irAEs in ICI clinical trials, including hypothyroidism, hyperthyroidism, hypophysitis, primary adrenal hypofunction (PAI), and type 1 diabetes (5).

Little is known about severe adrenal insufficiency (AI) related to ICIs, with an incidence rate of ≤1% (6–9). AI usually manifests as grade 1–2 irAEs, while adrenal crisis (AC) manifesting as grade 3–4 irAEs is rare. The presentation of AI is usually non-specific. The main clinical symptoms include fatigue, anorexia, and nausea, which may be misdiagnosed as complications of a malignant tumor. When AI is not recognized, misdiagnosis or delayed diagnosis may lead to life-threatening AC (10, 11). A history of previous AC is a susceptible factor for patients with AI to experience AC again (10). Severe symptoms of adrenal crisis may lead to a decline in confidence and discontinuation of immunotherapy. Therefore, it is of great clinical significance to identify and treat AI in time.

This case report describes a middle-aged man with non-invasive urothelial carcinoma who manifested AC characterized by recurrent syncopal episodes after treatment with a PD-1 inhibitor, tislelizumab. Syncope under the category of undifferentiated symptom diseases necessitates a significant investment of time, finances, and effort to pinpoint the precise etiology (12). We present this case to underscore the importance of pre-medication education and regular post-usage monitoring of relevant diagnostic parameters. Elevating the awareness of healthcare practitioners regarding adverse drug reactions contributes to minimizing the progression of such reactions, ultimately reducing the temporal and financial costs incurred by patients.

Case description

A 58-year-old male patient was admitted to our department due to recurring syncopal episodes for more than 3 months. He was also suffering from high fever, confusion, fatigue, anorexia, nausea, and vomiting. The patient's family once monitored his blood pressure after syncope with a systolic blood pressure of 50–60 mmHg and a blood glucose level of 4.6 mmol/L. In addition to physical symptoms, the patient was under great mental stress at the time of admission.

Three years ago, he was diagnosed with urothelial carcinoma and underwent minimally invasive surgery. The tumor recurred 6 months after resection, and on May 20, 2022, he underwent complete transurethral resection of bladder tumor (TURBT). Histological diagnosis was low-grade non-invasive papillary urothelial carcinoma. Cancer tumor staging showed no metastasis or local invasion, and the last contrast-enhanced multislice computed tomography (CT) was normal. The patient received treatment with tislelizumab (approximately eight cycles). The first four cycles of immunotherapy were from May 25, 2022, to July 27, 2022, with the infusion of tislelizumab (200 mg, injection d1 3 weeks) without specific discomfort. Further details and information are presented in Figure 1. He has a smoking history for more than 30 years. Previous endocrine disorders were unclear.

On admission, his body temperature (BT) was 35.5°C, and his blood pressure (BP) was 117/77 mmHg. His physical examination revealed increased breath sounds and a positive Murphy's sign. Combined with the results of previous laboratory examinations and clinical manifestations, we first consider the possibility of Adams–Stokes syndrome attack, viral myocarditis, transient ischemic attack (TIA), pulmonary embolism, sepsis, vasovagal syncope, insulinoma, PD-1-related adverse reactions, and so on.

Laboratory data revealed a high level of high-sensitivity C-reactive protein. We further performed contrast-enhanced multislice CT, which showed interstitial infiltrates and exaggerated lung markings. The level of serum sodium was normal. Other abnormal data are shown in Table 1. Diurnal rhythm changes of serum adrenocorticotropic hormone (ACTH) and cortisol suggested extremely low cortisol and ACTH and inconsistent diurnal rhythm (Table 2), considering that the patient had hypoadrenalism. In view of the low level of ACTH and no abnormalities seen on adrenal CT, the diagnosis of central hypoadrenalism was confirmed. Given the history of immunotherapy, we considered the possibility of immune-related hypophysitis (irH). Consequently, we comprehensively evaluated the endocrine system of patients, as irH could involve the hypothalamic pituitary thyroid axis and gonadal axis in addition to the cumulative hypothalamic–pituitary–adrenal (HPA) axis. Thyroid function tests revealed that although thyroglobulin was slightly elevated, free triiodothyronine (FT3), free thyroxine (FT4), and thyroid-stimulating hormone (TSH) were normal, and thyroid peroxidase and thyrotropin receptor antibodies were negative, suggesting normal pituitary thyroid function. The results of the sex hormone test showed that luteinizing hormone and progesterone were mildly elevated, and the remaining indexes were within normal limits, suggesting normal pituitary–gonadal function. To this point, the patient's etiology could be clarified, as irH triggered isolated adrenocorticotropic hormone deficiency (IAD). The common clinical manifestations of IAD were fatigue, nausea and vomiting, weight loss, hypoglycemia, hyponatremia, and refractory hypotension. The patient's symptoms were highly consistent with IAD.

Finally, we performed pituitary MR imaging (Figure 2), which revealed a normal pituitary gland. The patient's family revealed that the patient experienced absolute low blood pressure (<100 mmHg) and hyperthermia with confusion during the syncopal episode; thus, adrenal crisis was the most reasonable diagnosis. After administration of hydrocortisone sodium succinate (0.15 g iv drip bid) and continuous fluid resuscitation, the patient's condition gradually improved. After discharge, he continued to be given prednisone 10 mg qd (8a) and 5 mg qd (5p) orally. After 3 months' follow-up, the patient did not have syncope again, and the symptoms of fever, fatigue, anorexia, nausea, and vomiting improved significantly. In addition, there was no recurrence of adrenal crisis or other immune-related adverse symptoms during the follow-up period.

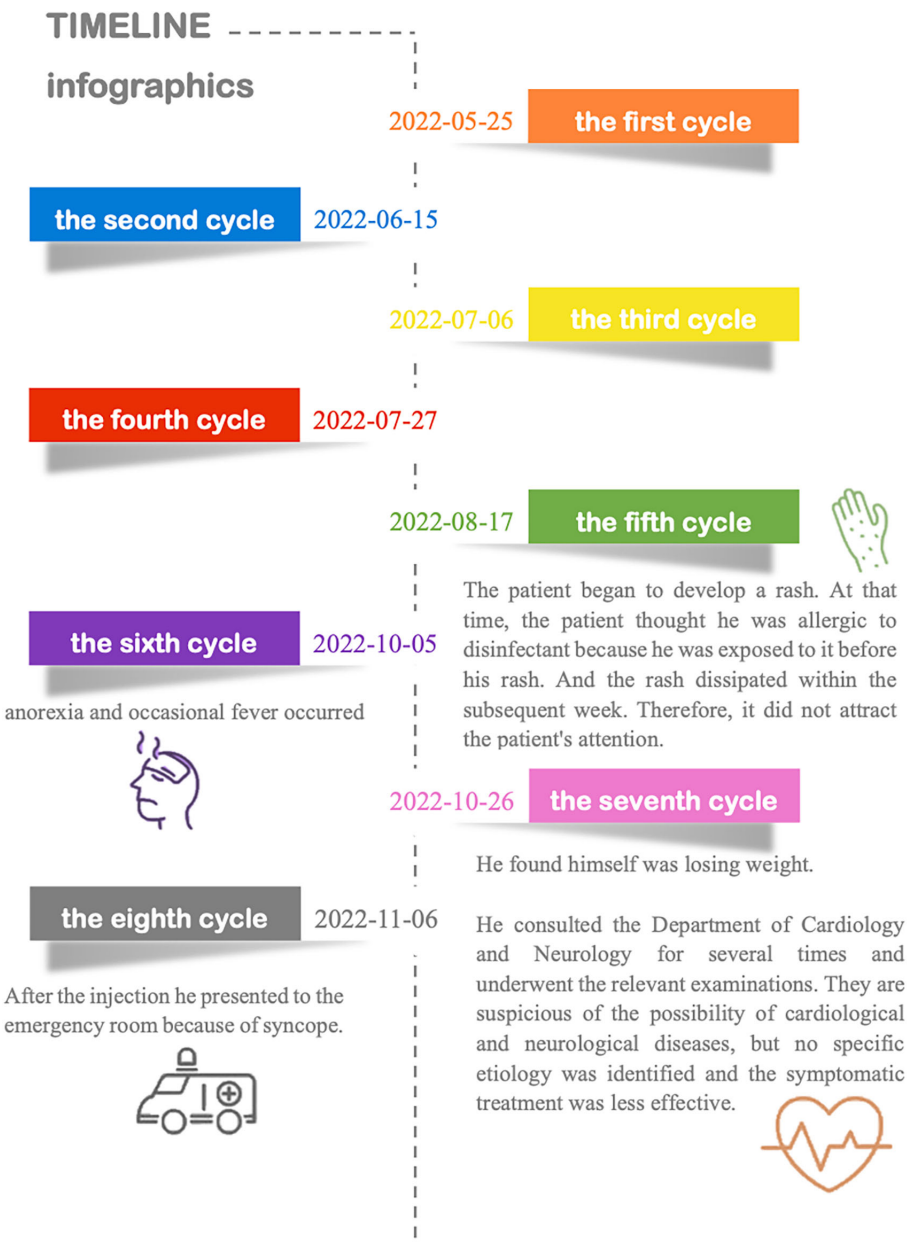

FIGURE 1
Timeline of symptoms.

Discussion

Here, we introduced a case of adrenal crisis after treatment with PD-1 (tislelizumab), which was a 3–4 grade irAE related to PD-1. Several cases of immunotherapy-induced adrenal crisis have been reported, most of which manifested as high-grade fever, persistent hyponatremia, or acute abdomen, while recurrent syncope is rare, and non-specific symptoms made the diagnosis of diseases difficult. Due to enormous psychological pressure, despite its immense clinical benefits, the patient stopped treatment. Clinical physicians should develop an awareness of irAEs in order to identify the events timely and reduce incidences of discontinuation of ICIs.

According to the American Society of Clinical Oncology (ASCO) Guideline (13), a routine endocrine examination should be taken to evaluate the endocrine gland or organ. In this case, we confirmed the diagnosis of central hypoadrenocorticism through endocrine examination. We then traced the patients' medical history to figure out the potential cause. The patient had no previous history of taking, inhaling, or injecting steroids and no history of opioid use. In addition to being treated with PD-1 for eight cycles, there were no other relevant reasons and incentives. Therefore, we considered whether there were PD-1-related adverse drug reactions. Among them, irH has attracted our attention, which is defined by the occurrence, in patients treated with ICIs, of functional defect of one or more pituitary axes with or without

TABLE 1 Laboratory measurements.

Parameter	Value	Normal range
High-sensitivity C-reactive protein (mg/L)	42.98	0–10
Blood glucose (mmol/L)	4.01	3.90–6.10
Sodium (mmol/L)	142	137–147
Potassium (mmol/L)	3.69	3.50–5.30
Glomerular filtration rate (ml/min)	105.35	
Progesterone (nmol/L)	0.590	<0.474
Luteinizing hormone (mIU/ml)	9.04	1.70–8.60
Thyroglobulin (ng/ml)	95.06	1.40–78.0
Ferritin (ng/ml)	818.00	13–400
Neuron-specific enolase (ng/ml)	16.50	0.0–16.3

slight pituitary MRI abnormalities (14). The exact pathogenesis of irH is still unknown. CTLA-4 and PD-1/PDL-1-related hypophysitis are currently known to have different clinical features, which may suggest different underlying mechanisms. CTLA-4-related hypophysitis manifests as frequent impaired TSH and luteinizing hormone/follicle-stimulating hormone (LH/FSH) secretion accompanied by impaired ACTH secretion (15) and a greater propensity for type II hypersensitivity reactions associated with off-target effects of CTLA-4 in the pituitary (16). In contrast, PD-1-associated hypophysitis is less frequent (17), and most patients have a specific impairment of ACTH only, presenting as IAD (18). The pituitary gland of autopsy cases showed evidence of type IV hypersensitivity by cytotoxic T lymphocytes (16). Different clinical presentations are presented depending on the specific target gland axis of injury.

Due to the lack of specific clinical manifestations and accurate onset time, the diagnosis of irH is difficult. At present, the diagnosis is mainly based on biochemical and imaging examinations. Specific immune biomarkers for its diagnosis are not currently available, with the most common biochemical evidence being a deficiency of pituitary hormones. Imaging can rely on pituitary MRI to provide diagnostic evidence: pituitary enlargement, stalk thickening, and enhancement with allogeneic or heterologous contrast media are present on MRI in 77% of patients with IRH, whereas 23%–33% of patients do not show abnormalities on MRI (16). Multiple studies have suggested that hypophysitis induced by PD-1/PD-L1 inhibitors may lack the typical pituitary enlargement compared to CTLA-4 inhibitors (19–21). Therefore, imaging studies showing a normal appearance of the pituitary gland do not rule out

TABLE 2 Diurnal rhythm changes of serum ACTH and cortisol.

	8 a.m.	4 p.m.	0 a.m.
Cortisol (μg/dl)	0.16	0.20	0.15
ACTH (pg/ml)	4.47	2.37	2.16

ACTH, adrenocorticotropic hormone.

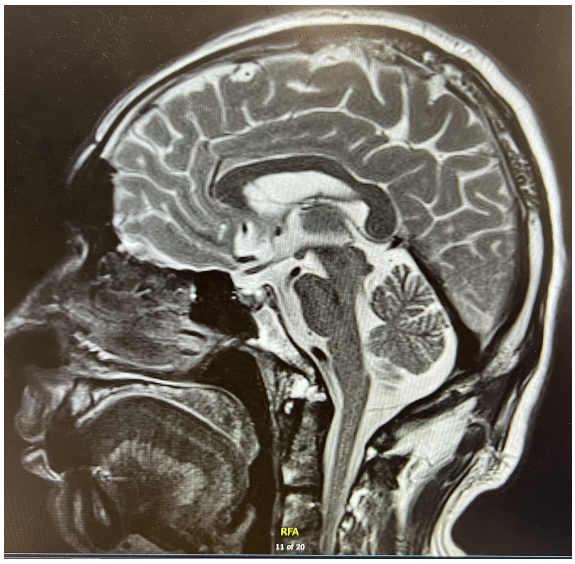

FIGURE 2
Post-contrast T2-weighted MR image of the pituitary gland.

hypophysitis (22). In addition, the diagnosis of hypophysitis may lag a few weeks after imaging shows pituitary enlargement (23).

According to the 2022 National Comprehensive Cancer Network (NCCN) guidelines for irH, MRI should be performed if the patient has symptoms during treatment (24). A recent study suggested that brain MRI after receiving ICI therapy should be compared with previous results to monitor changes in pituitary size, which may foreshadow that impending anterior pituitary hormone dysfunction is about to occur (22). An enlarged pituitary gland, as indicated by imaging studies, is important to exclude metastatic disease in addition to suspecting hypophysitis (23). Several studies showed that ICI-related central adrenocortical dysfunction appears to be permanent (22, 23, 25–27). However, most of these reports were central hypoadrenocorticism caused by another immune checkpoint inhibitor CTLA-4 drug. So far, there is a lack of histocytological evidence to prove whether pituitary adrenal axis function could be restored (28, 29). To sum up, hormone replacement therapy should not be delayed by waiting for a pituitary gland MRI when an endocrine examination prompts central hypoadrenocorticism (30).

The initial clinical manifestations of IAD lack specificity, which delays diagnosis and eventually progresses to adrenal crisis, threatening the patient's life. The main clinical manifestations of adrenal crisis are severe hypotension or hypovolemic shock, acute abdomen symptoms, vomiting, hyperthermia or hypothermia, and hypoglycemia. Among them, hypotension is a core symptom in the diagnosis of adrenal crisis. However, seemingly normal blood pressure could not rule out a crisis. According to the adverse event evaluation criteria (Common Terminology Criteria for Adverse Events (CTCAE)), adrenocortical insufficiency is usually grade 1–2 irAEs, while grade 3–4 irAEs, especially adrenal crisis, are rarely reported. In a large meta-analysis study containing 160 clinical trials and 40,432 patients, Jingli et al. found that among patients using ICIs, the incidence of all-grade and severe-grade

hypoadrenalism was 2.43% and 0.15%, respectively (6). Our case had grade 3–4 irAEs induced by tislelizumab who presented with adrenal crisis. The symptoms of our patient were unremarkable and could be easily overlooked if an irAE had not been suspected. Although adrenal crisis is rare, it is a life-threatening side effect of ICIs that requires immediate recognition and treatment with intravenous glucocorticoids. Therefore, a deep understanding of irAEs as well as adrenal crisis, early diagnosis, and treatment is significantly important. When an immunotherapy-related adrenal crisis occurs, an initial intravenous or intramuscular bolus of 100 mg hydrocortisone in addition to supportive fluid therapy is required, as well as a continuous intravenous infusion of 200 mg hydrocortisone q24h (daily) or an intravenous or intramuscular bolus of 50 mg hydrocortisone q6h (or 50 mg four times daily) (10).

The recommended duration is 24–48 h until the patient can take oral hydrocortisone (11). Glucocorticoid replacement therapy should be the primary treatment when the patient's condition is stable. Our patient had no history of underlying endocrine diseases such as diabetes, so there were no specific restrictions on the dose of cortisol to be administered. In patients with diabetes, choosing the appropriate cortisol dose that in turn maximizes benefits and reduces associated side effects is a challenge. Considering that high-dose cortisol may aggravate the underlying disease or lead to new disease (31), the potential benefit of high-dose glucocorticoid treatment should be balanced against efficacy loss due to anticancer immunotherapy. Although this issue remains controversial (27), the dose of cortisol should be reduced appropriately. For adults, the oral maintenance dose of hydrocortisone needs to be 15–25 mg per day (32). The hydrocortisone dose should then be gradually reduced according to the patient's clinical manifestations, with close monitoring of blood pressure and recurrence of clinical symptoms (33).

For patients who develop endocrine diseases that can be controlled using hormone replacement therapy, there is no need to discontinue ICIs despite grade 3–4 irAEs (34, 35). Theoretically, during the treatment period of ICIs, at the same time as the immune system's reduced tolerance to triggering irAEs, its ability to recognize and kill cancer cells is enhanced, so the occurrence of irAEs may be a positive predictor of treatment response (22). Our patient underwent imaging examinations that showed no metastasis and recurrence of the tumor, indicating that this patient achieved a complete response to the treatment with tislelizumab. Meanwhile, several studies have shown a positive correlation between the development of irAEs as a result of ICI therapy and improved tumor response and survival. However, for grade 3–4 irAEs, life-threatening side effects require urgent hospitalization for corresponding symptomatic supportive care. After adverse reaction disappearance, the restoration of ICIs requires consideration of many situations, such as previous tumor reactions, treatment duration, toxicity type and severity, toxicity resolution time, availability of alternative therapies, and patient's condition (13). Multiple studies have confirmed a significantly increased incidence of irAEs with the combination of ICIs, and it is not recommended to switch to a new ICI (27, 36–38). After a consult with an oncologist on this patient's condition, the oncologist recommended an antibody–drug conjugate (ADC) therapy. At our later follow-up visit, the patient decided to discontinue ICI therapy and switch to ADC to continue the anti-tumor treatment.

ADCs are composed of monoclonal antibodies, cytotoxic payloads, and linkers (39, 40). The efficacy and toxicities of an ADC as a cytotoxic therapy are contingent upon the critical contributions of each component (40, 41). Although high specificity and low toxicity are expected for this novel compound, unpredictable toxicity still exists and demands prompt consideration (40, 42). In the subsequent follow-up, our patient still exhibited adrenal insufficiency, but common side effects attributable to ADC drugs were not observed, such as thrombocytopenia, liver or ocular toxicity, and peripheral neuropathy (42). At present, a subset of clinical trials is underway for combined regimens involving ADC drugs and immune checkpoint inhibitors (43, 44). Additionally, it underscores the importance of clinicians exercising caution with respect to the drug toxicities induced by the combined regimen.

In addition, a growing number of clinical cases prove that endocrine diseases such as late-onset AC still occur after the termination of ICI therapy (45, 46), which also proves that the anti-tumor effects of ICIs can be long-term *in vivo* and expressed (46, 47). Therefore, it is recommended to be always alert to the possibility of irAEs even after the discontinuation of ICIs. Current medical examination methods cannot distinguish between immunological- and non-immunological-related causes and specific immunological biomarkers deprivation, making it difficult for clinicians to detect irAEs (48). Because of their specificity of presentation, atypical timing, and clinical coexisting with other diseases, irAEs may be more difficult to diagnose and identify (48–50). Especially for immune checkpoint inhibitors, risk factors predicting these events have yet to be determined. It is a challenge to predict who will develop severe or permanent toxicity (1). Before giving treatment to patients with PD-1/PD-L1 inhibitors, it is necessary to inquire about the history of endocrine diseases and autoimmune diseases in detail, conduct reasonable baseline screening, regularly monitor changes in endocrine indicators, increase vigilance against possible related symptoms and signs, and detect and promptly handle irAEs as soon as possible (22, 51). Once the dose of hormones and the types of anti-tumor drugs are determined, it is necessary to further provide patients with knowledge of common adverse reactions to ICI and conduct regular follow-up visits. We believe that self-education and management of such patients play an important role in the progress of the disease (52). Timely identification limits toxicities while maximizing anti-tumor efficacy to reduce the interruption of immunotherapy.

When reviewing the patient's history, it was found that the patient had skin manifestations of irAEs 5 months before admission. However, these failed to capture the attention of both the patient and clinicians. Despite that the most common organ system was involved (1, 5), the initial warning symptom was ignored, resulting in consequential outcomes. According to the American Society of Clinical Oncology Clinical Practice Guideline, timely and latest education about immunotherapies should be provided throughout treatment and survivorship (34). However,

our patient was only informed that this drug has durable therapeutic effects before treatment, accompanied by a spectrum of side effects affecting different organ systems. The detailed elaboration on these side effects was withheld due to multifarious factors. This case confirms significant deficiencies in our current approaches to education and management. This also poses a question: following a comprehensive explanation of the toxicities associated with ICIs and ADCs, which pharmaceutical approach do patients exhibit a greater inclination to for anti-tumor therapy? However, considering the preexistence of significant side effects, further inquiry at this juncture might compromise the objectivity of the responses from the patients.

With the increase in clinical practice of tumor immunotherapy, the occurrence of immune-related adverse reactions will constantly increase. Specialists of different departments may receive referrals for patients suffering from specific symptoms of adverse events in their field of expertise. However, as irAEs are multisystem damage with more non-specific symptoms, except for specialists, general practitioners should play a greater role in the management of cancer treatment. Identifying and characterizing irAEs is a cornerstone in ascertaining the impact of cancer treatment on patients and healthcare professionals (53). Cancer survivors are often troubled by the long-term consequences of cancer and its treatment (54). Because primary care is an integrated and accessible healthcare service, most patients consult general practitioners initially once they have symptoms. Lower-grade irAEs can be identified and controlled so as to divert medical resource pressure and financial pressure away from tertiary healthcare toward primary healthcare. As gatekeepers to further services, general practitioners should play a greater role in improving the quality of care for cancer survivors.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

HW: Writing – original draft. AZ: Writing – review & editing. JC: Writing – review & editing. CZ: Writing – review & editing. TL: Writing – review & editing. HY: Resources, Writing – review & editing. YG: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Clinical benefit analysis of PD-1 inhibitors in patients with advanced, recurrent or metastatic cervical cancer: a meta-analysis and systematic review

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Purpose: This study aims to comprehensively evaluate the efficacy and safety of programmed cell death protein-1 (PD-1) in patients with advanced, recurrent, or metastatic cervical cancer (ARMCC) and identify the population that may benefit the most.

Methods: We conducted a search of PubMed, EMBASE, and the Cochrane Collaboration Library from their inception to September 2023. We extracted and analyzed the results related to the efficacy and safety of PD-1 in patients with ARMCC. The primary endpoints included the overall objective response rate (ORR) and adverse events (AEs), while the secondary endpoints encompassed the 1-year overall survival (OS) rate, 1-year progression-free survival (PFS) rate, as well as OS and PFS. We used a random effects model to conduct a meta-analysis on single-group rates, and the Mantel-Haenszel method was utilized to compare the ORR and the incidence of AEs.

Results: Our study included a total of 21 trials involving 2,097 patients. The ORR of the combination of PD-1 inhibitors with chemotherapy was 56.36%, the combination of PD-1 inhibitors with anti-angiogenic agents was 38.72%, the combination of PD-1 inhibitors with Cytotoxic T-lymphocyte antigen 4 inhibitors was 25.60%, and PD-1 inhibitor monotherapy was 15.99%. The subgroup analysis showed that the group of patients with squamous cell carcinoma (SCC) exhibited a significantly higher ORR compared to the non-SCC group in patients who received PD-1 inhibitors combined with other anti-tumor drugs (Odds Ratio =2.43, P=0.002). Additionally, the group of patients with a programmed death-ligand 1 combined positive score (PD-L1 CPS) ≥1 exhibited a significantly higher ORR compared to the PD-L1 CPS <1 group in patients who received PD-1 inhibitor monotherapy (OR=4.14, P=0.02). PD-1 inhibitor monotherapy or PD-1 inhibitors combined with chemotherapy did not significantly increase the incidence of all grades of adverse events (Relative Risk=0.99, p=0.788) or the incidence of serious adverse events (RR=0.99, p=0.788) compared to chemotherapy alone.

Conclusion: PD-1 inhibitors demonstrate outstanding efficacy in the treatment of patients with ARMCC. Patients with SCC may benefit more from treatments including PD-1 inhibitors in combination with other anti-tumor drugs, and PD-L1 CPS ≥1 can be considered a favorable indicator of immune therapy response. Importantly, the use of PD-1 inhibitor monotherapy or PD-1 inhibitors in combination with chemotherapy did not lead to an increased incidence of AEs compared with chemotherapy alone, indicting safety during treatment.

Systematic Review Registration: PROSPERO (CRD42023457945).

KEYWORDS

cervical cancer, programmed cell death protein-1, objective response rate, adverse events, combination

1 Introduction

Cervical cancer (CC) is one of the most common malignancies among women and ranks fourth among all cancer-related deaths worldwide (1). In 2020, there were over 600,000 newly diagnosed cases of CC, with approximately 342,000 deaths, and the number of women under the age of 65 years being diagnosed with CC is also steadily increasing (2). In clinical settings, the treatment of patients with advanced, recurrent, or metastatic cervical cancer (ARMCC) is even more challenging. Despite various treatment options currently available for CC, including surgery, radiation therapy, chemotherapy, targeted therapy, and combination therapies (3), these approaches have limited survival rates and treatment effectiveness in patients with ARMCC. Therefore, there is an urgent need to develop novel therapeutic strategies.

Programmed cell death protein-1 (PD-1) inhibitors, which are a type of immunotherapy, have made significant breakthroughs in cancer treatment in recent years (4). PD-1 inhibitors inhibit the interaction between tumors and immune cells, enabling the patient's immune system to better recognize and attack cancer cells. This novel class of drugs has been widely used to treat various types of cancers, including melanoma, lung cancer, and renal cell carcinoma, and has demonstrated remarkable clinical efficacy (5–7).

Some clinical studies have demonstrated the significant efficacy and survival advantages of PD-1 inhibitors in the treatment of cervical cancer, and current research is increasingly focusing on whether the combination of PD-1 inhibitors with other anti-tumor drugs can have better therapeutic effects in the treatment of CC.

However, no studies have investigated which specific anti-tumor drug, when combined with PD-1 inhibitors, yields the most effective results in the treatment of cervical cancer. This study aimed to provide comprehensive evidence of the efficacy of PD-1 inhibitors combined with other anti-tumor drugs in treating patients with ARMCC and to identify the patient population that benefits the most. Additionally, a comprehensive analysis of all adverse events (AEs) mentioned in the included studies was performed. This approach can provide clinicians with more accurate data and guidance when making decisions relating to treatment, ultimately leading to improved treatment strategies.

2 Materials and methods

2.1 Data sources and search strategy

This study was rigorously evaluated using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (8). PubMed, EMBASE, and the Cochrane Collaboration Library databases were searched from their inception to September 2023, and the language was restricted to English. An additional search of the gray literature was performed using Google Scholar, OpenGrey, ClinicalTrials.gov, and The Cochrane Central Register of Controlled Trials.

We adjusted the medical subject headings terms combined with the related text words to comply with the rules for searching for relevant studies in each database. Our search strategy was as follows: (Cervical OR Cervix OR Cervical Neoplasm, Uterine OR Neoplasm, Uterine Cervical OR Uterine Cervical Neoplasm OR Neoplasms, Cervical OR Cervical Neoplasms OR Cervical Neoplasm OR Neoplasms, Cervix OR Cervix Neoplasm OR Neoplasm, Cervix OR Cervix Neoplasms OR Cancer of the Uterine Cervix OR Cancer of the Cervix OR Cervical Cancer OR Cancer, Cervical OR Cervical Cancers OR Uterine OR Cervical Cancer OR Cancer, Uterine Cervical OR Cervical Cancer, Uterine OR Uterine Cervical Cancers OR Cancer of Cervix OR Cervix Cancer OR Cancer, Cervix) and (PD-1 OR PD-1 inhibitors OR Programmed cell death protein-1 inhibitor). For example, the search query in PubMed was (PD-1[Title/Abstract] OR PD-1 inhibitors[Title/Abstract] OR Programmed cell death protein-1 inhibitors[Title/Abstract] OR PD-L1[Title/Abstract] OR PD-L1 inhibitors[Title/Abstract] OR Programmed Death-Ligand 1

inhibitors[Title/Abstract]) AND (Cervical[Title/Abstract] OR Cervix [Title/Abstract] OR Cervical Neoplasm, Uterine[Title/Abstract] OR Neoplasm, Uterine Cervical[Title/Abstract] OR Uterine Cervical Neoplasm[Title/Abstract] OR Neoplasms, Cervical[Title/Abstract] OR Cervical Neoplasms[Title/Abstract] OR Cervical Neoplasm [Title/Abstract] OR Neoplasms, Cervix[Title/Abstract] OR Cervix Neoplasm[Title/Abstract] OR Neoplasm, Cervix[Title/Abstract] OR Cervix Neoplasms[Title/Abstract] OR Cancer of the Uterine Cervix [Title/Abstract] OR Cancer of the Cervix[Title/Abstract] OR Cervical Cancer[Title/Abstract] OR Cancer, Cervical[Title/Abstract] OR Cervical Cancers[Title/Abstract] OR Uterine[Title/Abstract] OR Cervical Cancer[Title/Abstract] OR Cancer, Uterine Cervical[Title/Abstract] OR Cervical Cancer, Uterine[Title/Abstract] OR Uterine Cervical Cancers[Title/Abstract] OR Cancer of Cervix[Title/Abstract] OR Cervix Cancer[Title/Abstract] OR Cancer, Cervix [Title/Abstract]).

2.2 Study selection

Two independent researchers (Jing-ping Xiao and Yun-zi Wang) filtered the titles and abstracts of all of the retrieved studies to identify potentially relevant studies. The full texts of the retrieved studies that met the inclusion criteria were evaluated. Each of these discrepancies was resolved through discussion, and if conflicts remained, a third reviewer (Ji-sheng Wang) was consulted.

2.3 Inclusion and exclusion criteria

The inclusion criteria for the studies in the systematic review on the efficacy and safety of PD-1 inhibitors for the treatment of patients are as follows: (1) interventions included the use of PD-1 inhibitors; (2) Patients were ≥18 years of age; and (3) patients had a histological diagnosis of ARMCC. (4) The following outcomes were reported: objective response rate (ORR), 1-year overall survival (OS) rate, 1-year progression-free survival (PFS) rate, hazard ratios (HRs) of OS or PFS, and AEs. Editorials, meeting reports, and letters to the editors were excluded from the review. The focus was solely on primary research studies that reported specific outcomes and AEs to ensure the reliability and relevance of the findings.

2.4 Data extraction

Two researchers, Jing-ping Xiao and Yun-zi Wang, independently screened the studies using the predefined inclusion criteria. Any discrepancies were resolved through a consensus between the two researchers. From each included study, relevant information, such as study characteristics, baseline characteristics, and predefined outcomes, including ORR, 1-year OS rate, 1-year PFS rate, HRs for OS or PFS, and AEs (if applicable), were directly extracted from the original report.

2.5 Quality assessment

Two researchers, Jing-ping Xiao and Yun-zi Wang, independently used the Cochrane Risk of Bias Tool to assess the quality of eligible randomized controlled trials (RCTs) (9). The researchers also utilized the Institute of Health Economics Quality Appraisal (IHE QA) checklist (10) to evaluate the quality of eligible observational studies, which included 20 items. If a study met 14 or more items on the Delphi checklist, it was considered acceptable. Additionally, the Newcastle-Ottawa Scale (NOS) (11) was used to evaluate the quality of eligible expansion cohort studies by assessing selection, comparability, and exposure. The NOS scale included nine points, and a score of 7 or higher was considered indicative of high quality, while a score of 4–6 indicated good quality, and a score of 3 or less indicated low quality. Any discrepancies were resolved through discussion involving a third reviewer (Ji-sheng Wang) if conflicts remained.

2.6 Data synthesis and analysis

The primary endpoints included ORR and AEs, while the secondary endpoints included 1-year OS rate, 1-year PFS rate, OS, and PFS. The random-effects model was used to conduct a meta-analysis of single-group rates, including ORR, 1-year OS rate, and 1-year PFS rate. The Mantel-Haenszel method was used to compare the ORR stratified by programmed death-ligand 1 combined positive score (PD-L1 CPS) or histological types of squamous cell carcinoma (SCC) as well as the incidence of AEs in RCTs. The results were reported as odds ratio (OR) and relative risk (RR) with a corresponding 95% confidence interval (CI). If the I^2 value was greater than or equal to 50%, a random-effects model was used to merge the results; otherwise, the fixed-effects model was used. I^2 statistics were used to assess heterogeneity across the included trials, and I^2 values of 25%, 50%, and 75% indicated low, moderate, and high inconsistencies, respectively. The continuity correction method was applied by adding a correction of 0.5 to cells with zero values. Stata (version 14) software was used to analyze all results, and statistical significance was defined as a two-sided p-value of <0.05.

3 Results

3.1 Literature search

Figure 1 displays the process of selecting eligible studies. Initially, 1180 studies were identified through searches of the PubMed, Cochrane, and EMBASE databases. After removing duplicates, 722 studies remained. After reviewing titles and abstracts, 46 studies were selected for full-text review. Finally, 19 studies that met the inclusion criteria were included in this meta-analysis (4, 12–29).

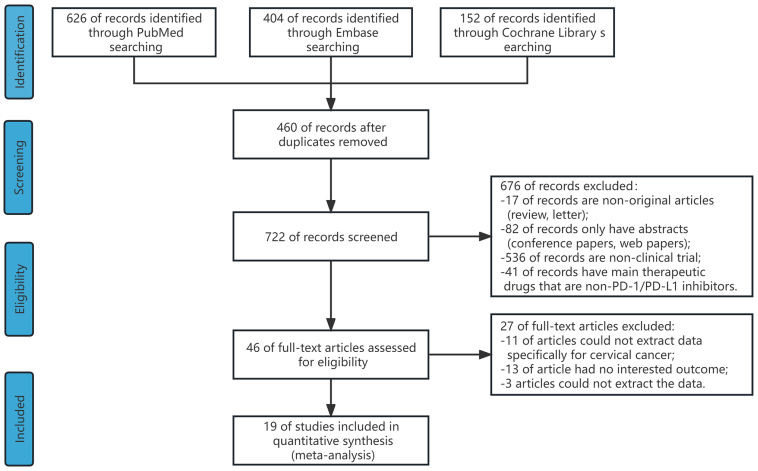

FIGURE 1
The flow diagram of studies included in this meta-analysis.

3.2 Study characteristics and quality

This meta-analysis included 21 trials from 19 studies, involving 2097 patients with ARMCC. Among them, there were 15 observational studies (4, 12, 13, 15–19, 21, 22, 24, 25, 27–29), one expansion cohort study (23), and three RCTs (14, 20, 26). All studies included at least one group that received treatment with PD-1 inhibitors. Among the 21 trials, 10 involved the use of PD-1 inhibitor monotherapy (12, 13, 15, 19, 22–26, 29), five combined PD-1 inhibitors with chemotherapy (4, 12, 14, 18, 20), five combined PD-1 inhibitors with anti-angiogenic agents (16, 17, 27–29), and one combined PD-1 inhibitors with Cytotoxic T-lymphocyte antigen 4 (CTLA-4) inhibitors (21). Seven trials reported a correlation between the PD-L1 CPS and efficacy in patients with ARMCC (13, 20–22, 24, 25, 27). Eight trials reported a correlation between histological type and efficacy in patients (12, 21–23, 25, 26, 28, 29) (Table 1).

As shown in Table 2, all 15 observational studies scored greater than 14 points. Supplementary Table 1 shows that all three RCTs were of high quality. Supplementary Table 2 shows that the quality assessment of the expansion cohort study yielded a score of nine points. It is noteworthy that all 19 studies mentioned above met the inclusion criteria.

3.3 Efficacy

3.3.1 ORR in ARMCC patients

Twenty trials comprising 2040 patients were eligible for the ORR. The analyses based on intervention types indicated that the ORR of the combination of PD-1 inhibitors with chemotherapy was 56.36% (95% CI 39.48% to 73.25%), the combination of PD-1 inhibitors with anti-angiogenic agents was 38.72% (95% CI 7.84% to 69.60%), the combination of PD-1 inhibitors with CTLA-4 inhibitors was 25.60 (17.95, 33.25), and PD-1 inhibitor monotherapy was 15.99% (95% CI 11.29% to 20.70%) (Figure 2).

3.3.2 Comparison of the ORR among different histological types of ARMCC patients

The ORR among patients with different histological types of ARMCC was analyzed in eight trials comprising 1080 patients. The results demonstrated that there was no significant difference in the ORR between the SSC and non-SSC groups in patients who received PD-1 inhibitor monotherapy (OR=1.48, 95% CI 0.81-2.71, P=0.203, I^2=0%). However, the SSC group exhibited a significantly higher ORR compared to the non-SSC group in patients who received PD-1 inhibitors combined with other anti-tumor drugs (OR=2.43, 95% CI 1.40-4.23, P=0.002, I^2=0%) (Figure 3).

3.3.3 Comparison of the ORR among different PD-L1 CPS of ARMCC patients

The ORR among different PD-L1 CPS of patients was analyzed in seven trials involving 498 patients. The results demonstrated that the PD-L1 CPS ≥1 group exhibited a significantly higher ORR compared to the PD-L1 CPS <1 group in patients who received PD-1 inhibitor monotherapy (OR=4.14, 95% CI 1.19-14.40, P=0.02, I^2=0%) (Figure 4). Additionally, the PD-L1 CPS ≥1 group exhibited a higher ORR compared to the PD-L1 CPS <1 group, but there was no statistical difference in patients who received PD-1 combined with other anti-tumor drugs (OR=2.17, 95% CI 0.95-4.96, p=0.067, I^2=0%) (Figure 4).

3.3.4 1-year OS rate and 1-year PFS rate in ARMCC patients

Eight trials including 328 patients were eligible for inclusion based on the 1-year OS rate. The analysis based on different intervention types indicated that the 1-year OS rate in ARMCC patients who received PD-1 inhibitors combined with chemotherapy was 87.39% (95% CI 59.64%-115.14%), that in ARMCC patients who received PD-1 inhibitors combined with anti-angiogenic agents was 67.18% (95% CI 48.57%-85.79%), and that in ARMCC patients who received PD-1 inhibitor monotherapy was 50.0% (95% CI 39.0%-61.0%) (Figure 5).

TABLE 1 Characteristics of studies included in this meta-analysis.

Trials name	year	Study type	Intervention drugs	Intervention types	Number of patients	Stage	PD-L1 CPS≥1%	PD-L1 CPS<1%	PD-L1 CPS unknown	Age, median (range)	Squamous cell carcinoma (%)	Follow-up (m), median (range)
Frenel	2017	NRCT Single arm	Pembrolizumab	PD-1 inhibitors monotherapy	24	Advanced or metastatic	24	0	0	42(26-62)	23 (95.8)	11(1.3-32.2)
Tamura	2019	NRCT Single arm	Nivolumab	PD-1 inhibitors monotherapy	20	Recurrent or advanced	15	5	0	50(32-68)	14 (70)	NR
Chung	2019	NRCT Single arm	Pembrolizumab	PD-1 inhibitors monotherapy	98	Advanced	82	15	1	46(24-75)	92 (93.9)	10.2(0.6-22.7)
Friedman	2020	NRCT Single arm	Atezolizumab + Bevacizumab	PD-1 inhibitors + Anti-angiogenic agent	11	Recurrent or metastatic or persistent	NR	NR	NR	48(31-55)	6(54.5)	NR
Rischin	2020	NRCT	Cemiplimab	PD-1 inhibitors monotherapy	10	Recurrent or metastatic	NR	NR	NR	55(31-76)	4 (40)	5.6(0.8-16.2)
O'Malley	2021	NRCT Single arm	Balstilimab	PD-1 inhibitors monotherapy	140	Recurrent or metastatic or persistent	85	38	17	53 (25–81)	85 (60.7)	14.6 (9.9–38.8)
Miller	2021	NRCT Single arm	Pembrolizumab	PD-1 inhibitors monotherapy	14	Recurrent	13	0	1	59 (22-77)	11 (78.5)	14.4 (3.3–39.0)
Huang	2021	NRCT Single arm	Camrelizumab + Apatinib	PD-1 inhibitors + Anti-angiogenic agent	32	Recurrent or metastatic or persistent	35	6	1	50 (33–63)	21 (65.6)	NR
Santin	2021	NRCT Single arm	Nivolumab	PD-1 inhibitors monotherapy	25	Recurrent or persistent	17	5	3	45 (20-79)	15(60)	32(2-41.5)
Colombo	2021	RCT	Pembrolizumab + Paclitaxel + Cisplatin or Carboplatin Versus Paclitaxel + Cisplatin or Carboplatin	PD-1 inhibitors + Chemotherapy Versus Chemotherapy alone	617	Recurrent or metastatic or persistent	273	35	0	51(25-82)	235 (76.3)	22(15.1-29.4)
O'Malley	2022	NRCT Single arm	Balstilimab + Zalifrelimab	PD-1 inhibitors + CTLA-4 inhibitor	125	Recurrent or/ and metastatic	67	33	25	50 (24-76)	89 (71.2)	21 (11.8-32.1)

(Continued)

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TABLE 1 Continued

Trials name	year	Study type	Intervention drugs	Intervention types	Number of patients	Stage	PD-L1 CPS≥1%	PD-L1 CPS<1%	PD-L1 CPS unknown	Age, median (range)	Squamous cell carci-noma (%)	Follow-up (m), median (range)
Xia	2022	NRCT Single arm	Camrelizumab + Famitinib	PD-1 inhibitors + Anti-angiogenic agent	33	Recurrent or metastatic	10	9	14	50 (43–55)	33(100)	13.6(10-23.6)
Ma	2022	NRCT Single arm	Sintilimab or Tislelizumab or Camrelizumab + Paclitaxel + Cisplatin	PD-1 inhibitors + Chemotherapy	85	FIGO IVB stage or recurrent or metastatic	30	55	0	52 (46-62)	68 (80)	23.4 (22.19–24.62)
Cheng A	2022	NRCT Single arm	Camrelizumab or Sintilimab	PD-1 inhibitors	24	Recurrent or metastatic	NR	NR	NR	52 (22-78)	UTE	18 (2-28)
Cheng B	2022	NRCT Single arm	Camrelizumab or Sintilimab + Paclitaxel + Cisplatin or Carboplatin	PD-1 inhibitors + Chemotherapy	26	Recurrent or metastatic	NR	NR	NR	52 (22-78)	UTE	18 (2-28)
Tewari	2022	RCT	Cemiplimab Versus Pemetrexed or Topotecan or Irinotecan or Gemcitabine or Vinorelbine	PD-1 inhibitors Versus Chemotherapy	608	Recurrent or metastatic	82	44	178	51 (22–81)	240 (78.9)	18.2 (6.0 - 38.2)
Xu	2022	NRCT Single arm	Sintilimab + Anlotinib	PD-1 inhibitors + Anti-angiogenic agent	42	Recurrent or metastatic	42	0	0	53(36-67)	35 (83.3)	10.9(0.03-19.2)
An	2023	NRCT Single arm	Serplulimab + Nab-Paclitaxel	PD-1 inhibitors + Chemotherapy	21	Recurrent or/ and metastatic	21	0	0	50.8 (31–64)	20 (95.2)	14.6(0.2-21.7)
Nishio	2023	RCT	Pembrolizumab + Paclitaxel + Cisplatin or Carboplatin Versus Paclitaxel + Cisplatin or Carboplatin	PD-1 inhibitors + Chemotherapy Versus Chemotherapy alone	57	Recurrent or metastatic or persistent	30	5	0	54 (26–82)	27(77.1)	23.2 (16.4-27.8)
Zheng A	2023	NRCT Single arm	Tislelizumab + Bevacizumab or Apatinib	PD-1 inhibitors + Anti-angiogenic agent	44	Recurrent or metastatic	NR	NR	NR	54 (32-70)	UTE	11.3 (2.2-28.7)
Zheng B	2023	NRCT Single arm	Tislelizumab	PD-1 inhibitors monotherapy	41	Recurrent or metastatic	NR	NR	NR	54 (32-70)	UTE	11.3 (2.2-28.7)

RCT, randomized controlled trial; NRCT, not RCT; PD-1, programmed cell death protein-1; PD-L1, programmed cell death ligand-1; ICIs, Immune Checkpoint Inhibitors; CPS, combined positive score; ECOG, Eastern Cooperative Oncology Group; NR, not report; UTE, Unable to extract.

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TABLE 2 The IHE AQ checklist for evaluating the quality of eligible observational studies.

trails name	year	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	⑪	⑫	⑬	⑭	⑮	⑯	⑰	⑱	⑲	⑳	scores
An	2023	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Cheng	2022	yes	no	no	no	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	16
Chung	2019	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Frenel	2017	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Friedman	2020	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	no	yes	yes	yes	18
Huang	2021	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	partial	yes	yes	yes	18
Ma	2022	yes	no	yes	no	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	partial	yes	yes	yes	16
Miller	2021	yes	yes	no	no	no	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	no	no	yes	yes	14
O'Malley	2021	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
O'Malley	2022	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Santin	2020	yes	no	no	no	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	16
Tamura	2019	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Xia	2022	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Xu	2022	yes	yes	yes	yes	yes	yes	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	19
Zheng	2023	yes	no	no	no	yes	no	yes	yes	yes	yes	unclear	yes	yes	yes	yes	yes	yes	yes	yes	yes	15

①: Was the hypothesis/aim/objective of the study clearly stated?
②: Was the study conducted prospectively?
③: Were the cases collected in more than one centre?
④: Were patients recruited consecutively?
⑤: Were the characteristics of the patients included in the study described?
⑥: Were the eligibility criteria (i.e. inclusion and exclusion criteria) for entry into the study clearly stated?
⑦: Did patients enter the study at a similar point in the disease?
⑧: Was the intervention of interest clearly described?
⑨: Were additional interventions (co-interventions) clearly described?
⑩: Were relevant outcome measures established a priori?
⑪: Were outcome assessors blinded to the intervention that patients received?
⑫: Were the relevant outcomes measured using appropriate objective/subjective methods?
⑬: Were the relevant outcome measures made before and after the intervention?
⑭: Were the statistical tests used to assess the relevant outcomes appropriate?
⑮: Was follow-up long enough for important events and outcomes to occur?
⑯: Were losses to follow-up reported?
⑰: Did the study provided estimates of random variability in the data analysis of relevant outcomes?
⑱: Were the adverse events reported?
⑲: Were the conclusions of the study supported by results?
⑳: Were both competing interests and sources of support for the study reported?
The criteria for quality rating scores are as follows: 1 point for a 'yes' answer, 0 for an 'unclear', 'partial' or 'no' answer.
The quality of included studies was evaluated based on 20 items from the Delphi checklist. If the literature≥14 items of the Delphi checklist, it was considered to meet acceptable quality criteria.
IHE QA, Institute of Health Economics Quality Appraisal.

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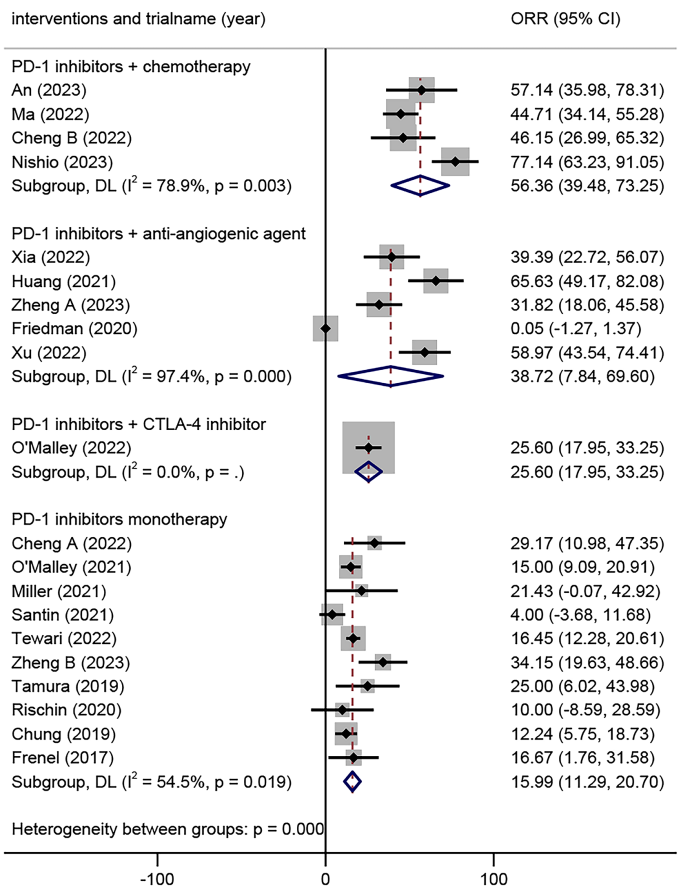

FIGURE 2
Overall objective response rates of different combinations of PD-1 inhibitors in the treatment of advanced, recurrent, or metastatic cervical cancer. PD-1, Programmed Cell Death Protein 1.

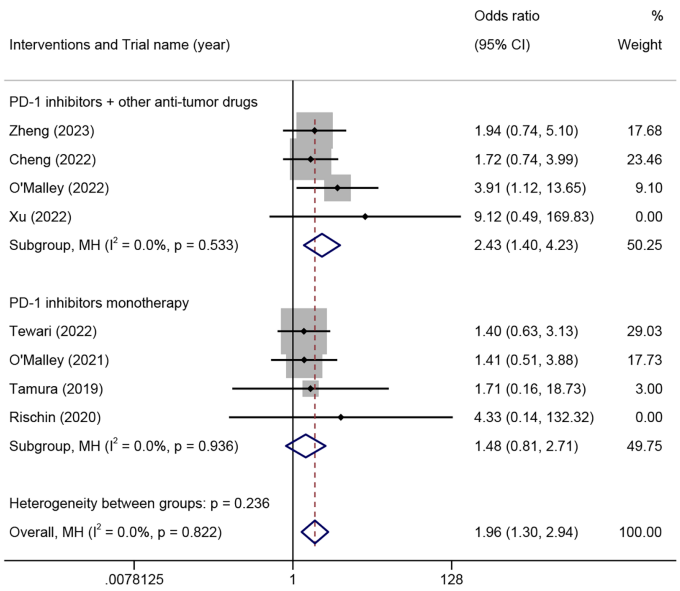

FIGURE 3
The odds ratio of objective response rates in squamous cell carcinoma and non-squamous cell carcinoma patients treated with different combinations of PD-1 inhibitors for advanced, recurrent, or metastatic cervical cancer. PD-1, programmed cell death protein 1.

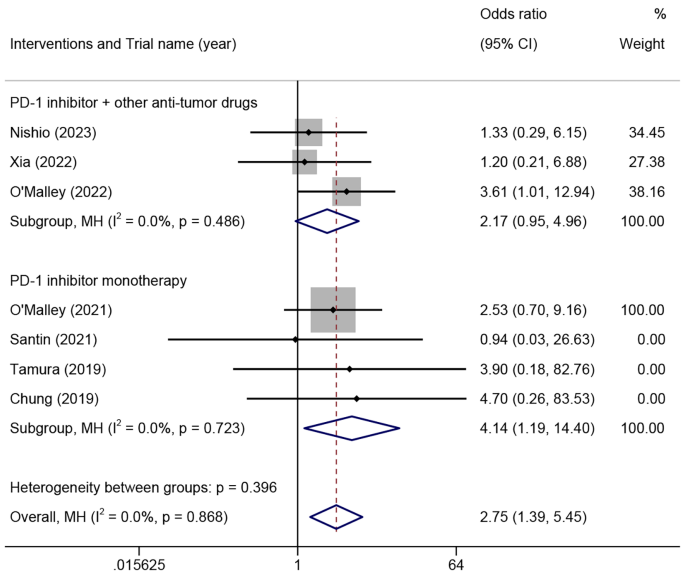

FIGURE 4
The odds ratio of objective response rates in PD-L1 combined positive score ≥1 and <1 patients treated with different combinations of PD-1 inhibitors for advanced, recurrent, or metastatic cervical cancer. PD-L1, programmed death-ligand 1; PD-1, programmed cell death protein 1.

Five trials including 177 patients were eligible for inclusion based on the 1-year PFS rate. The analysis based on different intervention types indicated that the 1-year PFS rate in patients with ARMCC who received PD-1 inhibitors combined with chemotherapy was 58.85% (95% CI 42.96%-74.75%), that in patients with ARMCC who received PD-1 inhibitors combined with anti-angiogenic agents was 48.48% (95% CI 31.43%-65.54%), and that in patients with ARMCC who received PD-1 inhibitor monotherapy was 17.61% (95% CI -12.97%-48.18%) (Supplementary Figure 1).

3.3.5 The HRs for OS and PFS in RCTs

Two RCTs including 674 patients were eligible for OS and PFS analyses. The OS was significantly higher in ARMCC patients who

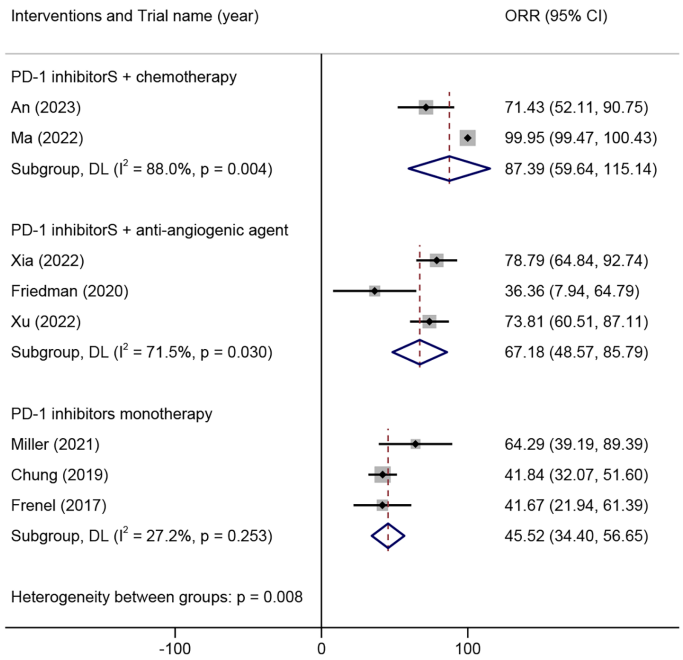

FIGURE 5
1-year overall survival rate of different combinations of PD-1 inhibitors in the treatment of advanced, recurrent, or metastatic cervical cancer. PD-1, programmed cell death protein 1.

received PD-1 inhibitors with chemotherapy compared to those who received chemotherapy alone (HRs=0.65, 95% CI 0.53-0.81, p=0.000, I^2=10%) (Supplementary Figure 2). Similarly, the PFS in ARMCC patients who received PD-1 inhibitors with chemotherapy was significantly higher compared to those who received chemotherapy alone (HRs=0.63, 95% CI 0.52-0.77, p=0.000, I^2=0%) (Supplementary Figure 3).

3.4 Safety

3.4.1 Overall incidence of AEs

This review included 17 studies reporting AEs, with 85 different types of AEs included in 4049 cases.

In the analysis based on the intervention type, the top five AEs in patients who received PD-1 inhibitors combined with chemotherapy were anemia (19.70%; 95% CI, 12.91%-26.48%), neutropenia (18.18%; 95% CI, 11.60%-24.76%), leukopenia (12.88%; 95% CI, 7.16%-18.59%), hypothyroidism (9.85%; 95% CI, 4.77%-14.93%), and constipation (9.09%; 95% CI, 4.19%-14.00%) (Figure 6A). The top five AEs in patients who received PD-1 inhibitors combined with anti-angiogenic agents were hyperglycemia (22.09%; 95% CI, 15.89%-28.29%), hypothyroidism (19.19%; 95% CI, 13.30%-25.07%), anemia (15.70%; 95% CI, 10.26%-21.13%), diarrhea (15.70%; 95% CI, 10.26%-21.13%), and elevated aspartate aminotransferase levels (15.12%; 95% CI, 9.76%-20.47%) (Figure 6B). The top five AEs in patients who received PD-1 inhibitor monotherapy were asthenia (20.38%; 95% CI, 16.54%-

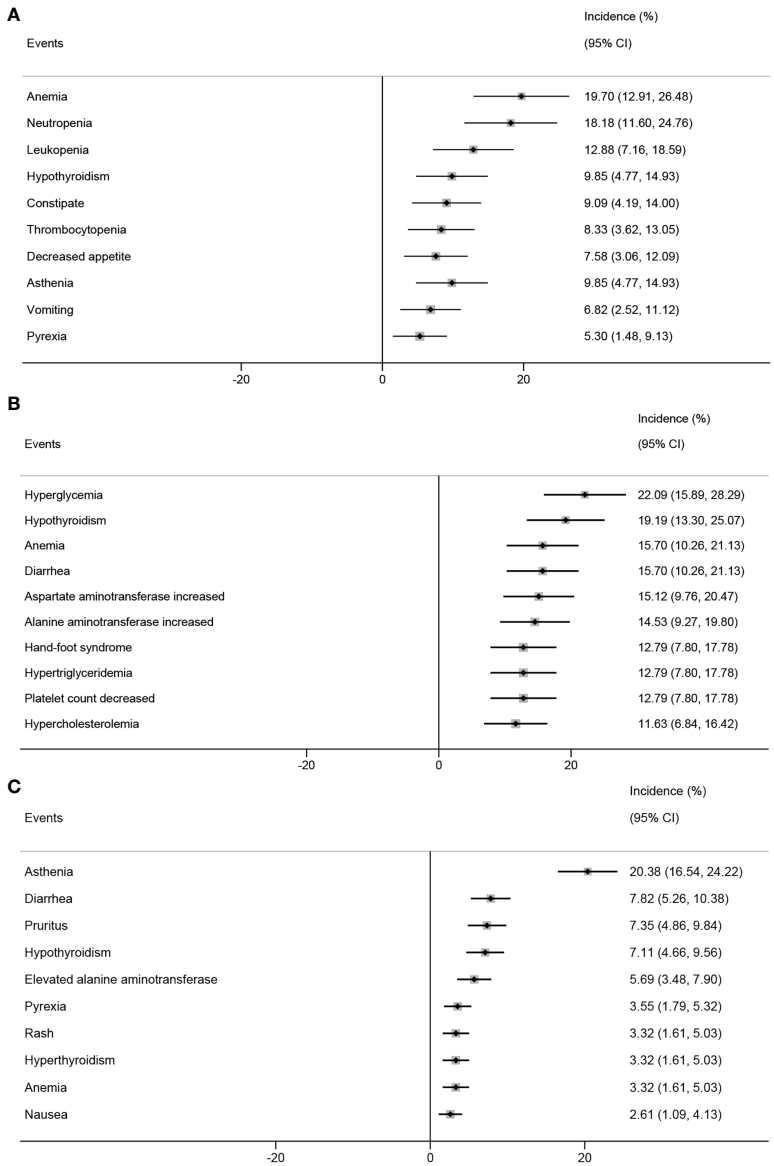

FIGURE 6
Top 10 incidence of adverse events in patients with advanced, recurrent, or metastatic cervical cancer treated with different combinations of PD-1 inhibitors. PD-1 inhibitors combined with chemotherapy **(A)**, with anti-angiogenic agent **(B)**, monotherapy **(C)**. PD-1, programmed cell death protein 1.

24.22%), diarrhea (7.82%; 95% CI, 5.26%-10.38%), pruritus (7.35%; 95% CI, 4.86%-9.84%), hypothyroidism (7.11%; 95% CI, 4.66%-9.56%), and elevated alanine transaminase (ALT) levels (5.69%; 95% CI, 3.48%-7.90%) (Figure 6C).

3.4.2 Incidence of immune-related AEs

PD-1 inhibitors block the immune checkpoint pathway, reactivate cellular immunity, and cause autoimmune-mediated AEs. This study included 10 studies reporting 33 different types of ir-AEs involving 314 cases.

In the analysis based on the intervention type, the top three ir-AEs in patients who received PD-1 inhibitors combined with chemotherapy were hypothyroidism (6.06%; 95% CI, 1.99%-10.13%), hyperthyroidism (2.27%; 95% CI, 0.18%-4.82%), and pruritus (2.27%; 95% CI, 0.18%-4.82%) (Figure 7A). The top three ir-AEs in patients who received PD-1 inhibitors combined with anti-angiogenic agents were hypothyroidism (24.24%; 95% CI, 9.62%-38.86%), immune-mediated hypothyroidism (6.06%; 95% CI, 0.37%-14.20%), and autoimmune thyroiditis (3.03%; 95% CI, 0.36%-8.88%) (Figure 7B). The top three ir-AEs in patients who received PD-1 inhibitor monotherapy were hypothyroidism (8.47%; 95% CI, 5.66%-11.27%), hyperthyroidism (2.91%; 95% CI, 1.22%-4.60%), and diarrhea (1.32%; 95% CI, 0.42%-2.47%) (Figure 7C).

3.4.3 Overall incidence of AEs in RCTs

In this meta-analysis including three RCTs, the results indicated that PD-1 inhibitor monotherapy or PD-1 inhibitors combined with chemotherapy did not significantly increase the incidence of all grades of AEs (RR=0.99, 95% CI 0.91-1.08, p=0.788, I^2=0.0%) (Figure 8A) or the incidence of serious AEs (grade≥3) (RR=0.99, 95% CI 0.89-1.10, p=0.788, I^2=0.0%) when compared to chemotherapy alone (Figure 8B).

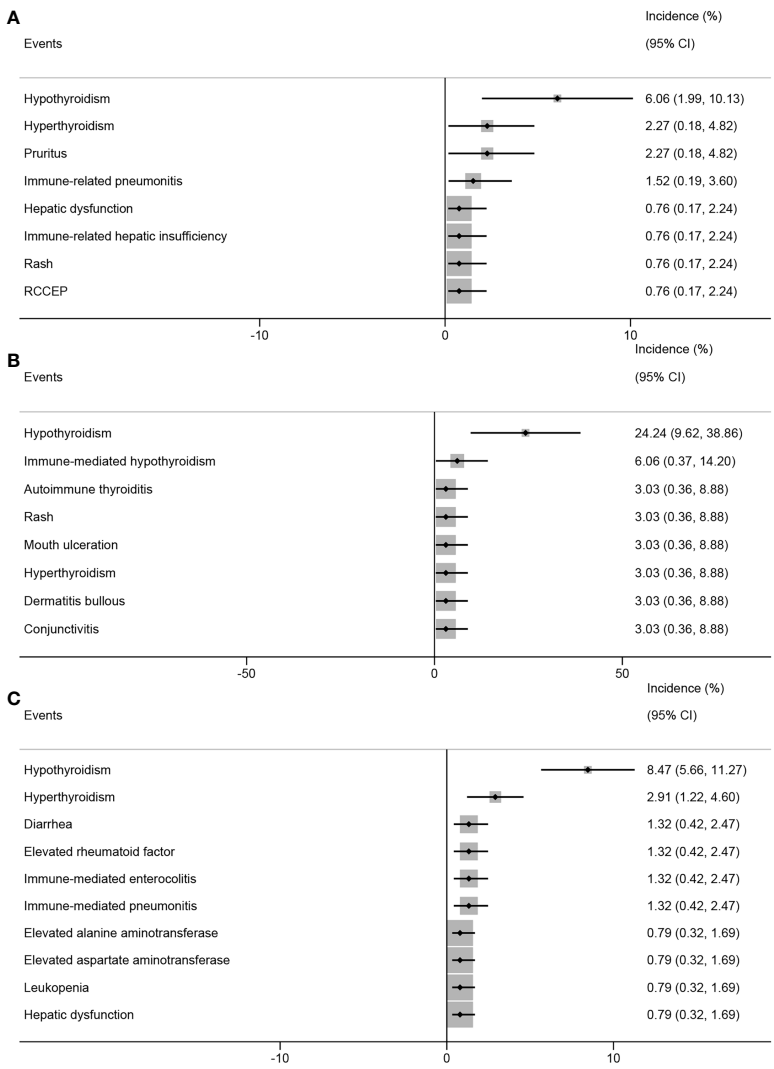

FIGURE 7
Top 8 incidence of immune-related adverse events in patients with advanced, recurrent, or metastatic cervical cancer treated with different combinations of PD-1 inhibitors. PD-1 inhibitors combined with chemotherapy **(A)**, with anti-angiogenic agent **(B)**, monotherapy **(C)**. PD-1, programmed cell death protein 1.

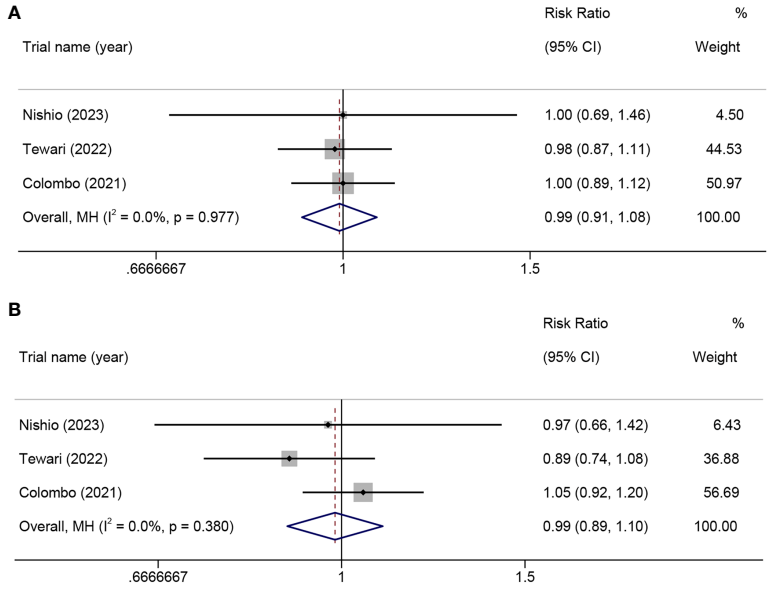

FIGURE 8
In a randomized controlled trial, the risk ratio of adverse events in patients with advanced, recurrent, or metastatic cervical cancer treated with PD-1 inhibitors combined with chemotherapy versus chemotherapy alone. all-grade adverse events **(A)**, grade ≥3 adverse events **(B)**. PD-1, programmed cell death protein 1.

3.5 Publication bias

No significant publication bias was detected in the funnel plot, and the p-values of Egger's test for the pooled ORR in different histological types or PD-L1 CPS of patients with ARMCC were not significant (p=0.083 and p=0.709, respectively) (Supplementary Figures 4, 5).

4 Discussion

The binding of PD-L1, which is expressed on tumor cells or within the tumor microenvironment, to PD-1 on T cells leads to the suppression of T cell function, allowing cancer cells to evade immune surveillance and promote tumor development. Conversely, PD-1 inhibitors can reverse T cell dysfunction or apoptosis and maintain peripheral immune system tolerance, thereby exerting anti-tumor effects. This treatment strategy has shown significant success in the treatment of various malignant tumors and is transforming cancer therapy (5–7). As a significant category, PD-1 inhibitors have been approved for the treatment of various malignancies, including non-small cell lung cancer, malignant melanoma, and CC (30–33).

However, the therapeutic efficacy of PD-1 inhibitors for the treatment of CC, particularly ARMCC, remains controversial (20, 26). The key controversial issues include the following:1) Can the combination of PD-1 inhibitors with other treatment modalities significantly improve the therapeutic efficacy? For example, a combination of chemotherapy, anti-angiogenic agents, or other immunotherapies can effectively control tumor growth and spread. 2) Does the use of PD-1 inhibitors lead to severe adverse events? These adverse events can significantly affect the quality of life of patients with CC. Therefore, we conducted this study to address these issue. To the best of our knowledge, this study represents the first comprehensive analysis on the effectiveness and safety of PD-1 inhibitors combined with other anti-tumor drugs for the treatment of patients with ARMCC.

We found that, among the several treatment strategies for PD-1 inhibitor therapy in ARMCC, the combination of PD-1 inhibitors with chemotherapy exhibited the highest ORR. The combination of PD-1 inhibitors with anti-angiogenic agents exhibited the second highest ORR, followed by the combination of PD-1 inhibitors and CTLA-4 inhibitors. Finally, PD-1 inhibitor monotherapy resulted in the lowest ORR. In terms of the 1-year OS and 1-year PFS, various treatment strategies involving PD-1 inhibitor therapy in ARMCC have yielded similar results.

Based on the above results, it can be inferred that the treatment outcomes in ARMCC tend to favor the combination of PD-1 inhibitors with other anti-tumor drugs, such as chemotherapy, anti-angiogenic agents, or CTLA-4 inhibitors, rather than PD-1 inhibitor monotherapy. Evidence supports the suggestion that chemotherapy drugs and anti-angiogenic agents are able to disrupt tumor cells and release immunostimulatory tumor antigens, thereby enhancing immunogenicity (34). Our findings also confirmed that combination therapy enhanced the anti-tumor effects of PD-1 inhibitors.

According to a stratified analysis based on histological types and PD-L1 positive expression in ARMCC, the results revealed that in PD-1 monotherapy, the SCC and non-SCC groups did not exhibit a significant difference in terms of ORR. However, the group with a CPS≥1 exhibited a significantly higher ORR compared to the CPS<1 group. Additionally, when PD-1 inhibitors were used in combination with other anti-tumor drugs, the SCC group exhibited a significantly higher ORR than the non-SCC group.

Moreover, the CPS≥1 group exhibited a higher ORR than the CPS<1 group, although the difference was not statistically significant. These findings suggest that SCC patients may benefit more from combined treatment with PD-1 inhibitors and other anti-tumor drugs, and patients with positive PD-L1 expression exhibit a better response to immunotherapy, which suggests that PD-L1 may be a potential biomarker for predicting clinical outcomes in patients with cervical cancer. Although a previous study indicated that PD-L1 expression levels did not enhance the OS and PFS of patients with ARMCC treated with PD-1 inhibitors (31), it is important to note that assessing the impact of drugs on OS and PFS can be confounded by the complex nature of the causes of death in patients with ARMCC, potentially introducing bias into the results. In contrast, ORR reflects the proportion of tumors that experience a rapid reduction or disappearance in volume within a short period of time, which provides a better indication of the therapeutic effect of drugs on tumors.

To enhance the credibility of our research findings, we conducted a meta-analysis of RCTs. The findings demonstrated that, compared to patients undergoing chemotherapy alone, the combination of PD-1 inhibitors with chemotherapy can significantly improve OS and PFS in patients with ARMCC. These results further support the superior efficacy of PD-1 inhibitors combined with chemotherapy compared to chemotherapy alone, and that more patients with ARMCC could benefit from them.

We also conducted a safety analysis and found that, on average, each patient with ARMCC who received PD-1 inhibitor treatment experienced approximately two adverse events. It is crucial to communicate these statistical data to patients before initiating PD-1 inhibitor therapy. The top five AEs in patients who received PD-1 inhibitor monotherapy were asthenia, diarrhea, hypothyroidism, pruritus, and elevated alanine aminotransferase levels. Similarly, patients who received PD-1 inhibitors in combination with chemotherapy experienced anemia, neutropenia, leukopenia, hypothyroidism, and constipation. Furthermore, patients who received PD-1 inhibitors combined with anti-angiogenic agents experienced hyperglycemia, hypothyroidism, anemia, diarrhea, and elevated aspartate aminotransferase levels. The top three ir-AEs in patients who received PD-1 inhibitor monotherapy were hypothyroidism, hyperthyroidism, and diarrhea. Similarly, patients who received PD-1 inhibitors in combination with chemotherapy experienced hypothyroidism, hyperthyroidism, and pruritus. Lastly, patients who received PD-1 inhibitors in combination with anti-angiogenic agents experienced hypothyroidism, immune-mediated hypothyroidism, and autoimmune thyroiditis.

We included three RCTs in the safety analysis to assess whether the use of PD-1 inhibitors would increase the incidence of AEs. The results showed that PD-1 inhibitor monotherapy or PD-1 inhibitors in combination with chemotherapy did not increase the overall or severe AEs rates compared to chemotherapy alone. These findings are similar to a previous large-scale meta-analysis, which indicates the reliability of our analysis, despite our focus on only analyzing the AEs reported in the included literature (35).

This study has some limitations. First, most of the included studies were single-arm trials, which introduced a certain risk of bias and confounding factors owing to the lack of a control group. Even though we attempted to mitigate these risks through subgroup and stratified analyses, significant heterogeneity remained in some of the results. Second, in some of the included studies, data such as OS and PFS could not be utilized as the patients did not reach the median survival time, resulting in a less comprehensive survival analysis. Third, only one study on the use of PD-L1 inhibitors in combination with CTLA-4 inhibitors was included. Therefore, it is crucial to continue to monitor research related to PD-L1 inhibitors combined with CTLA-4 inhibitors to obtain more data that can be used to validate our results. Fourth, variations in PD-1 inhibitor use across studies in terms of therapy lines, combination regimens, treatment durations, and dosages may have increased outcome heterogeneity. Despite the significant heterogeneity, this study still holds value and significance.

5 Conclusion

Our study revealed that PD-1 inhibitors demonstrate outstanding efficacy in the treatment of patients with ARMCC. Patients with SCC may benefit more from treatments including PD-1 inhibitors in combination with other anti-tumor drugs. Additionally, PD-L1 may be a potential biomarker for predicting clinical outcomes in patients with cervical cancer. Importantly, the use of PD-1 inhibitor monotherapy or PD-1 inhibitors in combination with chemotherapy did not lead to an increased incidence of AEs compared with chemotherapy alone, indicting safety during. Furthermore, identifying more subgroups of cervical cancer that benefit from PD-1 inhibitors is a direction worth researching.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

YW: Writing – original draft, Writing – review & editing. JW: Writing – original draft. JD: Writing – original draft. XT: Writing – original draft. JX: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1305810/full#supplementary-material

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Oncological outcomes of sequential laparoscopic gastrectomy after treatment with camrelizumab combined with nab-paclitaxel plus S-1 for gastric cancer with serosal invasion

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Objective: To explore the oncological outcomes of sequential laparoscopic gastrectomy after treatment with camrelizumab in combination with nab-paclitaxel plus S-1 for the treatment of gastric cancer with serosal invasion.

Methods: This study is a retrospective cohort study and retrospectively analyzed the clinicopathological data of 128 patients with serosal invasion gastric cancer (cT4NxM0) who received nab-paclitaxel + S-1(SAP) or camrelizumab + nab-paclitaxel + S-1 (C-SAP) regimen and underwent laparoscopy assisted gastrectomy in Fujian Union Hospital from March 2019 to December 2020. The patients were divided into SAP group and C-SAP group. The 2-years overall survival rate, 2-year recurrence free survival rate recurrence rate and initial recurrence time were compared between the two groups.

Results: A total of 128 patients were included, including 90 cases in SAP group and 38 cases in C-SAP group. There were no significant differences in age, gender, gastrectomy method, surgical approach, R0 resection, nerve invasion, vascular invasion, total number of harvested lymph nodes, number of positive lymph nodes and major pathologic response (MPR) rate between the two groups (P>0.05). However, the proportion of ypT0, ypN0 and pCR rate in C-SAP group were significantly higher than those in SAP group (P<0.05). The 2-year OS of C-SAP group (80.7%) was higher than that of SAP group (67.8%), and the difference was not statistically significant (P = 0.112); At 2 years after operation, the recurrence rate of C-SAP group (44.3%) was lower than that of SAP group (55.8%) (P = 0.097); Further analysis showed that the average time to recurrence in the C-SAP group was 18.9 months, which was longer than that in SAP group 13.1 months (P = 0.004); The 2-year recurrence free survival rate in C-SAP group

was higher than that in SAP group (P=0.076); There was no significant difference in the overall survival time after recurrence between the two groups (P= 0.097).

Conclusion: Camrelizumab combined with neoadjuvant chemotherapy can improve the proportion of ypT0, ypN0 and pCR in patients, while prolonging the initial recurrence time of patients in the C-SAP group, but did not increase the immunotherapy/chemotherapy related side effects and postoperative complications.

KEYWORDS

Gastric cancer with serous invasion, Camrelizumab, Neoadjuvant chemotherapy, 2-years overall survival rate, 2-year recurrence free survival rate

Introduction

Gastric cancer is the fifth most common malignancy (1) and the third leading cause of cancer-related death(s) worldwide. However, neoadjuvant therapy for locally advanced gastric cancer remains controversial. A previous study (2) reported that neoadjuvant therapy (S-1 combined with nab-paclitaxel) was safe and effective for locally advanced gastric cancer. To improve the feasibility of radical surgery for primary gastric lesions, it is important to develop a treatment scheme with a high tumor remission rate and low toxicity. Accumulating evidence supports the use of immune checkpoint inhibitors for advanced gastric cancer. The KEYNOTE-059 (3) and Check-mate 649 trials confirmed that the programmed death (PD)-1 monoclonal antibody has a significant survival benefit and is safe for advanced, recurrent, or metastatic gastric or gastroesophageal junction adenocarcinoma. Based on the results of KEYNOTE-059 and Check-Mate 649, pembrolizumab and nivolumab were approved as third- and first-line treatments for unresectable and advanced metastatic gastric cancer, respectively. However, their application in patients with locally advanced gastric cancer remains rare.

According to the 8th Edition of the American Joint Committee on Cancer TNM Staging System for Gastric Cancer (4), the clinical stage of patients with cT4aN+M0 gastric cancer was stage III and the 5-year survival rate was 25.9%–43.4%; the clinical stage of cT4bN+M0 patients was stage IVA, and the 5-year survival rate after palliative surgery was only 5%–14.1%. In addition, direct surgical treatment of these patients was associated with low safety and a low resection rate. Preoperative neoadjuvant therapy can effectively improve the resection rate and long-term survival.

Recently, significant progress has been made in neoadjuvant chemotherapy for patients with locally advanced resectable gastric cancer, with Li et al. (5) reporting that laparoscopic distal gastrectomy was safe for patients after neoadjuvant chemotherapy. However, the fibrogenic reaction and cytotoxicity induced by chemotherapy lead to loss of the normal tissue plane, which introduces new technical challenges. However, whether the combined application of immunotherapy affects the perioperative period is unclear. Patients with serous gastric cancer invasion fall between those with locally advanced resectable and those with locally advanced unresectable gastric cancers. Currently, the long-term survival of patients with serous invasion of gastric cancer treated with immune + neoadjuvant chemotherapy combined with surgery has not been reported. As such, this study aimed to explore the oncological outcomes of sequential laparoscopic gastrectomy after treatment with camrelizumab in combination with nab-paclitaxel plus S-1 for the treatment of gastric cancer with serosal invasion to provide evidence-based support for the comprehensive treatment of this patient population.

Methods

Study design

This study is a retrospective cohort study. This study was conducted in Fujian Union Hospital from March 2019 to December 2020 retrospectively. The clinicopathological data of patients with serosal invasion gastric cancer (cT4NxM0) who received nab-paclitaxel + S-1(SAP) or camrelizumab + nab-paclitaxel + S-1 (C-SAP) regimen and underwent laparoscopy assisted gastrectomy were retrospectively analyzed.

Participants

Inclusion criteria: 1. gastric adenocarcinoma confirmed by gastroscopy and pathology before operation; 2. the clinical staging of CT and other imaging evaluation included: cT4; 3. lymph nodes N1 to N3; 4. patients with M0 without distant metastasis from liver, lung, peritoneum and other places evaluated by preoperative CT imaging were included; 5. The ECOG score was 0-2, and the blood

indexes, liver and kidney function, cardiopulmonary function could tolerate chemotherapy or surgery. Exclusion criteria: 1. incomplete pathological diagnosis data; 2. patients with gastric stump cancer; 3. gastric cancer patients undergoing emergency surgery; 4. combined with other malignant tumors. Finally, 128 patients were included. Flow diagram was described in Supplementary Figure 1.

Outcome measures/end points

The primary end-point was the 2-years overall survival rate, and the secondary end-points included 2-year recurrence free survival rate, initial recurrence time, pCR, MPR and safety.

Neoadjuvant therapy

We divided the patients into two groups according to the different neoadjuvant drug treatments: the SAP group (nab-paclitaxel + S-1), and C-SAP group (camrelizumab + nab-paclitaxel + S-1). The specific scheme was as follows:

The cycle of Nab-paclitaxel + S-1 chemotherapy consisted of the following: Day 1: Intravenous Nab-paclitaxel 260 mg/m^2 over 30 min. Dose reductions (220 mg/m^2, 180 mg/m^2, or 150 mg/m^2) were permitted in patients with severe haematological or non-haematological toxicity. Day 1–14: S-1 at 120 mg/day for surface area ≥ 1.5m^2, 100 mg/day for surface area between 1.25 and 1.5m^2, and 80 mg/day for surface area < 1.25 m^2 were given 2 times daily. The next chemotherapy was repeated on the 22nd day.

The cycle of Camrelizumab consisted of the following: Day 1: Intravenous Camrelizumab 200mg.

Tumor regression grade (TRG)

Tumor regression grade according to Becker criteria (6, 7) included "Grade 1a" (Complete tumor regression i.e., 0% residual tumor per tumor bed), "Grade 1b" (Subtotal tumor regression i.e., <10% residual tumor per tumor bed) "Grade 2" (Partial tumor regression i.e., 10–50% residual tumor per tumor bed), "Grade 3" (Minimal or no tumor regression i.e., >50% residual tumor per tumor bed). MPR is defined as: TRG1a+TRG1b. Pathological complete response: pCR is defined as no invasive disease within an entirely submitted and evaluated gross lesion and histologically negative nodes based on central review.

Postoperative pathological staging

TNM staging was performed according to the 8th edition of AJCC staging standard in 2016. Methods of lymph node treatment: after the specimens were isolated, the lymph nodes of each group were collected and subpackaged, fixed with 10% formalin solution, sent for pathological examination, and sorted by experienced pathologists. The depth of tumor invasion was divided into T1 (invasion of lamina propria, muscularis or submucosa), T2 (invasion of muscularis propria), T3 (invasion of subserosa, but not invasion of visceral peritoneum or adjacent organ), T4a (invasion of serosa) and T4b (invasion of adjacent organ). The staging of lymph node metastasis was divided into N0 (no regional lymph node metastasis), N1 (1 or 2 regional lymph node metastasis), N2 (3 to 6 regional lymph node metastasis), N3a (7 to 15 regional lymph node metastasis), and N3b (≥16 regional lymph node metastasis) according to the 8th edition AJCC staging of gastric cancer. Similarly, ypTNM staging was divided into stage I, stage II, stage III and IV according to the 8th Edition AJCC staging of gastric cancer. If the patient is diagnosed as cT4b before suegery and the postoperative pathology indicates ypT3 or below, it is considered as R0 resection.

Efficacy evaluation of solid tumor

Tumor response was assessed response evaluation criteria in solid tumors (RECIST), version 1.1 (8): target lesion evaluation criteria (1) CR: all (non lymph node) target lesions diSAPpeared, emphasizing that the short diameter of all original pathological lymph nodes (including target lesions and non target lesions) was<10mm after treatment. (2) PR: the total length and diameter of all target lesions decreased by 30% or more. (3) SD: the change is between PR and PD. (4) PD: the total length and diameter of all target lesions increased by at least 20%, and it was emphasized that the absolute value of the total length and diameter increase was more than 5mm; Or new lesions appear. When lymph nodes were evaluated as target lesions, it was judged that the sum of the long diameters of CR target lesions did not include lymph nodes, as long as the short diameters of all lymph nodes were<10mm; When judging PR, SD and PD, the short diameter of lymph nodes was added to the long diameter of all other target lesions for comparison before and after treatment.

Surgical indications

After neoadjuvant therapy every 2 cycles, all patients need to review abdominal enhanced CT. Fasting for 6-12 hours before the examination. In addition, the patient drank 600-1000 ml of water to expand the stomach before CT examination. Generally, after 4-6 cycles of neoadjuvant therapy, the operation plan is formulated after multidisciplinary consultation according to the tumor regression grade.

The scope of gastric resection was selected according to the Japanese "Regulations on the treatment of gastric cancer" and the lymph node dissection around the stomach was performed. According to the Japanese Gastric Cancer Treatment Guidelines (5th edition) (9), we perform D2 lymph node dissection. For the distal stomach, we perform lymph node dissection for No.1,3,4sb,5,6,7,8a,9,11p,12a. For the entire stomach, we perform lymph node dissection for No.1-7,8a,9,11p,12a. Standard lymphadenectomy sequences and resection methods were performed as described in the Laparoscopic Gastrectomy for Gastric Cancer (10). LN dissection at station 10 was performed as

a selective dissection: (1) When the primary tumor is located in the upper or middle part of the stomach and invades the greater curvature, and (2) When preoperative imaging or intraoperative findings show enlarged lymph nodes in the splenic hilum area, a lymph node dissection for the No.10 region is performed. When No. 14v nodes were highly suspicious for tumor involvement, a lymph node dissection for the No.14v region is performed. This is also a selective dissection.

Surgery related complications and side events of chemotherapy or immunotherapy

The incidence of surgical complications is based on the number of patients who received surgical treatment as the denominator, and the number of patients with any of the following intraoperative/postoperative complications is the numerator ratio. The standard of intraoperative/postoperative complications refers to the early and late surgical complications mentioned in the intraoperative and postoperative observation items. The severity of complications was graded according to Clavien–Dindo (11) complication scoring system. IIIa and above were serious complications, Supplementary Table 1.

The adverse Events of chemotherapy and immunotherapy are classified into grade 0-IV according to the Common Terminology Criteria for Adverse Events (CTCAE) version 5.0 (12), Supplementary Table 2.

Postoperative follow-up

The endpoints of this study include OS and RFS, where OS is defined as the time from surgery to death from any cause. The outcomes include tumor related death, non tumor related death, loss of follow-up, and survival. RFS is defined as the time from the beginning of surgery to the recurrence or death of gastric cancer. According to the follow-up strategy of the Japanese gastric cancer treatment guidelines, patients are followed up every 3 months for the first 2 years and every 6 months for the following 3-5 years. The follow-up routine examination items include physical examination, laboratory examination (CA19-9, CEA, CA72-4), chest X-ray, abdominal ultrasound, CT, and annual gastroscopy examination. The follow-up methods include outpatient follow-up, phone calls, letters, and doorstep visits. The last follow-up date is due to January 2023.

Ethic

The human research involved in this study has obtained approval from the Ethics Committee of ethics committee of Fujian Union Hospital (Ethics registration number: 2020YF013-01). The study was conducted in accordance with local laws, regulations, and institutional requirements. Detailed information about medication was provided to the patients, and treatment informed consent forms were signed and provided before administration.

Statistics method

All of the data were analyzed by SPSS software (SPSS, Chicago, IL, USA), version 25.0. The chi-square test or Fisher's exact test was used for comparisons of categorical variables. The independent sample t test or the Mann-Whitney U test was used for comparisons of continuous variables. Apply COX regression analysis to analyze factors that affect overall survival rate. RFS and OS were estimated using the Kaplan Meier method, RFS and OS were compared using a log-rank test. When $P<0.05$, it indicates that the difference is statistically significant.

Results

Participants

A total of 128 patients were included in this study and divided into two groups: S-1 + nab-paclixatel (SAP), n = 90; and camrelizumab-SAP (C-SAP), n = 38. The median follow-up was 22 months (range, 1–39 months). There were no statistical differences in age, sex, Eastern Cooperative Oncology Group (i.e., "ECOG") score, Borrmann type, tumor location, preoperative adjuvant cycles, or tumor differentiation type between the two groups ($P > 0.05$) (Table 1).

Pathological response

The proportion of ypT0 patients in the C-SAP group was 21.1%, which was significantly higher than that in the SAP group (5.6%) ($P = 0.008$). The proportion of ypN0 in the C-SAP group (63.2%) was significantly higher than that in the SAP group (38.9%), and the difference was statistically significant ($P = 0.012$). The proportion of those with pathological complete response (pCR) in the C-SAP group (18.4%) was statistically greater than that in SAP group (5.6%) ($P = 0.03$). The proportion of patients with TRG grade 1a+1b in the C-SAP group was 39.5%, which was similar to that in the SAP group (25.6%) ($P = 0.115$). The proportion of nerve invasion in the C-SAP and SAP groups was 28.9% and 42.2%, respectively ($P = 0.158$). The proportion of vascular invasion in the C-SAP and SAP groups was 26.3% and 36.7%, respectively ($P = 0.241$). The mean (± SD) number of harvested lymph nodes in the C-SAP and SAP groups was 41.6 ± 18.2 and 43.3 ± 14.1, respectively ($P = 0.58$). The number of positive lymph nodes in the C-SAP and SAP groups 2.3 ± 5.1 and 4.2 ± 3.1, respectively ($P = 0.13$). Finally, the proportion of those achieving partial response (PR) in the SAP and C-SAP groups was 92.2% and 90.9% ($P = 0.982$) (Table 2).

Intraoperative and postoperative clinicopathological results

The mean estimated blood loss in the C-SAP and SAP groups was 70.3 ± 120.9 ml and 41.9 ± 21.6 ml, a difference that was

TABLE 1 Demographic data before surgery.

Baseline Variable	C-SAP group(n=38)	SAP group(n=90)	P* value
Gender			0.586
Male	21(55.3)	45(50.0)	
Female	17(44.7)	45(50.0)	
Age			0.586
<65	29(76.3)	66(73.3)	
≥65	9(23.7)	24(26.7)	
ECOG			0.772
0	34(89.5)	82(91.1)	
1	4(10.5)	8(8.9)	
Tumor size			
median(cm)	5.5	5.1	0.550
Borrmann type			0.857
2-3	29(76.3)	70(77.8)	
4	9(23.7)	20(22.2)	
Neoadjuvant cycle			0.639
≤3	13(34.2)	27(30.0)	
≥4	25(65.8)	63(70.0)	
Tumor location			0.241
Upper	20(52.6)	44(48.9)	
Middle	12(31.6)	20(22.2)	
Lower	6(15.8)	26(28.9)	
Differentiation			0.582
Well/moderate	12(3.6)	33(36.7)	
Poor/ undifferentiated	26(68.4)	57(63.3)	

statistically significant (P = 0.008). The proportion of total gastrectomy in the C-SAP and SAP groups was 89.5% and 86.7%, respectively, with no statistical difference (P = 0.661). Two (5.3%) patients in the C-SAP group were converted to open laparotomy, with no conversion to open surgery in the SAP group (P = 0.109). In the SAP group, 1 (1.1%) patient underwent left partial hepatectomy. The R0 resection rate in the C-SAP and SAP groups was 97.4% and 98.9%, respectively (P = 0.58). Operative duration in the C-SAP and SAP groups was 201.3 ± 62.6 min and 208.6 ± 63.5 min, respectively (P = 0.867). The mean time to start liquid diet in the C-SAP and SAP groups was 3.5 ± 1.2 and 3.6 ± 1.0 days, respectively, (P=0.867). The mean time to start semifluid in the C-SAP and SAP groups was 5.1 ± 0.9 and 5.3 ± 0.7 days, respectively (P = 0.851). The mean length of postoperative hospital stay in the C-SAP and SAP groups was 9.0 ± 5.0 and 9.2 ± 11.9 days, respectively (P = 0.794) (Table 3).

Surgery-related complications and chemotherapy/immunotherapy-related side effects

The proportions of overall postoperative complications were 24.2% and 22.1% in the C-SAP and SAP groups, respectively, with no statistically significant difference (P=0.801). The proportion of grade I-II complications in the C-SAP and SAP groups was 18.2% and 18.9%, respectively (P = 0.923). The proportion of grade III complications in the C-SAP and SAP groups was 6.1% and 3.2%, respectively (P = 0.826). Grades IV and V complications did not occur in either group. There was no statistically significant difference in the incidence of complications such as pneumonia, abdominal infection, postoperative bleeding, and anastomotic leakage between the two groups (P > 0.05) (Supplementary Table 3).

The side effects of neoadjuvant therapy were also analyzed. The most common (grade 3, 4) side effects included declines in neutrophil and white blood cell counts, and increased plasma aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels. The proportion of patients exhibiting a decrease in white blood cell count (grade 3, 4) in the C-SAP and SAP groups was 20.0% and 21.1%, respectively (P = 0.984). The proportion of patients exhibiting a decrease in neutrophil count (grade 3, 4) was 22.9% in the C-SAP group and 23.3% in the SAP group (P = 0.955). The proportion of anemia (grade 3, 4) in the C-SAP and SAP groups was 3.0% and 2.1% respectively P = 1.000). The proportion of thrombocytopenia (grade 3, 4) in the C-SAP and SAP groups was 5.7% and 3.3%, respectively (P = 0.619). The proportion of patients exhibiting an increase in plasma AST/ALT level in the C-SAP group was 25.7% and 13.3% in the SAP group (P = 0.096). The proportion of febrile neutropenia in the C-SAP and SAP groups was 2.9% and 6.7 (P = 0.649) (Supplementary Table 4).

Two-year overall survival rate after surgery

Analysis revealed that the two-year overall survival (OS) rate in the C-SAP group (80.7%) was higher than that of the SAP group (67.8%) (P = 0.112) (Figure 1A). Further stratified analysis revealed that, among M0 patients, the two-year OS rate in the C-SAP group (79.9%) was similar to that of the SAP group (71.9%) (P = 0.703) (Figure 1B). Among M1 patients, the two-year OS rate in the C-SAP group (85.7%) was significantly better than that of the SAP group (0%) (P = 0.002) (Figure 1C).

The effect of risk factors on the prognosis of the population is illustrated in Supplementary Figure 1. The OS rate of ypT0 patients (100.0%) was significantly higher than that of ypT1-T4 patients (68.5%) (P = 0.044) (Supplementary Figure 2A). The OS rate of ypN0 patients (74.6%) was higher than that of ypN1-N3b patients (66.2%) (P = 0.21) (Supplementary Figure 2B). The OS rate of M0 patients (79.1%) was significantly higher than that of M1 patients (34.9%) (P < 0.001) (Supplementary Figure 1C). The OS rate of patients with TRG 1a+1b (76.2%) was higher than that of patients

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TABLE 2 Difference of response between two groups.

Baseline Variable	C-SAP group(n=38)	SAP group(n=90)	P* value
TRG			0.014
TRG1a	9(23.7)	5(5.6)	
TRG1b	6(15.8)	18(20.0)	
TRG2	7(18.4)	31(34.4)	
TRG3	16(42.1)	36(40.0)	
subgroup analysis			0.115
TRG1a-1b	15(39.5)	23(25.6)	
TRG2-3	23(60.5)	67(74.4)	
ypT stage			0.059
T0	8(21.1)	5(5.6)	
T1	3(7.9)	11(12.2)	
T2	4(10.5)	12(13.3)	
T3	16(42.1)	51(56.7)	
T4a	6(15.8)	11(12.2)	
T4b	1(2.6)	0	
ypN stage			0.146
N0	24(63.2)	35(38.9)	
N1	6(15.8)	21(23.3)	
N2	3(7.9)	13(14.4)	
N3a	3(7.9)	16(17.8)	
N3b	2(5.3)	5(5.6)	
ypTNM stage			0.106
pCR	7(18.4)	4(4.4)	
I	4(10.5)	14(15.6)	
II	12(31.6)	33(36.7)	
III-IV	15(39.6)	39(43.3)	
ypT stage			0.008
T0	8(21.1)	5(5.6)	
T1-T4b	30(78.9)	85(94.4)	
ypN stage			0.012
N0	24(63.2)	35(38.9)	
N1-N3b	14(36.8)	55(61.1)	
pCR rate			0.03
pCR	7(18.4)	4(4.4)	
I-IV stage	31(81.6)	86(95.6)	
Nerve invasion			0.158
No	27(71.1)	52(57.8)	
Yes	11(28.9)	38(42.2)	
Vascular invasion			0.241
No	28(73.7)	57(63.3)	
Yes	10(26.3)	33(36.7)	
Harvested lymph nodes			0.58
median	41.6±18.2	43.3±14.1	
Positive lymph nodes			0.13
median	2.3±5.1	4.2±3.1	
Radiological response			0.982
PR	35(92.1)	83(92.2)	
SD	3(7.9)	7(7.8)	

with TRG 2+3 (70.8%) (P = 0.788) (Supplementary Figure 2D). The OS rate of patients who underwent ≤ 3 preoperative chemotherapy cycles (75.0%) was higher than that of those who underwent ≥ 4 cycles (70.4%) (P = 0.765) (Supplementary Figure 2E). The OS rate of patients who underwent ≤ 3 postoperative chemotherapy cycles (64.0%) was lower than that of those who underwent ≥ 4 cycles (76.2%), a difference that was statistically significant (P = 0.042) (Supplementary Figure 2F).

Univariate Cox regression analysis revealed that that ypT0, ≥ 4 postoperative chemotherapy cycles, and M0 were closely associated with patient prognosis. Further multivariate Cox analysis revealed that ≥ 4 postoperative chemotherapy cycles (hazard ratio [HR] 0.418 [95% confidence interval (CI) 0.207–0.891]; P = 0.023) and M1 (HR 5.304 [95% CI 2.464–11.417]; P < 0.001) were risk factors for long-term survival (Supplementary Table 5).

Postoperative recurrence outcomes

The two-year recurrence free survival (RFS) rate was higher in the C-SAP group (62.0 %) than in the SAP group (46.2%) (P = 0.361) (Figure 2).

There was no significant difference in the proportion of recurrence between the C-SAP and SAP groups after 2 years (44.3% versus [vs.] 55.8%, respectively; P = 0.294). However, the average time to recurrence in the C-SAP group was longer (18.9 months) than that in the SAP group (13.1 months) (P = 0.004) (Table 4).

The two-year recurrence patterns of the C-SAP and SAP groups are detailed in Supplementary Table 6, with distant metastasis being the most common, followed by peritoneal metastasis, and local recurrence being the least common.

There was no statistically significant difference in OS after recurrence between the two groups (P = 0.097) (Supplementary Figure 3).

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TABLE 3 Clinicopathological results after surgery.

Baseline Variable	C-SAP group(n=38)	SAP group (n=90)	P* value
Type of gastrectomy			0.661
Partial	4(10.5)	12(13.3)	
Total	34(89.5)	78(86.7)	
Surgical approach			0.109
Laparoscopy	36(94.7)	90(100)	
Conversion to open laparotomy	2(5.3)	0	
Combination organ dissection			
Partial Left liver		1(1.1)	
Extent of resection			0.526
R0	37(97.4)	89(98.9)	
R1	1(2.6)	1(1.1)	
Operation time (min)	201.3±62.6	208.6±63.5	0.867
Estimated blood loss (ml)	70.3±120.9	41.9±21.6	0.002
Time to start liquid diet(days)	3.5±1.2	3.6±1.0	0.862
Time to start semifluid diet (days)	5.1±0.9	5.3±0.7	0.851
Postoperative hospital stay (days)	9.0±5.0	9.2±11.9	0.794

Computed tomography scans for continued immunotherapy after recurrence in the C-SAP group are shown in Supplementary Figure 4.

Discussion

To our knowledge, this is the first study to explore oncological outcomes of sequential laparoscopic gastrectomy after camrelizumab combined with nab-paclitaxel and S-1 (i.e., "C-SAP") for the treatment of gastric cancer with serosal invasion. The results revealed that camrelizumab combined with neoadjuvant chemotherapy improved the proportion of ypT0, ypN0, and pCR in patients but did not increase immune/chemotherapy-related side effects and postoperative complications. Although it failed to significantly improve the two-year OS and RFS rates, it prolonged the average time to recurrence in patients in the C-SAP group.

Baseline characteristics of the two groups of patients in this study were comparable in terms of general clinical data. The R0 resection rate, operative duration, postoperative recovery, immune/chemotherapy-related side effects, and postoperative complications were not significantly different between the groups.

We compared immune/chemotherapy related side effects in the two groups of patients and found that their incidence was similar; as such, immunotherapy did not result in more side effects. This is similar to the side effects of chemotherapy reported in the ABSOLUTE trial (13) and those for immune/chemotherapy reported in the Neo-PLANET study (14). Therefore, camrelizumab combined with neoadjuvant chemotherapy appears to be safe.

Surgical safety after neoadjuvant therapy is the focus of attention. Li et al. (5) reported that laparoscopic distal gastric cancer surgery appears to have better postoperative safety than open distal gastric cancer surgery in patients with locally advanced gastric cancer receiving neoadjuvant chemotherapy. Li et al. (5) reported that 6 patients (13%) in the laparoscopic surgery group and 20 (40%) in the open surgery group experienced grade II complications. Grade III complications five patients (11%) in the laparoscopic surgery group and two patients (4%) in the open surgery group; Grade IV complications occurred in only 1 patient (2%) in the laparoscopic surgery group, without grade V complications. The proportion of grade II complications in the present study was 18.2% in the C-SAP group and 18.9% in the SAP group, with no statistical difference. The proportion of Grade III complications was 6.1% in the C-SAP group and 3.2% in the SAP group, with no statistical difference. There were no grade IV and grade V complications in either group. Therefore, camrelizumab in combination with neoadjuvant chemotherapy did not increase the incidence of postoperative complications. Intraoperative blood loss was greater in the C-SAP group than in the SAP group. Therefore, more attention should be devoted to intraoperative safety in patients undergoing immunotherapy to avoid intraoperative bleeding.

In the present study, the proportions of those with ypT0, ypN0, and ypCR were higher in the C-SAP group than in the SAP group. The combination of immunotherapy and chemotherapy can effectively alter the overall tumor microenvironment, as well as immune tolerance and immunosuppression, to maintain an effective and persistent antitumor immune response. Increasing evidence supports the use of immunotherapy in combination with chemotherapy for cancer treatment. Chemotherapeutic drugs promote programmed death (PD)-1/PD-ligand1 (PD-L1) expression through multiple signaling pathways (15). Chemotherapy drugs are based on interferon-gamma (IFN- γ) dependent and non-IFN-γ dependent pathways. Depending on the pathway, these drugs can upregulate the expression of PD-L1 by activating different signaling pathways (such as RAS/RAF, PI3K/AKT, JAK/STAT3) and release specific immunosuppressive cytokines, thus weakening the anti-tumor immune response. Therefore, chemotherapy combined with PD-1/PD-L1 inhibitors can enhance antitumor efficacy; as such, the combination of camrelizumab resulted in better tumor response.

The effects of neoadjuvant immunotherapy on several solid tumors have been evaluated. Several phase II studies by the American Society of Clinical Oncology (ASCO) and European Society for Medical Oncology (ESMO) have reported promising pCR rates. Sintilimab combined with the FLOT regimen (fluorouracil + oxaliplatin + docetaxel + leucovorin) (16) and the XELOX regimen (oxaliplatin + capecitabine) (17) for neoadjuvant treatment of gastric cancer resulted in postoperative pCR rates of 18.8% and 23.1%, and major pathologic response (MPR) rates of

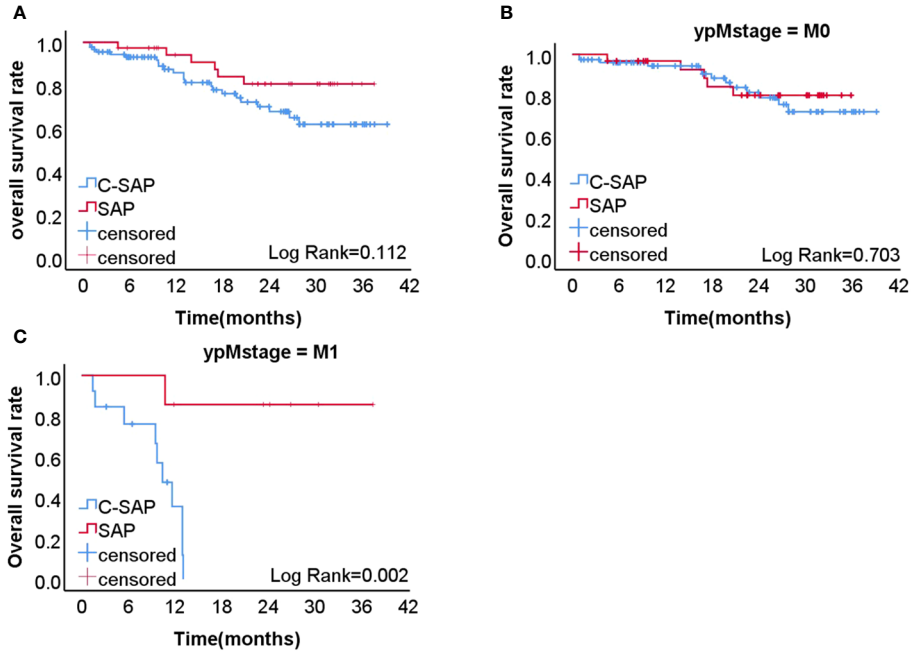

FIGURE 1
(A) the 2-years overall survival rate of C-SAP group (80.7%) was higher than that of SAP group (67.8%) (P=0.112). **(B)** In M0 patients, the 2-years overall survival rate of C-SAP group (79.9%) was similar to that of SAP group (71.9%) (P=0.703). **(C)** In M1 patients, the 2-years overall survival rate of C-SAP group (85.7%) was significantly better than that of SAP group (0%)(P =0.002,).

62.5% and 53.8%, respectively. The pCR rate for camrelizumab combined with FOLFOX was 8.8% (18). A phase II, single-center, two-arm study (ChiCTR2000030610) enrolled 61 patients who were randomly divided into neoadjuvant camrelizumab + FLOT and neoadjuvant FLOT groups; pCR rates were 11.5% (3/26) and 4.8% (1/21), respectively. Our results revealed that the pCR rate for neoadjuvant chemotherapy combined with camrelizumab was 18.3% and 4.4%, which was higher than that of the neoadjuvant chemotherapy group, with MPR rates of 39.5% and 25.6%, respectively. Recently, a randomized phase II clinical study, Neo-PLANET (NCT03631615) (14) reported the results of the treatment of 36 patients with locally advanced gastric or esophagogastric junction adenocarcinoma with camrelizumab combined with neoadjuvant chemoradiotherapy. Compared with our study, Neo-PLANET reported higher pCR (36.4%) and MPR (48.5%) rates.

In this study, the two-year OS rate of patients in the SAP group treated with two-drug chemotherapy (nab-paclitaxel + S-1) was 67.8%. The two-year OS rate was similar to that of patients receiving the three-drug FLOT regimen (68%) but higher than that of patients receiving the ECF/ECX regimen (59%), which was reported in the phase III clinical study FLOT-4 (19, 20). For patients with gastric cancer invading the serosa, although there is no large randomized

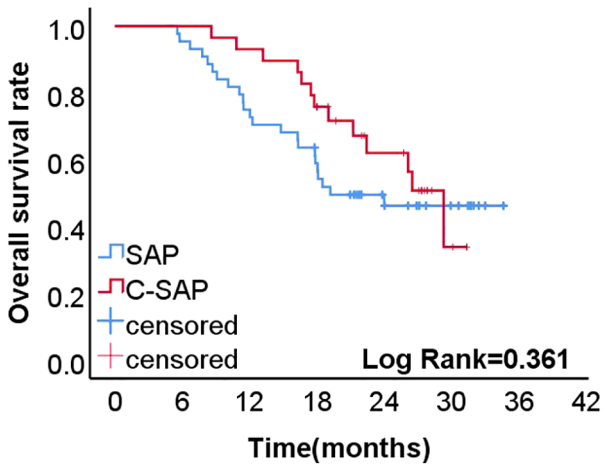

FIGURE 2
The 2-year recurrence free survival rate of C-SAP group (62.0%) was higher than that of SAP group (46.2%)(P=0.361).

TABLE 4 Recurrence within 2 years after surgery.

Baseline Variable	C-SAP group(n=30)	SAP group (n=43)	P* value
Recurrence within 2 years			0.294
Yes	13(43.3)	24(55.8)	
No	17(56.7)	19(44.2)	
Initial recurrence time (month)	18.9	13.1	0.004

controlled trial to further confirm long-term survival with the use of nab-paclitaxel plus S-1, the two-drug regimen is a better choice for Asian populations with poor general conditions.

In this study, the two-year OS and RFS rates were 80.7% and 62.0%, respectively, in patients treated with camrelizumab combined with preoperative chemotherapy (i.e., "C-SAP"). The phase II clinical study Neo-PLANET (14) reported two-year OS and RFS rates of 76.1% and 66.9%, respectively, after sequential gastrectomy after camrelizumab in combination with neoadjuvant chemoradiotherapy, which was similar to the results of our study. Compared with the Neo-PLANET study (14), radiotherapy was not used in the present study; therefore, the related side effects of radiotherapy were reduced. Because the sample sizes in this study and the Neo-PLANET study (14) were small, larger sample sizes are needed to accumulate more evidence. Although there was no statistical difference in the two-year OS and RFS rates between the two groups in this study, the 2-years OS and 2-years RFS of patients in the C-SAP group were higher than those in the SAP group, and the survival curve demonstrated advantages, which appeared to yield long-term survival benefits. We also found that the initial time to recurrence in the C-SAP group was 18.9 months, which was longer than that in the SAP group (13.1 months) (P = 0.004). The two-year recurrence rate was lower in the C-SAP group (44.3%) than in the SAP group (55.8%) (P = 0.076). The combination of camrelizumab appeared to prolong the time to recurrence time and reduce the recurrence rate. Studies, such as KEYNOTE-059 (3) and Check-mate 649, have confirmed the survival benefits of immunotherapy for nonresectable advanced metastatic gastric cancer. Combined with the results of this study and the conclusions of Check-mate 649 and other studies, gastric cancer surgery after a specific number of cycles of immunotherapy combined with preoperative chemotherapy in a specific M1 population may benefit patients. However, more evidence-based studies are needed to support this conclusion.

According to Japanese gastric cancer treatment guidelines (6th edition) (21), it is recommended to consider surgical resection after chemotherapy for oligometastasis (such as 16a2/b1 group omental lymph nodes and solitary liver metastasis). For other stage IV gastric cancer patients, if they have a good response to chemotherapy and the response is sustained, conversion surgery can be considered if R0 resection is achievable. We believe that combination immunotherapy can improve the pathological response rate, enabling some patients to achieve pathological complete response (pCR) or major pathological response (MPR), thereby creating the possibility of cure for oligometastatic gastric cancer patients.

The effectiveness of chemotherapy combined with immunotherapy has been demonstrated in advanced first-line treatment and perioperative period, giving us hope for its breakthrough in the conversion treatment of stage IV gastric cancer. The preliminary exploration was conducted in the CO-STAR study (22) by Chinese researchers, which included 56 cases of unresectable stage IV metastatic gastric cancer patients who received treatment with camrelizumab combined with apatinib and chemotherapy to evaluate the feasibility of surgery. The results showed a high response rate and conversion rate for the camrelizumab combined with apatinib and chemotherapy regimen: ORR 61.7%, R0 resection rate 96.6%. For stage IV gastric cancer, although immunotherapy combined with chemotherapy may have a higher conversion rate and can prolong patient survival, further research is needed to determine its feasibility and safety.

As we all know, dMMR/MSI-H patients are the population that benefits from immunotherapy. According to NCCN Gastric Cancer Guidelines, Version 2. 2022 (23), regardless of the HER-2 status, dMMR/MSI-H population should choose a treatment strategy mainly based on immunotherapy. This includes first-line treatment options, and second-line treatment options recommend immunotherapy, including pembrolizumab, nivolumab plus ipilimumab. Pembrolizumab has indications for MSI-H/dMMR solid tumors. The pooled analysis of KN-059, KN-061, and KN-062 studies targeting gastric cancer found that MSI-H gastric cancer patients can achieve good therapeutic effects regardless of treatment line. In the first-line KN-062 study (24) for gastric cancer, MSI-H patients treated with pembrolizumab showed significant superiority over chemotherapy in terms of ORR (57.7 vs. 36.8%), PFS (11.2 vs. 6.6 months), and 2-year survival rate (71 vs. 26%). Due to the low incidence and lack of large-scale high-level evidence in the field of dMMR/MSI-H gastric cancer, the Level I recommendation is temporarily unavailable.

Limitations

The present study had several limitations, the first of which were its single-center, retrospective design and inherent selection bias. Second, the follow-up period in this study was < 5 years. Whether it is necessary to screen the population according to PD-L1 expression and MSI status, how to screen the real benefit population, and how to adjust postoperative treatment according to neoadjuvant efficacy are unresolved issues in clinical practice that need to be confirmed by the results of an ongoing RCT.

Conclusion

This study is the first report long-term survival results for of sequential laparoscopic gastrectomy after camrelizumab in combination with nab-paclitaxel plus S-1 for the treatment of gastric cancer with serosal invasion Camrelizumab combined with

neoadjuvant chemotherapy improved the proportion of ypT0, ypN0, and pCR in patients, while prolonging the initial time to recurrence of patients in the C-SAP group, but did not increase immunotherapy/chemotherapy-related side effects and postoperative complications. Although it failed to significantly improve two-year OS and RFS rates after surgery, the survival curve exhibited advantages. This study provides clinical evidence supporting the use of PD-1 inhibitors in the neoadjuvant treatment of gastric cancer.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by ethics committee of Fujian Union Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

J-LL: Conceptualization, Methodology, Writing – original draft, Writing – review & editing. ML: Writing – review & editing. G-TL: Writing – review & editing. QZ: Writing – review & editing. JL: Writing – review & editing. CZ: Writing – review & editing. JX: Writing – review & editing. J-BW: Writing – review & editing. CH: Writing – review & editing. PL: Conceptualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1322152/full#supplementary-material

SUPPLEMENTARY FIGURE 2
(A–E) shows the effect of risk factors on the overall survival rate.

SUPPLEMENTARY FIGURE 3
No statistically significant difference in the overall survival time after recurrence between the two groups (P=0.097).

SUPPLEMENTARY FIGURE 4
CT for continued immunotherapy after patient recurrence in C-SAP group.

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Achilles' Heel of currently approved immune checkpoint inhibitors: immune related adverse events

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Immunotherapy has revolutionized the cancer treatment landscape by opening up novel avenues for intervention. As the use of immune checkpoint inhibitors (ICIs) has exponentially increased, so have immune-related adverse events (irAEs). The mechanism of irAEs may involve the direct damage caused by monoclonal antibodies and a sequence of immune responses triggered by T cell activation. Common side effects include dermatologic toxicity, endocrine toxicity, gastrointestinal toxicity, and hepatic toxicity. While relatively rare, neurotoxicity, cardiotoxicity, and pulmonary toxicity can be fatal. These toxicities pose a clinical dilemma regarding treatment discontinuation since they can result in severe complications and necessitate frequent hospitalization. Vigilant monitoring of irAEs is vital in clinical practice, and the principal therapeutic strategy entails the administration of oral or intravenous glucocorticoids (GSCs). It may be necessary to temporarily or permanently discontinue the use of ICIs in severe cases. Given that irAEs can impact multiple organs and require diverse treatment approaches, the involvement of a multidisciplinary team of experts is imperative. This review aims to comprehensively examine the pathogenesis, clinical manifestations, incidence, and treatment options for various irAEs.

KEYWORDS

PD-1 inhibitors, PD-L1 inhibitors, CTLA-4 inhibitors, immune-related adverse events, ICIs, ICIS

1 Introduction

Immunotherapy has emerged as a promising avenue for new cancer treatments by boosting the patient's immune system (1). Immune checkpoint inhibitors (ICIs) such as those targeting programmed cell death protein 1 (PD-1) or its primary ligand (PD-L1), as well as the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) signaling, have demonstrated encouraging therapeutic effects against various types of solid tumors.

Ipilimumab was the first CTLA-4 inhibitor approved by the US Food and Drug Administration (2). It was followed by PD-1 inhibitors (e.g., pembrolizumab, nivolumab, and cemiplimab) and PD-L1 inhibitors (e.g., atezolizumab, durvalumab, and avelumab) have also been approved for a variety of indications. The National Medical Products Administration has approved an expanded range of drugs in this category. Currently, there are 10 PD-1 inhibitors (e.g., pembrolizumab, nivolumab, toripalimab, sintilimab, camrelizumab, tislelizumab, penpulimab, zimberelimab, serplulimab, and adebrelimab). Additionally, there are 4 PD-L1 inhibitors (e.g., atezolizumab, durvalumab, envafolimab, and sugemalimab). Furthermore, there were CTLA-4 inhibitors (ipilimumab and tremelimumab) and a combination inhibitor of PD-1 and CTLA-4 (cadonilimab).

Whether solid or non-solid tumors, ICIs play a vital role in cancer treatment, due to their well-established clinical benefits. The utilization of these agents is expected to increase significantly in the upcoming years (3). ICIs work by interacting with immune cells through signaling pathways, impairing their ability to recognize and eliminate cancer cells (4). Although effective against cancer, this approach can also result in immune-related adverse events (irAEs), mainly affecting the skin, endocrine glands, liver, lungs, gut, and potentially other organs. This susceptibility represents a significant drawback of this particular therapeutic agent, known as the Achilles' Heel of immunotherapy (5). Understanding the underlying mechanisms is essential for prompt diagnosis and, more importantly, appropriate therapeutic management. Therefore, this review aims to present the pathogenesis, clinical manifestations, incidence, and treatment strategies of various irAEs through 49 clinical trials from ICIs encompassing solid and non-solid tumors, retrospective analyses, and case reports. Hopefully, this will help provide a deeper understanding of irAEs.

2 Mechanism

The emergence and intensity of irAEs could potentially be influenced by various immune mechanisms. Existing evidence suggests that during the later stages of the immune response (6), ICIs can facilitate the infiltration of T-cells into peripheral tissues, which in turn, might explain the occurrence of irAEs in PD-1/PD-L1 blockade (7). Furthermore, ICIs have been shown to reduce the survival and inhibitory function of regulatory T (Treg) cells while concurrently augmenting cytokine production (8).

Several proposed mechanisms have been put forth to elucidate irAEs (Figure 1).

One such mechanism revolves around the direct effect of monoclonal antibodies. It has been postulated that some irAEs may arise due to the complement-mediated direct injury caused by

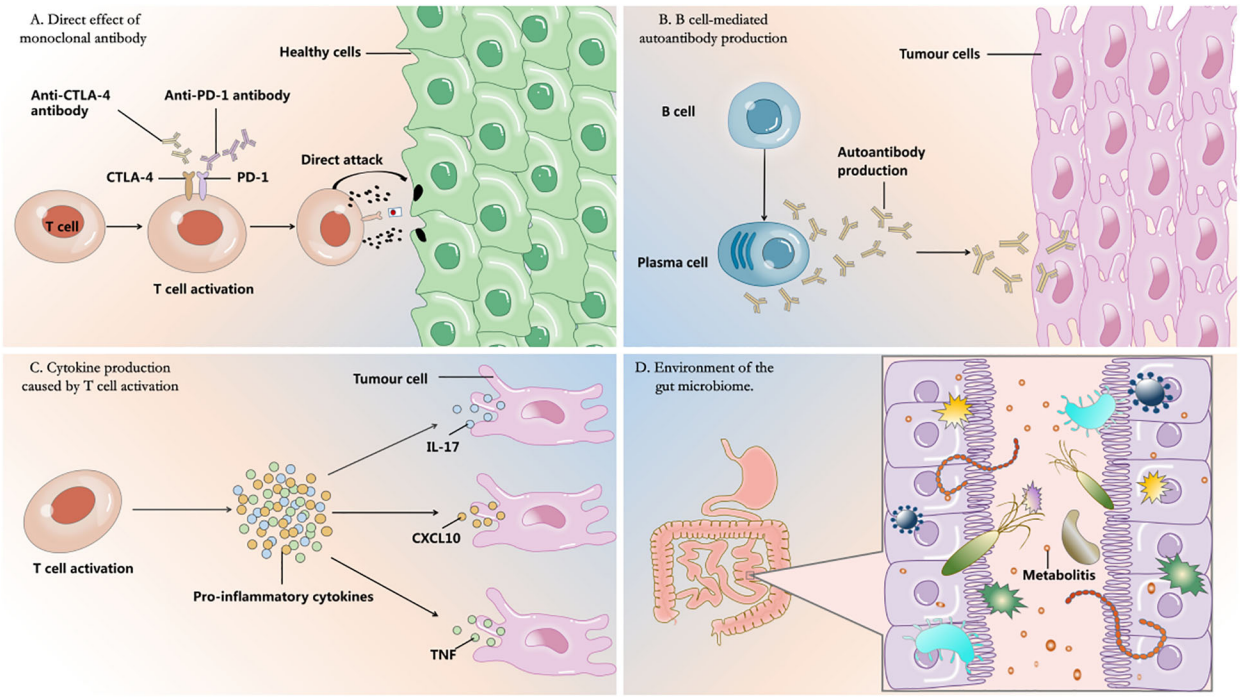

FIGURE 1
Four proposed mechanisms for the development of irAEs.

monoclonal antibody therapies (9). PD-L1, a molecule mainly expressed in the endothelium of the myocardium, plays a pivotal role in regulating immune-mediated cardiac injuries (10). In a patient who succumbed to myocarditis following combination therapy of ICIs, there were observations of a tenfold increase in PD-L1 expression in the cardiac tissue as compared to unaffected muscle tissue (11).

Another crucial aspect involving irAEs is the heightened production of autoantibodies by B cells following immunotherapy. It is possible that individuals who develop grade ≥3 irAEs, might have an increased presence of self-reactive B cells in the bloodstream after undergoing immunotherapy (12). Through immunotherapy-induced activation, T cells foster greater interactions with B cells, subsequently leading to the production of autoantibodies. For instance, the interactions between follicular T cells and B cells in germinal centers play a vital role in the development of humoral immunity, and any disruptions in these interactions have been linked to autoimmune diseases (13). Research has demonstrated that patients with antithyroid antibodies experience more severe thyroid dysfunction when subjected to PD-1 therapy (14).

Thirdly, the occurrence of irAEs can be elucidated by the fact that the activation of T cells stimulates the production of cytokines. Research has demonstrated that the depletion of Treg cells, which play a crucial role in maintaining peripheral tolerance, is observed during the administration of ICIs and contributes to the manifestation of irAEs (15). This depletion is hypothesized to transpire through the differentiation process of T helper 17 (Th17) cells into Treg cells (16–18), subsequently leading to an imbalance between Treg cells and Th17 cells which has been implicated in the development of irAEs (19). Th17 cells are renowned for their secretion of pro-inflammatory cytokines such as IL-17A, IL-21, and IL-22, which have been implicated in the pathogenesis of autoimmune diseases like rheumatoid arthritis and psoriatic arthritis (20). However, the influence of other pro-inflammatory cytokines on the manifestation of irAEs has not been comprehensively explored. Nonetheless, the analysis of serum cytokine levels has demonstrated a significant elevation in various levels of several pro-inflammatory cytokines among irAE patients, including IL-1Ra, CXCL10, and TNF-α, as well as soluble IL-2 receptors (21). A documented case report has proposed that the use of anti-TNF agents effectively manages irAEs in patients undergoing ICI therapy, suggesting a potential role of TNF in the development of irAEs (22).

Finally, recent research has revealed that the gut microbiota, specifically *Bifidobacterium*, *Bacteroides fragilis*, and *Akkermansia muciniphia*, play a vital role in enhancing the effectiveness of ICIs and impacting their toxicity (23–25). This is accomplished by modifying metabolites derived from nutrients in the host, maintaining the integrity of the gut mucosa barrier, and participating in immune-modulation (26). Various techniques for manipulating A. muciniphila in the gut microbiota have been described, such as fecal microbiota transplantation (FMT), probiotics, prebiotics, and dietary interventions((27). For example, in a study conducted by Wang Y. et al (28), successful treatment of immune-related colitis was achieved by utilizing FMT to restore the gut microbiota of oncology patients, suggesting that reshaping the gut microbiota could alleviate immune-related colitis. Additionally, promising results have emerged from recent clinical trials highlighting the significance of *Akkermansia* in immunotherapy for non-small cell lung cancer (NSCLC) (29). Notably, individuals with higher levels of *Bacteroides fragilis* are found to have a reduced risk of colitis, while those with an abundance of *Firmicutes* face an increased risk (30, 31).

Here, in Figure 1, we depict the immune mechanisms driving irAEs including: A. direct effect of monoclonal antibody; B. B cell-mediated autoantibody production; C. cytokine production caused by T cell activation; D. environment of the gut microbiome.

3 Immune-related adverse events

ICIs primarily target the immune system for combatting cancer. However, this mechanism unfortunately results in autoimmune-like toxicities, that are exclusive to ICIs and not observed with other targeted agents or cytotoxic chemotherapy (32). These toxicities have the potential to affect various tissues or organs such as the skin, endocrine system, liver, gastrointestinal tract, lungs, and rheumatoid/skeletal muscle. Although less common, the nervous system, blood, kidneys, heart, and eyes may also be affected. In rare instances, transfusion reactions may occur. While the majority of irAEs are mild and reversible, they can arise at any point, except for long-term endocrine irAEs (33–35). Severe irAEs are infrequent but can have significant consequences, especially when they impact the pericardium, lungs, and nervous system (36–38). A systematic review of 50 trials encompassing 5071 patients discovered that the median rate of grade 3/4 irAEs was 21% (39). The occurrence of irAEs caused by ICIs in 49 clinical trials involving solid or non-solid tumors is depicted in Table 1.

3.1 Immune-related dermatologic adverse events

Dermatologic irAEs are commonly observed in patients, impacting up to 50% of individuals. Most cases of dermatologic irAEs are mild reactions. The frequently reported dermatologic irAEs consist of erythema, rash, pruritus, reactive cutaneous capillary endothelial proliferation (RCCEP), and vitiligo (86). Numerous studies on camrelizumab have consistently identified RCCEP as an adverse event, with an incidence rate as high as 80% even when used as monotherapy (59, 61, 62). Nevertheless, severe cases (grade 3-5) of RCCEP are infrequent, occurring in less than 2% of patients. The rash can manifest with various clinical characteristics such as maculopapular or erythematous lesions. Data have indicated that the occurrence of rash in patients receiving nivolumab and pembrolizumab ranges from 34% to 40% (87). However, the risk of rash significantly increases when ipilimumab is combined with these drugs, and the overall prevalence of vitiligo is 8% (88–90). According to findings from CheckMate 914 (43), the incidence rate of rash in the treatment of Renal cell carcinoma with nivolumab plus ipilimumab was reported

TABLE 1 The incidence of immune-related adverse events in clinical trials with immune checkpoint inhibitors.

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
Nivolumab (n=337)	CheckMate 078	III	NSCLC	**Total:64%** Rash(12%), pruritus(8%), ALT elevation (9%), AST elevation(9%), thyroid disorder(9%), hypothyroidism(4%), GGT elevation(4%)	**Total:10%** Rash(1%), pneumonitis(1%), interstitial lungdisease(1%), ALT elevation(<1%), AST elevation (<1%), GGT elevation(<1%)	(40)
Nivolumab (n=418)	CheckMate 017&057	III	NSCLC	**Total:68%** Fatigue(17%), nausea(11%), decreased appetite(11%), asthenia(11%), diarrhea (9%), rash(8%), pruritus(7%), hypothyroidism(6%), arthralgia(6%)	**Total:11%** Fatigue(1%), pneumonitis(1%), diarrhea(1%), rash(<1%), nausea(<1%)	(41)
Nivolumab plus ipilimumab (n=300)	CheckMate 743	III	Malignant pleural mesothelioma	**Total:80%** Diarrhea(21%), pruritus(16%), rash (14%), fatigue(14%), hypothyroidism (11%), nausea(10%), decreased appetite(10%)	**Total:30%** Increased lipase(4%), increased lipase(4%), diarrhea(3%), increased amylase(2%), decreased appetite(1%), fatigue(1%), neutropenia(1%), thrombocytopenia(1%)	(42)
Nivolumab plus ipilimumab (n=404)	CheckMate 914	III	Renal cell carcinoma	**Total:59%** Pruritus(31%), fatigue(30%), diarrhea (24%), rash(21%), headache(17%), nausea(17%), hyperthyroidism(16%), arthralgia(16%), hypothyroidism(16%), decreased appetite(13%), cough(12%), asthenia(11%)	Diarrhea(4%), fatigue(1%), rash(1%)	(43)
Nivolumab (n=330)	ATTRACTION-2	III	Gastric or gastro-esophageal junction cancer	**Total:43%** Pruritus(9%), diarrhea(7%), rash(6%), fatigue(5%), decreased appetite(5%), nausea(4%), malaise(4%)	**Total:10%** Diarrhea(1%), fatigue(1%), decreased appetite(1%), AST increased(1%)	(44)
Nivolumab plus capecitabine/ leucovorin/ fluorouracil and oxaliplatin (n=782)	CheckMate 649	III	Gastric, gastro esophageal junction, and esophageal adenocarcinoma	**Total:94%** Nausea(41%), diarrhea(32%), peripheral neuropathy(28%), fatigue(26%), anaemia(26%), vomiting(25%), neutropenia(24%), decreased appetite (20%), thrombocytopenia(20%), PLT decreased(20%)	**Total:60%** Neutropenia(15%), NEU decreased(11%), anaemia(6%), lipase increased(6%), diarrhea (4%), peripheral neuropathy(4%), peripheral neuropathy(4%), fatigue (4%),nausea(3%)	(45)
Pembrolizumab (n=154)	KEYNOTE-024	III	NSCLC	**Total:77%** Diarrhea(16%), fatigue(14%), pyrexia (12%), pruritus(12%), rash(10%), nausea(10%), anorexia/decreased appetite(10%)	**Total:31%** Diarrhea(4%), fatigue(2%), rash (1%), anaemia(1%)	(46)
Pembrolizumab (n=637)	KEYNOTE-042	III	NSCLC	**Total:63%** Hypothyroidism(11%), fatigue(8%), pruritus(7%), rash(7%), ALT increased (7%), pneumonitis(7%), AST increased (6%), decreased appetite(6%), hyperthyroidism(6%), anaemia(6%)	**Total:18%** Pneumonitis(3%), ALT increased (1%), AST increased(1%), decreased appetite(1%), anaemia (1%), diarrhea(1%)	(47)
Pembrolizumab (n=314)	KEYNOTE-181	III	Esophageal cancer	**Total:64%** Fatigue(12%), hypothyroidism(11%), decreased appetite(9%), nausea(7%), asthenia(7%), diarrhea(5%)	**Total:18%** Decreased appetite(1%), diarrhea (1%), asthenia(1%), fatigue(1%), anemia(1%)	(48)
Pembrolizumab (n=110)	KEYNOTE-427	II	Renal cell carcinoma	**Total:80%** Pruritus(27%), fatigue(25%), diarrhea (19%), rash(15%), arthralgia(13%), hypothyroidism(10%)	Diarrhea(4%), AST increased(2%), asthenia(2%)	(49)
Pembrolizumab (n=300)	KEYNOTE-048	III	Head and neck squamous cell carcinoma	Fatigue(28%), anaemia(21%), constipation(20%), hypothyroidism (18%), nausea(16%), diarrhea(15%), weight decreased(15%), decreased appetite(15%)	Anaemia(5%), fatigue(3%), weight decreased(2%), hypokalaemia(2%), diarrhea(1%), asthenia(1%), mucosal inflammation(1%), decreased appetite(1%), rash(1%)	(50)

(Continued)

TABLE 1 Continued

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
Pembrolizumab plus platinum and 5-fluorouracil (n=276)	KEYNOTE-048	III	Head and neck squamous cell carcinoma	Anaemia(58%), nausea(51%), constipation(37%), neutropenia(34%), fatigue(34%), vomiting(33%), mucosal inflammation(31%), decreased appetite (29%), Thrombocytopenia(29%), diarrhea(28%)	Anaemia(25%), neutropenia(18%), NEU decreased(11%), mucosal inflammation(10%), thrombocytopenia(9%), Stomatitis (8%), fatigue(7%), hypokalaemia (7%), nausea(6%)	(50)
Cemiplimab (n=193)	EMPOWER-CSCC-1	II	Cutaneous squamous cell carcinoma	**Total:99%** Fatigue(35%), diarrhea(27%), nausea (24%), pruritus(21%), arthralgia(18%), cough(17%), rash(17%), constipation (15%), vomiting(13%)	**Total:49%** Anemia(4%), fatigue(3%), diarrhea (1%), constipation(1%), vomiting (1%), arthralgia(1%), rash(1%), rash maculo-papular(1%)	(51)
Cemiplimab (n=355)	EMPOWER-Lung	III	NSCLC	**Total:57%** ALT increased (6%), AST increased (6%), decreased appetite(5%), anaemia (5%), rash(5%), diarrhea(4%), nausea (4%), arthralgia(4%), fatigue(4%)	**Total:14%** ALT increased (1%), AST increased(1%), anaemia(1%), rash (1%), fatigue(1%), increased blood alkaline phosphatase(1%), increased weight(1%), dyspnea (1%), neutropenia(1%)	(52)
Toripalimab plus gemcitabine-cisplatin(n=146)	JUPITER-02	III	Nasopharyngeal carcinoma	**Total:100%** Leukopenia(91%), anemia(88%), neutropenia(86%), nausea(69%), vomiting(67%), thrombocytopenia (64%), decreased appetite(53%), constipation(39%), AST increased(38%), ALT increased(36%)	**Total:89%** Leukopenia(62%), neutropenia (58%), anemia(47%), thrombocytopenia(33%), pneumonia(10%), natremia(9%), hyponatremia(9%), lymphopenia (9%), hypokalemia(7%)	(53)
Toripalimab plus paclitaxel and cisplatin(n=257)	JUPITER-06	III	Esophageal squamous cell carcinoma	**Total:99%** Anemia(78%), leukopenia(68%), neutropenia(67%), nausea(43%), neuropathy peripheral(40%), vomiting (40%), decreased appetite(39%), alopecia(35%), weight decreased(29%), hypoproteinemia(25%)	**Total:73%** Neutropenia(42%), leukopenia (20%), anemia(11%), pneumonia (6%), fatigue(4%), hyponatremia (4%), weight decreased(3%), rash (3%), hypokalemia(3%)	(54)
Sintilimab plus pemetrexed and platinum (n=266)	ORIENT-11	III	NSCLC	**Total:43%** Hypothyroidism(7%), rash(6%), AST increased(6%), ALT increased(6%), increased thyroid stimulating hormone (5%), hyperthyroidsm(5%), diarrhea (5%), pneumonitis(3%), decreased thyroid stimulating hormone(3%), increased amylase(3%)	**Total:6%** Pneumonitis(1%), increased amylase(1%)	(55)
Sintilimab plus platinum and gemcitabine (n=179)	ORIENT-12	III	NSCLC	Anemia(93%), WBC decreased(89%), NEU decreased(83%), platelet count decreased(73%), nausea(40%), asthenia (34%), vomiting(32%), decreased appetite(32%), constipation(31%)	NEU decreased(49%), PLT decreased(45%), WBC decreased (36%), anemia(34%), infectious pneumonitis(14%), hyponatremia (6%), asthenia(2%), vomiting(2%), rash(2%), hemoptysis(2%)	(56)
Sintilimab plus cisplatin and paclitaxel (n=327)	ORIENT-15	III	Esophageal squamous cell carcinoma	**Total:98%** Anemia(75%), WBC decreased(64%), NEU decreased(62%), nausea(47%), vomiting(34%), asthenia(33%), decreased appetite(28%), hypoaesthesia (23%), PLT decreased(21%)	**Total:60%** Asthenia(45%), NEU decreased (30%), WBC decreased(17%), anemia(13%, decrease in lymphocyte count(5%), PLT decreased(3%), decrease in lymphocyte count(3%), blood pressure increased(3%), pneumonia(3%)	(57)
Sintilimab plus bevacizumab biosimilar (n=380)	ORIENT-32	III	Hepatocellular carcinoma	**Total:99%** Proteinuria(42%), PLT decreased(41%), increased AST(36%), hypertension (32%), increased blood bilirubin(29%), increased ALT(26%), WBC decreased	**Total:55%** Hypertension(14%), PLT decreased(8%), proteinuria(5%), increased blood bilirubin(5%), increased γ-glutamyltransferase	(58)

(Continued)

TABLE 1 Continued

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
				(20%), pyrexia(17%), hypoalbuminaemia(17%), asthenia(16%)	(5%), elevated blood pressure(3%), abnormal liver function(3%), increased conjugated bilirubin (3%), NEU decreased(2%)	
Camrelizumab plus carboplatin and pemetrexed (n=205)	CameL	III	NSCLC	RECCP(78%), NEU decreased(71%), WBC decreased(71%), anaemia(66%), anaemia(66%), PLT decreased(46%), AST increased(45%), ALT increased (43%), nausea(36%), asthenia(31%), decreased appetite(30%)	NEU decreased(38%), WBC decreased(20%), anaemia(19%), PLT decreased(17%), ALT increased(5%), lymphocyte count decreased(4%), bone marrow toxicity(4%), asthenia(3%), GGT increased(3%)	(59)
Camrelizumab plus carboplatin and paclitaxel (n=193)	CameL-sq	III	Squamous NSCLC	WBC decreased(79%), NEU decreased (78%), RCCEP(69%), anemia(63%), PLT decreased(41%), asthenia(33%), hypoaesthesia(30%), decreased appetite (28%), nausea(24%)	NEU decreased(55%), WBC decreased(30%), anemia(10%), PLT decreased(7%), lymphocyte count decreased(4%), pneumonia (4%), RCCEP(2%), asthenia(2%), ALT increased(2%)	(60)
Camrelizumab plus gemcitabine and cisplatin(n=134)	CAPTAIN-1st	III	Nasopharyngeal carcinoma	WBC decreased(96%), anaemia(95%), NEU decreased(95%), PLT decreased (80%), nausea(70%), decreased appetite (64%), RECCP(58%), vomiting(56%), asthenia(49%), hypothyroidism(46%), constipation(45%)	WBC decreased(66%), NEU decreased(64%), PLT decreased (40%), anaemia(39%), lymphocyte count decreased(19%), hyponatraemia(10%), hypokalaemia(7%), pneumonia (6%), nausea(4%), AST increased (3%), hypophosphataemia(3%)	(61)
Camrelizumab (n=228)	ESCORT	III	Esophageal squamous cell carcinoma	**Total:94%** RECCP(80%), hypothyroidism(17%), anaemia(11%), asthenia(10%), WBC decreased(7%), diarrhea(6%), decreased appetite(5%), NEU decreased (4%)	**Total:19%** Anaemia(3%), diarrhea(1%), lymphocyte count decreased(1%), hyponatraemia(1%), death(1%)	(62)
Tislelizumab plus platinum and pemetrexed (n= 223)	RATIONALE 304	III	Nonsquamous NSCLC	Anemia(86%), lukopenia(82%), neutropenia(82%), thrombocytopenia (70%), ALT increased (48%), nausea (43%), AST increased(43%), fatigue (38%), decreased appetite(34%), vomiting(27%), musculoskeletal pain(25%)	Neutropenia(44%), leukopenia (22%), thrombocytopenia(19%), anemia(15%), increased ALT(4%), increased AST(2%), fatigue(1%), decreased appetite(1%)	(63)
Tislelizumab (n=256)	RATIONALE 302	III	Esophageal Squamous CellCarcinoma	AST increased(11%), anemia(11%), hypothyroidism(10%), fatigue(7%), decreased appetite(6%), diarrhea(5%), asthenia(5%), malaise(4%), weight decreased(3%), nausea(3%), leukopenia(3%)	/	(64)
Tislelizumab plus paclitaxel and carboplatin (n=120)	RATIONALE 307	III	Squamous NSCLC	Anemia(88%), alopecia(64%), NEU decreased(63%), WBC decreased(53%), leukopenia(48%), decreased appetite (43%), neutropenia(43%), ALT increased(42%), AST increased(36%), PLT decreased (34%)	NEU decreased(52%), neutropenia (33%), WBC decreased(23%), leukopenia(16%), anemia(8%), thrombocytopenia(6%), rash(3%), pain in extremity(3%), ALT increased(2%)	(65)
Tislelizumab plus nab-paclitaxel and carboplatin (n=118)	RATIONALE 307	III	Squamous NSCLC	Anemia(93%), alopecia(69%), NEU decreased(61%), WBC decreased(58%), leukopenia(56%), decreased appetite (44%), PLT decreased (44%), neutropenia(42%), ALT increased(35%), AST increased(34%)	NEU decreased(46%), WBC decreased(27%), neutropenia (27%), leukopenia(25%), anemia (23%), PLT decreased (14%), thrombocytopenia(13%), AST increased(2%), rash(2%), decreased appetite(1%)	(65)
Penpulimab (n=85)	AK105-201	II	Classical Hodgkin lymphoma	Hypothyroidism(35%), upper respiratory tract infection(28%), fever (27%), ALT increased(26%), hypertriglyceridemia(21%), reduced	Skin rash(4%), hyperlipidemia (4%), NEU decreased(2%), weight gain(1%), fever(1%), hypertrigly	(66)

(Continued)

TABLE 1 Continued

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
				leucocyte count(20%), rash(18%), AST increased(16%), anemia(16%), elevated TSH(15%)	(1%), reduced leucocyte count (1%), anemia(1%)	
Penpulimab plus carboplatin and paclitaxel (n=175)	AK105-302	III	Squamous NSCLC	/	**Total:63.6%**	(67)
Zimberelimab (n=85)	GLS-010-cHL	II	Classical Hodgkin lymphoma	Pyrexia(32%), hypothyroidism(21%), NEU decreased(20%), ALT increased (20%), WBC decreased(19%), weight increased(13%), blood bilirubin increased(12%), upper respiratory tract infection(11%), pruritus(11%), anemia (11%), AST increased(11%), hepatic function abnormal(11%)	Hepatic function abnormal(6%), hyperuricemia(5%), weight increased(4%), NEU decreased (4%), upper respiratory tract infection(2%), hypertriglyceridemia(2%), pyrexia (1%), lymphocyte count decreased (1%), hypokalemia(1%)	(68)
Serplulimab plus cisplatin and 5-fuorouracil (n=382)	Serplulimab-ESCC	III	Esophageal squamous cell carcinoma	**Total:99%** Anemia(76%), nausea(64%), WBC decreased(58%), NEU decreased(56%), PLT decreased(43%), vomiting(43%), appetite decreased(42%), asthenia(30%), blood creatinine increased(16%)	**Total:64%** NEU decreased(19%), anemia (18%), WBC decreased(11%), hyponatremia(5%), hypokalemia (4%), nausea(3%), vomiting(3%), appetite decreased(2%), AST increased(2%)	(69)
Serplulimab plus carboplatin and etoposide (n=389)	ASTRUM-005	III	Extensive-stage SCLC	**Total:70%** Anemia(22%), WBC decreased(20%), PLT decreased(15%), hypothyroidism (15%), nausea(13%), ALT increased (12%), hyperthyroidism(11%), AST increased (10%)	**Total:33%** NEU decreased(14%), WBC decreased(8%), PLT decreased (6%), anemia(5%), neutropenia (4%), leukopenia(3%), decreased lymphocyte count(2%), hyperglycemia(2%)	(70)
Adebrelimab (n=230)	CAPSTONE-1	III	Extensive-stage SCLC	**Total:100%** NEU decreased(95%), WBC decreased (94%), anaemia(85%), PLT decreased (83%), alopecia(44%), ALT increased (41%), nausea(40%), AST increased (35%), decreased appetite(30%), vomiting(26%)	**Total:86%** NEU decreased(76%), WBC decreased(46%), PLT decreased, anaemia(28%), ALT increased (2%), γ-glutamyltransferase increased(2%), decreased appetite (2%), hyponatraemia(2%), hypokalaemia(2%)	(71)
Atezolizumab (n=286)	IMpower110	III	NSCLC	**Total:90%** Anemia(15%), decreased appetite(15%), nausea(14%), asthenia(13%), fatigue (13%), constipation(12%), hyponatremia(6%), pneumonia(5%), hyperkalemia(4%)	**Total:34%** Anemia(2%), hyponatremia(2%), pneumonia(2%), hyperkalemia (2%), decreased appetite(1%), asthenia(1%), fatigue(1%), constipation(1%), neutropenia(1%)	(72)
Atezolizumab plus nab-paclitaxel (n=453)	IMpassion130	III	Triple-negative breast cancer	**Total:93%** Alopecia(57%), fatigue(47%), nausea (46%), diarrhea(32%), anaemia(28%), constipation(26%), cough(25%), headache(24%), neuropathy peripheral (22%), neutropenia(21%)	**Total:50%** Neutropenia(8%), neuropathy peripheral(6%), NEU decreased (5%), fatigue(4%), anaemia(3%), peripheral sensory neuropathy (2%), AST increased(2%), hypokalaemia(2%), pneumonia (2%), diarrhea(2%)	(73)
Atezolizumab plus carboplatin and etoposide (n=198)	IMpower133	III	Extensive-stage SCLC	**Total:95%** Rash(20%), hypothyroidism(13%), hepatitis(8%), Infusion-related reactions (6%), hyperthyroidism(6%), pneumonitis(3%), colitis(2%)	**Total:59%**	(74)
Atezolizumab plus	IMbrave150	III	Hepatocellular carcinoma	Proteinuria(29%), hypertension(28%), AST increase(16%), fatigue(16%),	Hypertension(12%), AST increase (5%), proteinuria(4%), PLT	(75)

(Continued)

TABLE 1 Continued

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
bevacizumab (n=329)				pruritus(14%), ALT increase(12%), decreased appetite(12%), diarrhea(11%), infusion-related reaction(11%), PLT decreased(10%), hypothyroidism(10%), rash(10%)	decreased(2%), fatigue(2%), ALT increase(2%), infusion-related reaction(2%), pneumonia(1%), gastrointestinal hemorrhage(1%), liver injury(1%), decreased appetite(1%), diarrhea(1%)	
Durvalumab plus tremelimumab and platinum–etoposide (n=266)	CASPIAN	III	Extensive-stage SCLC	**Total:89%** Neutropenia(43%), anaemia(38%), nausea(32%), alopecia(30%), decreased appetite(21%), constipation(20%), thrombocytopenia(20%), fatigue(20%), asthenia(14%), vomiting(14%)	**Total:64%** Neutropenia(32%), anaemia(13%), thrombocytopenia(9%), leucopenia (6%), febrile neutropenia(6%), hyponatraemia(5%), pneumonia (5%), diarrhea(3%)	(76)
Durvalumab plus platinum–etoposide (n=265)	CASPIAN	III	Extensive-stage SCLC	**Total:98%** Neutropenia(42%), anaemia(38%), nausea(34%), alopecia(32%), decreased appetite(18%), fatigue(18%), constipation(17%), asthenia(16%), thrombocytopenia(15%), vomiting (15%), leucopenia(15%)	**Total:65%** Neutropenia(24%), anaemia(9%), thrombocytopenia(6%), leucopenia (6%), NEU decreased(6%), febrile neutropenia(5%), hyponatraemia (4%), hypertension(3%), lipase increased(3%)	(76)
Durvalumab (n=475)	PACIFIC	III	Stage III SCLC	**Total:97%** Cough(35%), pneumonitis or radiation pneumonitis(34%), fatigue(24%), dyspnea(22%), diarrhea(18%), pyrexia (15%), decreased appetite(14%), nausea (14%), pneumonia(13%), arthralgia(12%)	**Total:30%** Pneumonia(4%), pneumonitis or radiation pneumonitis(3%), anemia(3%), dyspnea(1%), diarrhea(1%), asthenia(1%), musculoskeletal pain(1%)	(77)
Durvalumab plus tremelimuma (n=388)	HIMALAYA	III	Hepatocellular Carcinoma	Rash(32%), diarrhea(27%), fatigue (26%), pruritus(23%), musculoskeletal pain(22%), abdominal pain(20%), decreased appetite(17%), hypothyroidism(14%), pyrexia(13%), nausea(12%), insomnia (10%)	Diarrhea(6%), fatigue(3.9%), rash (2.8%), musculoskeletal pain (2.6%), abdominal pain(1.8%)	(78)
Durvalumab plus tremelimuma +chemotherapy (n=338)	POSEIDON	III	NSCLC	**Total:92.7%** Anemia(43.6%), nausea(37.6%), neutropenia(29.1%), decreased appetite (20.9%), fatigue(19.7%), thrombocytopenia(19.7%), rash(15.8%), vomiting(14.2%), diarrhea(13.9%), leukopenia(12.7%)	**Total:51.8%** Anemia(17.3%), neutropenia (16.1%), neutrophil count decreased(7.3%), thrombocytopenia(5.5%), leukopenia(2.7%)	(79)
Avelumab (n=344)	JAVELIN Bladder 100	III	Urothelial carcinoma	**Total:98%** Fatigue(18%), pruritus(17%), urinary tract infection(17%), diarrhea(17%), arthralgia(16%), asthenia(16%), constipation(16%), back pain(16%), nausea(16%), pyrexia(15%)	**Total:47%** Urinary tract infection(4%), anemia(4%), fatigue(2%), hematuria(2%), diarrhea(1%), arthralgia(1%), constipation(1%), back pain(1%), vomiting(1%), infusion-related reaction(1%)	(80)
Avelumab plus axitinib(n=434)	JAVELIN Renal 101	III	Renal cell carcinoma	**Total:100%** Diarrhea(62%), hypertension(50%), fatigue(41%), nausea(34%), PPES (33%), dysphonia(31%), decreased appetite(26%), hypothyroidism(25%)	**Total:71%** Hypertension(26%), diarrhea(7%), fatigue(3%), PPES (6%),ALT increased (6%), AST increased (4%), fatigue(3%)	(81)
Envafolimab (n=103)	Envafolimab	II	dMMR/MSI-H solid tumors	WBC decreased(17%), asthenia(17%), rash(16%), hypothyroidism(16%), hyperthyroidism(12%), NEU decreased (12%), anemia(12%)	Anemia(5%), NEU decreased(1%), rash(1%),	(82)
Sugemalimab plus carboplatin and paclitaxel (n=320)	GEMSTONE-302	III	NSCLC	**Total:100%** Anaemia(73%), NEU decreased(58%), WBC decreased(56%), PLT decreased (33%), AST increased(33%), ALT increased(32%), appetite decreased	**Total:64%** NEU decreased(33%), WBC decreased(14%), anaemia(13%), PLT decreased(10%), neutropenia (4%), γ-glutamyltransferase	(83)

(Continued)

TABLE 1 Continued

Treatment (patients)	Clincal trials	Phase	Tumor Types	IrAEs (any grade)	IrAEs (grade 3-5)	Reference
				(23%), nausea(22%), alopecia(19%), asthenia(16%)	increased(2%), leukopenia(2%), hepatic function atypical(2%), pneumonia(2%)	
Sugemalimab (n=255)	GEMSTONE-301	III	NSCLC	**Total:76%** Pneumonitis(19%), hypothyroidism (17%), hyperthyroidism(15%), ALT increased(13%), AST increased(12%), rash(7%), pruritus(6%), anaemia(5%), GGT increased(5%), hypertriglyceridaemia(4%), blood cholesterol increased(4%)	**Total:10%** Pneumonitis(3%), pneumonia (2%), hypothyroidism(1%), rash (1%), hypertriglyceridaemia(1%)	(84)
Cadonilimab (n=111)	AK104-201	II	Cervical cancer	**Total:96.4%** Anaemia(7.2%), decreased appetite (2.7%)	**Total:28.8%**	(85)

NSCLC, non-small cell lung cancer; SCLC, small cell lung cancer; dMMR, defective mismatch repair; MSI-H, microsatellite instability high; ALT, alanine aminotransferase; AST, aspartate aminotransferase; WBC, white blood cell count; PLT, platelet count; NEU, neutrophil count; TSH, thyroid stimulating hormone; RECCP, reactive cutaneous capillary endothelial proliferation; PPES, palmar-plantar erythrodysesthesia syndrome; GGT, gamma-glutamyltransferase.

to be 21%. It is important to note that another treatment regimen containing CTLA-4 inhibitors has a higher rash incidence. In the HIMALAYA study (78), the safety of durvalumab plus tremelimumab in the treatment of hepatocellular carcinoma (HCC) is currently under investigation, and the reported incidence of rash is 32%.

Dermatological irAEs typically arise during the initial two weeks of therapy and can be observed in any patient with cancer. Less frequently occurring dermatologic irAEs entail actinic keratosis and skin exfoliation, along with dermatitis acneiform, dry skin, and palmar-plantar erythrodysesthesia syndrome (PPES) (41, 51). Patients exhibiting grade 1 dermatologic irAEs, as stipulated by the Common Terminology Criteria for Adverse Events 5.0, are eligible for ICI treatment. However, in the event of a grade 3 rash, it becomes imperative to introduce prednisone, a systemic steroid, at a daily dose of 0.5–1 mg/kg and temporarily suspend ICI treatment (91). The primary approach to managing dermatologic irAEs involves providing supportive care. Utilizing medium to high-potency topical corticosteroids proves beneficial for treating the rash. Alternatively, pruritus symptoms can be relieved by using cold compresses, oatmeal baths, and systemic antihistamines such as hydrochloride and hydroxyzine hydrochloride (92). As a rule, RCCEP generally does not necessitate specialized treatment nor is it affected by GSCs. The majority of symptoms tend to spontaneously resolve within approximately 1.6 months after discontinuing camrelizumab. For large nodules and instances of bleeding, it is crucial to implement measures to promote hemostasis and prevent infection (93).

3.2 Immune-related endocrinopathies adverse events

Thyroid disorders, hypophysitis, insulin-deficient diabetes mellitus, and primary adrenal insufficiency (PAI) have been cited as irAEs caused by ICIs therapy (94). Most instances of thyroid irAEs present as painless thyroiditis accompanied by temporary thyrotoxicosis (95). In patients with severe thyrotoxicosis, there is often a subsequent period of hypothyroidism. Over 40% of patients experience permanent hypothyroidism and necessitating thyroid hormone replacement (96). Some individuals may develop primary hypothyroidism without prior thyrotoxicosis (95). Two observational studies examining thyroid irAEs found that between 42-53% of patients encountered immune checkpoint inhibitor-related thyroid irAEs (96, 97). The incidence of thyroid dysfunction in patients treated with a combination of PD-L1 inhibitors and CTLA-4 inhibitors has been reported as high as 56% (98). Research suggests that hypophysitis is frequently associated with CTLA-4 inhibitors, whereas PD-1 inhibitors are more commonly linked to thyroid dysfunction in comparison to PD-L1 inhibitors (95, 98). A clinical trial investigating zimberelimab for the treatment of classical Hodgkin lymphoma discovered a 21% incidence rate of hypothyroidism (68). Conversely, a phase 3 clinical study on sugemalimab as monotherapy in NSCLC reported a 17% incidence of hypothyroidism (84). PAI poses a significant clinical concern. The analysis of the 2020 WHO VigiBase report revealed immune-related PAI to be linked to a considerable level of morbidity, with over 90% of cases categorized as severe, the mortality rate was observed to be 7.3% (99).

Most cases of immune-related endocrinopathies typically occur within 12 weeks of initiating ICIs therapy. However, there have been reports of some endocrinopathies developing several months to years after starting ICIs treatment (100). A retrospective study (101) found that 67% of patients did not show any symptoms during the thyrotoxicosis phase, which lasted approximately 6 weeks. After around 10.4 weeks, 84% of patients developed hypothyroidism. The majority of immune-related thyroid complications are mild to moderate, and thyrotoxicosis only requires active surveillance without treatment (102). It is recommended to regularly monitor thyroid function, including levels of thyroid-stimulating hormone and free thyroxine after

completing 5-6 cycles of ICIs treatment (103). Symptoms of hyperthyroidism can be alleviated by orally administering receptor blockers such as propranolol, metoprolol, or atenolol (104). When thyroid-stimulating hormone levels exceed 10 mIU/L, treatment with levothyroxine is recommended. Typically, levothyroxine is initiated at a low dose of 25~50 μg/day or 1.6 μg/kg (102, 105). For overt hypothyroidism, levothyroxine is usually initiated at a low dose of 25-50 μg/day (106). However, in young and healthy patients, it may also be initiated at a full estimated replacement dose of 1.6 g/kg body weight (107). In elderly patients or those with heart disease, it is particularly important to initiate treatment with a lower initial dose of 12.5~25 μg/day and titrate slowly (107). In cases of a patient developing an acute adrenal crisis or severe illness, it is crucial to promptly administer stress doses of GCSs. Additionally, mineralocorticoid replacement therapy is necessary for the treatment of PAI. It is important to note that endocrine irAEs are often irreversible and may require lifelong hormone replacement therapy (92).

Regarding to immune-related diabetes, patients commonly display symptoms and indications of hyperglycemia or diabetic ketoacidosis (DKA) (102). Although rare, diabetes mellitus and PAI are endocrine toxicities that can be life-threatening if not promptly recognized and treated. A study conducted by Kotwal A. et al. (108) discovered that just 1.4% of patients who received treatment with ICIs for more than 6 years developed new-onset insulin-dependent diabetes or experienced significant deterioration of type 2 diabetes. Nevertheless, clinical trials have reported a slightly higher incidence rate, with hyperglycemia observed in 6% of patients treated solely with serplulimab (70). Another recent study revealed a noteworthy correlation between the utilization of metformin to regulate blood glucose levels and a 53% heightened risk of mortality following ICIs treatment (109). Hence, vigilant monitoring of blood glucose levels post-ICI usage is imperative to promptly detect ICI-related diabetes and prevent DKA (102). Moreover, it is essential to rule out the presence of ketoacidosis. When blood glucose levels are raised, promptly assessing glycosylated hemoglobin levels, and seeking consultation from an endocrinologist is recommended (34, 110).

3.3 Immune-related gastrointestinal adverse events

Gastrointestinal irAEs related to the digestive system, such as gastritis, colitis, and enterocolitis, typically manifest themselves approximately 6 to 8 weeks after starting treatment with ICIs (33). Symptoms affecting the upper digestive tract nausea, vomiting, dysphagia, pain in the upper abdomen. On the other hand, manifestations in the lower digestive tract can involve abdominal pain, hematochezia, constipation, and diarrhea (111). There have been instances where diarrhea and/or colitis may develop months after discontinuing immunotherapy, resembling symptoms similar to chronic inflammatory bowel disease (34). Among the various gastrointestinal irAEs associated with immune checkpoint inhibitors, colitis is the most common occurrence during CTLA-4 inhibitor therapy (112). Colitis tends to appear earlier, exhibit greater severity, and frequently necessitates discontinuation of medication. The reported incidence rates of colitis with CTLA-4 inhibitors and PD-1 inhibitors are approximately 27-54% and 19.2%, respectively (113). When both therapies are administered in combination, the incidence rate increases to 44.1% (88).

A study evaluating the safety of toripalimab in combination with gemcitabine and cisplatin (GP) treatment for advanced nasopharyngeal carcinoma reported incidence rates of nausea (69%), vomiting (67%), decreased appetite (53%), and constipation (39%) (53). In a clinical trial that examined the safety of combining avelumab and axitinib for advanced renal cell carcinoma, diarrhea emerged as a frequent side effect, with a reported incidence rate of 62% (81). Similarly, the KEYNOTE-048 study observed a high prevalence of gastrointestinal disorders (83%) in the pembrolizumab and chemotherapy group, wherein constipation was reported in 37% of cases. In comparison, the incidence of gastrointestinal disorders was lower at 57% in the pembrolizumab monotherapy, with constipation also reduced to 20% (50).

The incidence rates of colitis with CTLA-4 inhibitors and PD-1 inhibitors are 27-54% and 19.2%, respectively (113). When these therapies are combined, the incidence rate rises to 44.1% (88). A meta-analysis conducted by Wang DY. et al (114) investigated the incidence of immune-related colitis in patients with solid tumors. The study discovered that ICIs monotherapy with exhibited a 1.3% lower incidence of colitis (any grade) compared to alternative treatments. Severe colitis and severe diarrhea rates were 0.9% and 1.2%, respectively. However, the combination therapy of ipilimumab and nivolumab showed an increase in immune-related colitis (13.6%), severe colitis (9.4%), and severe diarrhea (9.2%). Another meta-analysis conducted in China (115), including more recent clinical trials, concluded that ICIs inhibitors posed a heightened risk of colitis across all grades when compared to chemotherapy. Notably, a solitary patient experienced bloody diarrhea after taking the 70th dose of nivolumab, suggesting a potential association between long-term nivolumab use and immune-related colitis (116). Moreover, reports suggest that raising the dosage of nivolumab or adding osimertinib after long-term stabilization of NSCLC can induce immune-related colitis (117–119).

Patients with grade 1 symptoms can be treated conservatively with a bland diet and oral hydration during episodes of acute diarrhea. For patients presenting with grade 2 symptoms, characterized by moderate diarrhea, it is recommended to start with immunotherapy cessation and initiate corticosteroid treatment as the primary approach. The dosing regimen involves administering oral prednisone or methylprednisolone at a dose of 1 mg/kg/day. If there is no improvement within 2-3 days, the corticosteroid dose should be increased to 2 mg/kg/day. In patients with more severe symptoms (grade 3 and above), the first step is to discontinue immunotherapy and then initiate intravenous methylprednisolone at a dose of 2 mg/kg/day. In cases where there is a persistent lack of response, the addition of a single dose of infliximab should be considered and starting with an initial dose of 5 mg/kg/day (34). Generally, most gastrointestinal irAEs can be effectively managed, but colitis often leads to discontinuation of therapy. When considering the reintroduction of immunotherapy

after gastrointestinal irAEs, it is crucial to evaluate the risks on an individual basis (35). Once there is an improvement in grade 2/3 diarrhea, immunotherapy can be resumed. However, if the irAEs are graded as G4, it is advisable to permanently discontinue the therapy (120).

3.4 Immune-related hepatic adverse events

Hepatic irAEs can occur at any time after the initial administration of ICIs, but they are most commonly observed between 8 to 12 weeks of starting the therapy. The main indicators of hepatic irAEs are increased levels of alanine aminotransferase (ALT) and/or aspartate aminotransferase (AST), with or without elevation in bilirubin. Patients may experience non-specific symptoms such as fever, fatigue, anorexia, and nausea. Elevation in bilirubin levels can lead to jaundice in the skin and sclera, as well as the presence of tea-colored urine (121). The occurrence of hepatic irAEs is more frequent in patients receiving combination therapy than in those undergoing monotherapy. The incidence of hepatic irAEs varies significantly depending on the type of ICIs, combination therapy, and tumor type (122).

Statistics have indicated that CTLA-4 inhibitors had a higher risk of hepatotoxicity, whereas PD-1 inhibitors appear to be associated with a lower risk (123). Patients with HCC who underwent ICIs therapy also had a higher incidence of ALT/AST elevation compared to patients with another solid tumor (124). Notably, when bevacizumab was combined with sintilimab and atezolizumab in the treatment of HCC, the incidence of AST elevation was 16% and 36%, respectively (58, 75). The ORIENT-32 study also reported a 29% increase in bilirubin levels in the blood. In a meta-analysis of non-HCC patients in the Chinese population (125), who underwent treatment with pembrolizumab, nivolumab, camrelizumab, toripalimab, tislelizumab, and sintilimab, the incidence of any grade of hepatic irAEs ranged from 7.4% to 14.0%. Monotherapy demonstrated an incidence rate of 6.9% to 13.1%, while combination therapy ranged from 12.2% to 37.8% (125).

The standard management of grade 1~2 hepatic dysfunction generally involves close monitoring to detect any worsening liver tests that may indicate a grade 3~4 irAEs at an early stage (126). In cases of grade 3~4 liver toxicity, high-dose intravenous glucocorticoids are administered for 24~48 hours, followed by an oral steroid taper with prednisolone at a dosage of 1~2 mg/kg over a minimum period of 30 days (127). It is recommended to wait until the liver function tests return to at least grade 1 before resuming immunotherapy. Unlike autoimmune hepatitis, hepatic irAEs occur when initiating higher doses of GSCs for a shorter duration, which does not require additional immunosuppression and retreatment with ICIs is not associated with relapse (128). If liver function tests do not improve or worsen within 48 hours of systemic steroid use, alternative medications such as mycophenolate mofetil (500 mg every 12 hours) or infliximab (5mg/kg/day) may be considered (129, 130). A case study reports some success with the use of mycophenolate mofetil in GSCs-refractory cases (131). Give additional doses of infliximab only if there is no improvement after the initial dose (132). However, caution should be exercised when using infliximab as it may increase the risk of severe liver injury (133).

3.5 Immune-related pulmonary adverse events

Pulmonary irAEs often manifest with symptoms such as dyspnea, cough, fever, or chest pain. While hypoxia is rare, approximately one-third of patients remain asymptomatic and only show abnormalities on imaging (134, 135). These events typically occur around 2.8 months after starting treatment, and most patients experience grade 1 to 2 symptoms (35). In a phase 3 trial of durvalumab in patients with stage III NSCLC, a high incidence of pneumonitis or radiation pneumonitis (including acute interstitial pneumonitis, interstitial lung disease, and pulmonary fibrosis) was reported, with pneumonia accounting for 13.1% of cases (77). A retrospective study of 205 NSCLC patients found that the incidence of immune-related pneumonia was 19% (136). It has been observed that patients with chronic immune-related pneumonia consistently show lymphocytosis in bronchoalveolar lavage fluid from the initial onset and throughout the steroid taper. Immunofluorescence has revealed rapid infiltration of CD8+ cells (137). Furthermore, patients with pre-existing pulmonary fibrosis have a higher risk of developing anti-PD-1-associated pneumonia (138). Additionally, an increase in blood absolute eosinophil count has been linked to a higher risk of immune-related pneumonitis (139).

Treatment of immune-related pneumonia includes discontinuing ICIs, systemic steroids, and immunosuppressive medications (140). Research indicates that 20% of cases experience a recurrence of immune-related pneumonia upon resuming ICIs (141). Moreover, some patients have developed recurrent pneumonia even after cessation of systemic steroid therapy and without resuming ICIs treatment (142). GSCs remain the primary treatment, and it is crucial to continue preventive measures against the recurrence of pulmonary irAEs for at least 4 weeks, followed by a gradual reduction. It is also important to consider measures to prevent fungal infection and osteoporosis. If a course of corticosteroid therapy fails to alleviate the severity of initial symptoms, the option of immunosuppression with infliximab may be considered (143).

3.6 Immune-related hematologic adverse events

Hematologic irAEs include hemolytic anemia, immune thrombocytopenia, lymphopenia, neutropenia, and aplastic anemia (144). These events typically occur around 10 weeks after starting ICIs therapy and can manifest at any time during treatment (145). Data from VigiBase revealed that immune thrombocytopenia had a median onset time of 41 days, while autoimmune hemolytic anemia had a median onset time of 55 days (146, 147). In a retrospective analysis by Kramer R. et al (148), involving 7,626

patients from 18 international cancer centers, hematologic irAEs were reported in 50 patients (0.6%). A meta-analysis of 47 separate studies with 9,324 patients reported that the incidence of anemia was 9.8% in grade, with grades 3 to 5 observed in 5% of cases (149). Although the reported rates of hemolytic anemia, aplastic anemia, and thrombocytopenia are relatively low, it is important to recognize that these conditions can lead to life-threatening situations, as evidenced by documented fatal cases (150–152). In the CAPSTONE-1 study conducted on patients with advanced small cell lung cancer receiving adebrelimab, a notably high incidence of hematological irAEs was observed. Approximately 95% of the patients experienced neutropenia, 94% experienced leukopenia, 85% experienced anemia, and 82% experienced thrombocytopenia (71).

Effective management is crucial in dealing with hematological irAEs. The diagnosis of immune thrombocytopenia can be challenging, and clinicians must be vigilant for symptoms such as easy bruising, petechiae, and spontaneous mucocutaneous bleeding. It is essential for patients to promptly report any of these symptoms (153). While steroids are commonly used to treat mild thrombocytopenia, they may not be sufficient for severe cases (152). Other available treatment options include recombinant human thrombopoietin (TPO), platelet transfusions for short-term and concurrent therapy, intravenous immunoglobulin (IVIG), and the utilization of immunosuppressants like azathioprine and rituximab.

In cases of steroid resistance, TPO receptor agonists such as eltrombopag, herombopag, or avatrombopag can be administered (154). An in-depth and descriptive observational study (144) revealed that 78% of immune-related thrombocytopenia cases were classified as grade 4. All patients underwent steroid treatment, with 67% of them also receiving IVIG. However, 22% of patients did not respond to these treatments and required replacement therapy involving a TPO receptor agonist or rituximab. The study also provided preliminary safety data on rechallenging patients with ICIs. Among the patients, 67% discontinued halted the use of ICIs treatment, while 33% were rechallenged. Out of this group, 33% experienced a relapse of immune-related thrombocytopenia. Currently, the optimal treatment for hematologic irAEs is still under investigation.

3.7 Immune-related cardiovascular adverse events

Cardiovascular irAEs can manifest in various ways, including myocarditis, pericarditis, arrhythmias, reduced ventricular function, vasculitis, venous thromboembolism, cardiac valvulitis, and pulmonary hypertension. Myocarditis is characterized by symptoms such as palpitations, chest pain, acute or chronic heart failure, pericarditis, and pericardial effusion (155).

A retrospective study (156) conducted in the United States involved 105 patients from 8 medical centers. The study revealed that the median onset time of immune-related myocarditis after immunotherapy was 27 days. The age of symptom onset was 65 years, and the estimated occurrence rate was 1.9%. Approximately 81% of cases occurred within the first three months of ICIs therapy. Similar results were found in a retrospective analysis conducted in China (157), which involved 2373 individuals receiving ICI monotherapy or combination therapy from 12 medical centers. The estimated event rate of immune-related myocarditis was 1.05%, but the median time of development was delayed to 38 days. Another real-world investigation (158), that included 2647 patients treated with ICIs, revealed cardiovascular irAEs in 89 patients (3.4%), with myocarditis accounting for approximately. 37.1% of cases. Despite immune-related myocarditis being generally rare, it is considered one of the most perilous irAEs due to its high fatality rate, ranging from 27% to 60% (134, 159). For instance, a study on ipilimumab–nivolumab combination therapy reported a mortality rate of 60% in cases of myocarditis (160).

The likelihood of cardiovascular events has been found to triple in cancer patients due to atherosclerosis (161). Furthermore, the combination of PD-1/PD-L1 inhibitors with CTLA-4 inhibitors is also associated with higher rates of cardiovascular irAEs. These irAEs exacerbate the condition, leading to earlier symptom manifestation and increased risk of mortality (162). The increase in cardiac biomarkers is strongly correlated with disease severity and frequently occurs before the onset of symptoms (163). Diagnostic tests primarily involve troponin measurement and electrocardiogram, while cardiac magnetic resonance imaging and endomyocardial biopsy are deemed the gold standard for diagnosis (164). Treatment options are determined based on risk stratification.

Palaskas NL. et al. (165) demonstrated that some patients with low-grade myocardial inflammation may continue ICIs treatment without immunosuppressive therapy. The first-line treatment suggests different doses of GSCs, while the second-line treatment includes the use of immunosuppressants such as IVIG and anti-thymocyte globulin. It should be noted that the second-line treatment is recommended for life-threatening situations or when GSCs are ineffective (166). However, high-dose infliximab should be avoided in patients with moderate to severe heart failure. Unlike other irAEs, restarting ICIs has been reported to be extremely dangerous (167).

3.8 Immune-related neurologic adverse events

Neurological irAEs demonstrate significant heterogeneity and occur relatively infrequently. These events can affect both the central and peripheral nervous systems, leading to conditions such as myositis, neuropathy, encephalopathy, and myasthenia gravis (38). Several phase 3 clinical trials have identified a higher occurrence of neurological irAEs. For instance, in patients with advanced nasopharyngeal carcinoma treated with toripalimab combined with GP, the incidence of peripheral neuropathy was 30%. Similarly, in patients treated with toripalimab, paclitaxel, and cisplatin for advanced esophageal squamous cell carcinoma, the incidence of peripheral neuropathy was 40% (53, 54). In a clinical trial (65) investigating the combination of tislelizumab, paclitaxel and carboplatin for advanced NSCLC, the occurrence rate of

hypoesthesia was reported to be 23%, notwithstanding the inclusion of both immunotherapy and chemotherapy in these treatment regimens. A comprehensive meta-study (168) merging data from 59 clinical trials revealed that neurological irAEs were documented in 6% of patients receiving PD-1 inhibitors, with the majority categorized as grade 1-2. Headache was the most frequently reported symptom, while grade 3 or higher neurological irAEs were observed in less than 1% of cases. Additional studies (169, 170) have reported estimated incidences of neurological irAEs ranging from approximately 1% to 12% in patients undergoing immunotherapy, primarily occurring within the initial 6 months of commencing ICIs. Furthermore, the peripheral nervous system is found to be more susceptible to these adverse events compared to the central nervous system.

To establish a conclusive link between peripheral neuropathy and ICIs, it is crucial to assess alternative potential origins in patients suspected of having neuropathy. It should be noted that these symptoms might also arise from other medications (171). Several factors should be considered when ruling out other possible causes, including the duration of drug use, presence of pre-existing neurological conditions, simultaneous irAEs and overlapping syndromes, and improvement upon discontinuation of the drug and/or initiation of GSCs (172). In addition, alternative immunomodulatory approaches, such as antirheumatic drugs, should be taken into account as well (173).

3.9 Immune-related musculoskeletal adverse events

Patients treated with ICIs have reported experiencing arthralgia and myalgia; however, there has not been a comprehensive report on the incidence of mild to moderate arthritis (174). According to a study, 13.3% of patients receiving PD-1 inhibitors experienced arthralgia, with a median onset time of 100 days. Specifically, arthralgia was observed in 18% of patients with advanced cutaneous squamous cell carcinoma who received cemiplimab monotherapy (51). In a study by Cappelli LC. et al (175) data from 52 trials of musculoskeletal irAEs revealed that arthritis was reported arthritis in 1–43% and myalgia in 2–20% of patients across 5 out of 33 clinical trials. To manage symptoms of myalgia or joint pain, nonsteroidal anti-inflammatory drugs (NSAIDs) or corticosteroids are generally recommended. Once symptoms improve to grade 1 or less, it is wise to gradually reduce the dose of corticosteroids over 4-6 weeks. If the corticosteroid dose cannot be reduced to 10 mg per day within 6-8 weeks, further consideration of antirheumatic drugs is recommended. Patients who experience symptoms persisting for more than 6 weeks or need a daily corticosteroid dose exceeding 20 mg that cannot be reduced to less than 10 mg daily within 4 weeks, should consult with a rheumatologist (176). In most patients, symptoms improved with the use of NSAIDs, while low-dose GSCs were required by 23.1% of patients and 7.6% required additional immunosuppressive therapy (177).

3.10 Other immune-related adverse events

In this section, other irAEs will also be discussed, including immune-related infusion reactions, ocular adverse reactions, and nephrotoxicity.

Infusion reactions related to ICIs are typically characterized by symptoms such as low-grade fever, chills, headache, or nausea, which can be ascribed to the nonspecific release of cytokines (178). A study involving patients with advanced renal cell carcinoma who received the combination of avelumab and axitinib, reported infusion reactions in 12.2% of patients, with grade 3 or higher reactions observed in 1.6% of cases (81). Manifestations of infusion reactions are usually mild, and mild fever and chills can be managed with NSAIDs. In certain cases, it may be advisable to consider reducing the dosage or discontinuing the infusion (34, 179).

The incidence of ocular irAEs is exceedingly low, less than 1%, and typically manifests within six months of ICI utilization (180). Ophthalmoplegia and uveitis are more prone to appear within the initial 10 weeks, while dry eye and other ocular irAEs may develop later (181). Among lung cancer patients receiving ICIs, the most prevalent ocular irAEs were ophthalmoplegia (40.51%), uveitis (20.25%), and dry eye syndrome (17.72%). Uveitis can usually be effectively treated with topical corticosteroids applied to the surface of the eye, although severe cases may necessitate GSCs administered throughout the body. Other treatment options include using subconjunctival GSCs, injecting dexamethasone directly into the eye, and injecting triamcinolone acetonide around the area near the eye (182). Prompt examination is crucial when symptoms of worsening vision, spots in vision, or redness of the conjunctiva appear (183). The occurrence of uveitis does not necessarily require suspension of immunotherapy. Symptomatic treatment of most ocular irAEs demonstrates exceptional and swift responses, with an overall remission rate as high as 92.31% (except for ophthalmoplegia) (184).

Acute kidney injury (AKI) is the common presentation for most cases of immune-related nephrotoxicity. It requires dialysis and results in abnormal levels of electrolytes (185). The median time to onset of immune-related nephrotoxicity usually occurs within a span of 3 to 4 months (186). Among patients receiving PD-1 inhibitors, the combined estimated rate of AKI was 2.2%. Additionally, interstitial nephritis had a combined estimated rate of 16.6% within this group (187). Nevertheless, the reported incidence of AKI may be higher than what is currently known. Evidence from case reports and cohort studies suggests a possibility of 10% to 30% in clinical practice. For instance, a cohort study reported an incidence of 16.5% (188), while real-world population data reported an incidence of 17% (189). It is important to note that patients with immune-related AKI often experience extrarenal toxicities, including rash, thyroiditis, and colitis, ranging from 40%–87% (188, 190, 191). After diagnosing immune-related AKI, clinicians should thoroughly assess the patient's medication history and discontinue nephrotoxic drugs. Symptomatic treatment usually involves corticosteroids, and if dialysis is required due to renal impairment, ICIs should be immediately discontinued (160).

4 Discussion

4.1 Association between irAEs and response to treatment

In 2018, a study conducted by Shafqat H. et al. (192) unveiled a connection between the occurrence of irAEs enhanced progression-free survival (PFS) in patients with various tumor types (192). Further investigations have provided additional evidence supporting the potential correlation between irAEs and clinical benefits. For instance, patients who experienced immune-related arthralgia exhibited better treatment responses, characterized by improved PFS and overall survival (OS) (177). Two studies (193, 194) involving lung cancer patients showcased improved clinical outcomes among individuals who encountered irAEs while undergoing nivolumab treatment. These patients exhibited a higher objective response rate (ORR) and increased PFS compared to those without irAEs. Additionally, a multicenter cohort study unveiled a connection between the progression of multisystem irAEs and improved OS (195). Interestingly, patients who developed late irAEs demonstrated a higher ORR than those with early irAEs (196).

A meta-analysis (197) encompassing 4971 subjects from 30 studies discovered a significant correlation between the development of irAEs and improved survival in tumor patients treated with PD-1 inhibitors. Notably, the group of patients who received ICIs as monotherapy showed a more prominent correlation in cancer outcomes compared to the group receiving combination therapy. Another meta-analysis (198) consolidated these findings, affirming a positive association between the occurrence of irAEs and enhancements in ORR, PFS, and OS, regardless of tumor site, type of ICIs, or irAEs status. It should be pointed out that grade 3 or 4 irAEs were associated with improved ORR, yet worse OS. However, a retrospective study reported contradictory findings, claiming that patients with immune-related constipation faced a significantly higher risk of disease progression, but no significant association with OS was observed (199).

4.2 Differences in adverse events between PD-1 inhibitors and PD-L1 inhibitors

Initially, Spagnuolo A. et al (200) discovered no significant distinction in irAEs between the two ICIs. Previous research indicates that patients who received PD-1 inhibitors had a higher occurrence of grade 3 or higher irAEs (201) and were more susceptible to pneumonia and thyroiditis (202). Conversely, PD-L1 inhibitors were associated with lower rates of cardiac complications and overall mortality compared to PD-1 inhibitors. They also exhibit a minimal risk of rash, elevated ALT, colitis, and hypothyroidism (203). Out of the 49 clinical trials analyzed (Table 1), it can be observed that immunotherapy generally leads to a higher incidence of anemia, neutropenia, leukopenia, and nausea. This pattern is particularly evident in regimens incorporating PD-1 inhibitors. On the other hand, regimens containing PD-L1 inhibitors tend to cause fatigue more frequently. Even when ICIs are administered as monotherapy, it is still observed that PD-1 inhibitor regimens have a higher incidence of anemia, followed by hyperthyroidism. Similarly, patients treated with PD-L1 inhibitors are more prone to experiencing fatigue, pneumonia, and cough. Combination regimens of PD-1/PD-L1 and CTLA-4 inhibitors were associated with higher rates of fatigue, nausea, rash, and diarrhea/colitis. A meta-analysis of clinical studies investigating regimens containing ipilimumab and tremelimumab found that irAEs primarily manifested as skin lesions (rash, pruritus, and vitiligo) and colitis, which aligns with our observed outcomes (204).

In terms of monotherapy, atezolizumab demonstrated a lower overall risk of any grade irAEs compared to pembrolizumab, while avelumab exhibited a lower risk of grade ≥3 irAEs (205). A comprehensive study involving 36 head-to-head phase 2/3 clinical trials revealed differences in the toxicity profiles of different PD-1/PD-L1 inhibitors (206). Specifically, nivolumab was more frequently correlated with endocrine toxicity, pembrolizumab displayed a higher prevalence of arthralgia, pneumonia, and hepatotoxicity, and atezolizumab showed a strong inclination towards symptoms such as hypothyroidism, nausea, and vomiting (206). These studies including CameL, CameL-sq, and ESCORT have confirmed that camrelizumab has a higher tendency to induce RCCEP (59, 61, 62), whereas adebrelimab was reported to give rise to various types of hematological irAEs in CAPSTONE-1 (71). These observations suggest that the pattern of irAEs varies among different PD-1/PD-L1 inhibitors, potentially owing to disparities in their capacity to stimulate immune cells (207). One specific difference to note is that PD-L1 inhibitors do not inhibit the interaction between PD-1 and PD-L2, which plays a role in suppressing the immune response. What's more, PD-L2 binds to the molecule b, regulating respiratory immunity (208). These factors might account for the discrepancy in the occurrence of particular irAEs between PD-1 inhibitors and PD-L1 inhibitors (209).

4.3 Strategies to limit irAEs

With the widespread use of ICIs, oncologists' understanding and management of irAEs are gradually improving. This review will highlight several strategies to alleviate irAEs.

The first step towards effectively limiting irAEs is to properly profile patients before treatment begins. Additionally, physicians and nurses must have accurate information about patients should serve as early indicators of irAEs. One important strategy is regular monitoring of patients throughout their treatment and during the follow-up period. Close monitoring of control indicators and organ functions is essential for the prompt detection, reporting, and treatment of irAEs (35). For instance, severe cutaneous irAEs, such as pruritus or rash, can signal the presence of other irAEs. Patients with dermatologic irAEs are more susceptible to the occurrence of gastrocolitis, while those with immune-related psoriasis are more prone to endocrine irAEs (210). Furthermore, certain irAEs such as diarrhea and colitis may manifest several months after the cessation of ICIs treatment (211). Therefore, long-term follow-up is crucial, as

there is a possibility of delayed onset of pneumonia or skin irAEs (212). Currently, it is recommended to follow up with patients for at least two years after completing ICIs treatment (33).

Secondly, symptomatic treatment plays a crucial role in managing irAEs. GSCs are commonly chosen to treat the main irAEs (35). Based on experience with nivolumab for irAEs, high-dose GSCs should be used cautiously due to potential exceptional reactions, although there are case reports of overall improvement in the condition (213, 214). For grade 1-2 irAEs, oral corticosteroids are typically prescribed. In cases where irAEs affect specific organs such as the heart, lungs, liver, and nervous system, high-dose intravenous GSCs are among the preferred prescriptions for prompt intervention. If GSCs prove to be ineffective, other immunosuppressants such as infliximab, mycophenolate mofetil, tacrolimus, and anti-thymocyte globulin may be taken into account (1). It has been found that glucocorticoid therapy was not necessary for hypothyroidism and other endocrine irAEs (such as diabetes mellitus); replacement hormone therapy is recommended (28, 215).

Thirdly, physicians must consider the possibility of continuation or cessation and subsequent reexposure of ICIs. If patients only experience mild cutaneous or endocrine irAEs, it is acceptable to continue ICIs (87). However, once severe or life-threatening irAEs occur, especially grade 3-4 cardiac, pulmonary, and neurotoxicity, it is imperative to permanently stop the administration of such ICIs (33). If irAEs are downgraded from grade 2 to grade 1, restarting ICIs becomes a viable option (216). Alternatively, replacing ICIs upon reboot is another strategy. An illuminating case report (217) demonstrated that a patient developed immune-related grade 3 colitis, requiring the discontinuation of ipilimumab. However, the patient subsequently received pembrolizumab for over 20 months without experiencing serious irAEs and achieved a partial objective response. When rechallenging with ICIs, it is of utmost importance to closely monitor the reemergence of the initial irAEs (218), as well as the patient's tumor response status. If irAEs resurface, it is advisable to permanently discontinue the use of such ICIs. A retrospective study (219) discovered that 14% of NSCLC patients had to terminate treatment due to irAEs when using ICIs. Among these patients, 56% were rechallenged with ICIs after the initial treatment. In the re-challenged patient cohort, 48% did not encounter any subsequent irAEs, while 26% experienced a recurrence of the initial irAEs and 26% developed new irAEs.

There is an ongoing debate regarding the best strategies for the management of irAEs. In addition to the previously mentioned mitigation approaches, it is important to consider additional strategies for managing these adverse events. These may include educating patients about their medications, improving guidelines for irAE management, standardizing the reporting of irAEs, and carefully selecting ICIs (220). Furthermore, Sullivan RJ. et al. (7) proposed several key approaches to alleviate irAEs, such as adjusting the dose and administration schedule of ICIs, developing alternative checkpoints, and altering the microbiota. These innovative approaches provide valuable insights for future investigations.

5 Conclusion

ICIs can induce unforeseen adverse effects on the body. The emergence and intensity of irAEs are influenced by various immune mechanisms. These mechanisms include the direct destruction of normal cells by monoclonal antibodies, the production of autoantibodies by B cells, T cell activation triggering cytokine pathways, and the influence of gut microbiota.

IrAEs exhibit different clinical manifestations, occurrence times, and impacts on different tissues and organs due to the variations in ICIs and cancer types. Currently, the treatment of irAEs has been mostly empirical, utilizing immune-based approaches for managing primary autoimmune diseases (9). Existing guidelines recommend the use of corticosteroids as the first-line treatment for the most severe forms of irAEs. However, a major limitation of these guidelines is the lack of stratification of irAEs based on the etiology of the immune histopathology (34, 35, 87, 133). While irAEs are generally rare and mostly mild to moderate, there have been cases where serious adverse reactions have resulted in fatal consequences. Therefore, early identification and diagnosis of certain non-specific irAEs, such as cardiac and endocrine irAEs, through regular examinations are crucial. In situations where a wide range of irAEs are present, consultation with experts from various disciplines may be necessary. Nevertheless, further research is required to determine the efficacy of these interventions in reducing the occurrence of irAEs.

Author contributions

TY: Writing – original draft. LY: Funding acquisition, Writing – review & editing. JZ: Investigation, Supervision, Writing – original draft. YC: Supervision, Visualization, Writing – review & editing. YF: Supervision, Validation, Writing – original draft. JT: Project administration, Resources, Writing – original draft. DL: Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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The occurrence of asthma in an extensive-stage small-cell lung cancer patient after combination therapy with atezolizumab and anlotinib: a case report

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Background: Extensive-stage small-cell lung cancer (ES-SCLC) is highly malignant, with early metastasis and high recurrence. Since therapeutic options are limited, ES-SCLC has a characteristically short survival period and extremely poor prognosis. A combination of immune checkpoint inhibitors (ICIs) and anti-angiogenic drugs can achieve promising efficacy and safety in patients with ES-SCLC as a second-line or subsequent treatment, extending survival to some extent. However, the clinical outcomes remain mostly unsatisfactory and are sometimes affected by treatment-related adverse events.

Case presentation: A 57-year-old woman with ES-SCLC was administered a combination therapy of atezolizumab (a PD-L1 inhibitor) and anlotinib [an oral multi-targeted tyrosine kinase inhibitor (TKI)]. She survived for 22 months, with no disease progression during the 28 courses of therapy. Unexpectedly, despite having no history of asthma, the patient developed asthma while receiving this regimen. This is possibly related to T-cell activation and the tumor immune microenvironment, which induce allergic inflammation after PD-L1 blockade.

Conclusions: This is the first report of an asthma-negative ES-SCLC patient who developed asthma after receiving atezolizumab plus anlotinib. Although this combination therapy may effectively extend survival in SCLC patients, asthmatic symptoms should be closely monitored.

KEYWORDS

atezolizumab, anlotinib, asthma, small-cell lung cancer, combined regimens

Introduction

Small-cell lung cancer (SCLC) is a highly malignant tumor with a poor prognosis, accounting for approximately 15% of all lung cancers, and is the leading cause of cancer-related deaths worldwide (1, 2). More than 50% of SCLC patients are diagnosed with extensive-stage (ES) disease (3). ES-SCLC is the most aggressive type of lung cancer, characterized by early metastasis, rapid proliferation rate, and high recurrence, with an average overall survival (OS) of only 2–4 months in its natural course (4, 5). After initial treatment with systemic chemotherapy and radiotherapy, current therapeutic strategies are limited to improving the long-term survival and reducing the mortality rate of ES-SCLC.

Comprehensive medical treatment should be a top priority for patients with ES-SCLC. Programmed death-ligand 1 (PD-L1) inhibitors and anti-angiogenic agents may represent new therapeutic strategies for ES-SCLC (6). The combination of immune checkpoint inhibitors (ICIs) with platinum-based chemotherapy has demonstrated sustained benefits in OS as a standard first-line option for current treatment (7). Anlotinib is a small-molecule tyrosine kinase inhibitor (TKI) that inhibits tumor neovascularization and negatively regulates tumor growth. Evidence indicates that anlotinib stimulates lymphocyte infiltration and migration in tumors, increasing the anticancer effects of PD-L1 inhibitors by reducing immunosuppression (8, 9). Several studies have reported promising efficacy and safety of the combination of ICIs and anlotinib as a second- or third-line treatment for ES-SCLC (10, 11).

Although immunotherapy offers some advantages over other anticancer regimens, its use is complicated by potentially lethal immune-related adverse events (irAEs), including skin toxicity (44%–68%), myocarditis (50%), colitis (10%–25%), nervous system toxicity (10%), and pneumonitis (9.6%) (12). However, to our knowledge, the development of bronchial asthma in patients with asthma-negative SCLC receiving immunotherapy has not yet been reported.

Herein, we report the case of an asthma-negative patient with ES-SCLC who experienced an asthma attack during treatment with atezolizumab in combination with anlotinib. Currently, the progression-free survival (PFS) of the patient has lasted for nearly 2 years. We have attempted to explain the reasons for this rare adverse effect.

Case presentation

A 57-year-old Chinese woman with a 6-month history of cough and 1 week of dyspnea was admitted to our hospital on 25 January 2022. She was in good health with no history of asthma, allergies, or smoking, and no family history of hereditary disease, asthma, or tumors. She was retired from school teaching and lived alone with no pets in a nonsmoking environment. Initial physical examination showed normal results. Chest computed tomography (CT) revealed a central-type tumor in the right lung with invasion of the right pulmonary vein and right atrium and multiple lymph node metastases in the mediastinum and hilar regions (Figure 1A). Abdominal CT showed left adrenal gland and liver metastases, while no metastasis was detected on brain and systemic bone imaging. The electrocardiogram findings were normal. Laboratory findings indicated significant elevation of tumor markers, including carcinoembryonic antigen (CEA) and neuron-specific enolase (NSE). Routine blood tests and IgE, eosinophil, serum cTnl, CK-MB, and D-dimer levels were all within the normal ranges. Lung biopsy was performed using fiberoptic bronchoscopy and endobronchial ultrasonography. Histopathological analysis revealed TTF-1 (+), Syn (+), CgA (+), CD56 (+), CK7 (−), napsin A (−), CK5/6 (−), Ki-67 (40%+), CK (+), P40 (−), P63 (−), and PD-L1 <5% (Figure 2A). The patient was diagnosed with ES-SCLC (T4N2aM1c2, stage IVB).

Owing to her resistance to chemotherapy, the patient was administered atezolizumab in combination with anlotinib as the

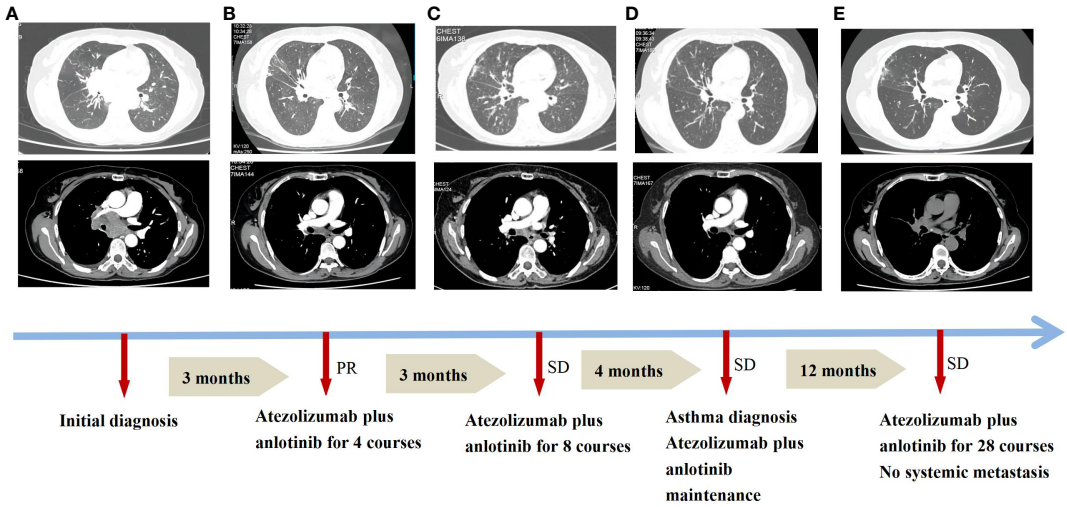

FIGURE 1
Treatment progress of atezolizumab plus anlotinib and asthma diagnosis in the patient. **(A)** Initial diagnosis. **(B)** Combined regimens for 4 courses. **(C)** Combined regimens for 8 courses. **(D)** Asthma diagnosis. **(E)** Combined regimens for 28 courses. PR, partial response; SD, stable disease. Efficacy was evaluated according to the Response Evaluation Criteria in Solid Tumours (RECIST).

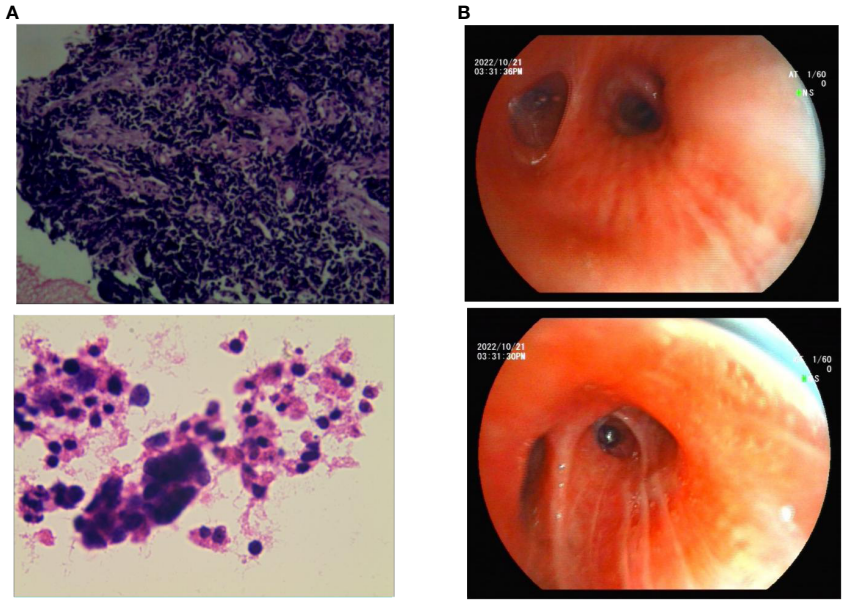

FIGURE 2
(A) Photograph of small-cell lung cancer. **(B)** Bronchoscopy during asthma diagnosis.

initial treatment strategy. After four courses of treatment, the tumor size decreased significantly (Figure 1B) and remained stable during subsequent treatment (Figure 1C). However, before the 12th course of treatment in October 2022, the patient experienced aggressive dyspnea (modified mMRC score ≥2) with wheezing rales in both lungs. She had no chest pain, hemoptysis, cough, sputum production, fever, urticaria, angioedema, abdominal pain, skin rash, or joint swelling and pain. Chest CT showed no change in tumor size and no pulmonary embolism (Figure 1D). Fiberoptic bronchoscopy revealed bronchial mucosal congestion, edema, and secretions, without obvious obstruction (Figure 2B). Bronchoalveolar lavage fluid tests for nucleic acid detection of respiratory pathogens (bacteria and viruses) and acid-fast bacilli and fungal smears yielded negative results. In turn, elevated eosinophil count ($1.28 * 10^9$/L) and IgE level (278 U/mL) were observed. The patient exhibited a positive response to a bronchodilation test, showing a 15% improvement in forced expiratory volume in 1 s (FEV1) and a 220-mL increase in the absolute FEV1 value in response to a beta-agonist. Pulmonary function test revealed 130 ppb of exhaled nitric oxide with no obstructive dysfunction. The patient was clinically diagnosed with asthma secondary to ICI treatment. She was started on systemic corticosteroids (methylprednisolone 40 mg/day for 5 days) and regular use of inhaled corticosteroids (ICS)/long-acting beta-agonists (LABA) (fluticasone propionate/salmeterol) and montelukast (10 mg/day). Her symptoms resolved with a decrease in eosinophil, IgE, and exhaled nitric oxide levels, along with normal lymphocyte counts during long-term therapy (Figure 3). Currently, her PFS has reached 22 months, with no systemic metastasis and a stable tumor status at the last follow-up (Figure 1E). In addition, reductions in serum CEA and NSE levels were recorded at the last follow-up. No tumor lysis syndrome or cytokine release syndrome was observed during treatment.

Discussion

SCLC is a high-grade neuroendocrine cancer that is characterized by intensive invasiveness and rapid progression. Approximately two-thirds of SCLC patients are initially diagnosed with distant metastasis, mainly involving the liver, adrenal gland, brain, and bones (13). In the case of ES-SCLC, short survival and poor outcome significantly impact the quality of life, with a median OS of only 6–10 months when treated with ICIs plus chemotherapy (14) and a 5-year survival rate of less than 5% (15). Recent studies have shown that the combination of programmed death-1 (PD-1)/PD-L1 inhibitors and anti-angiogenic therapy can improve outcomes in ES-SCLC, with a PFS and OS of 3.4–7.5 and 8.2 months, respectively (10, 11). However, reports on long-term survival in ES-SCLC are relatively rare and may be attributed to factors such as better physical status, the absence of liver or brain metastases, sensitivity to platinum-based chemotherapy, and adherence to close follow-up (16, 17). Currently, this patient has achieved a survival of 22 months following atezolizumab in combination with anlotinib therapy. However, the specific mechanisms underlying the antitumor actions of PD-L1 inhibitors combined with anlotinib in ES-SCLC have not been sufficiently investigated.

Anlotinib is a multi-targeted TKI that exerts marked inhibitory effects on tumor angiogenesis by inhibiting vascular endothelial growth factor (VEGF), fibroblast growth factor receptor (FGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR), and stem cell factor receptor (c-Kit) (18). In the clinical trial ALTER 1202, the SCLC group treated with anlotinib showed longer median PFS (4.1 *vs.* 0.7 months) and median OS (7.3 *vs.* 4.9 months) compared to the placebo group, reducing the risk of death by 47% (19). Anlotinib is currently the only antiangiogenic drug approved as third-line treatment for ES-SCLC in China. A recent study also showed that anlotinib was effective in SCLC as first-line maintenance therapy and second-line treatment, with no new

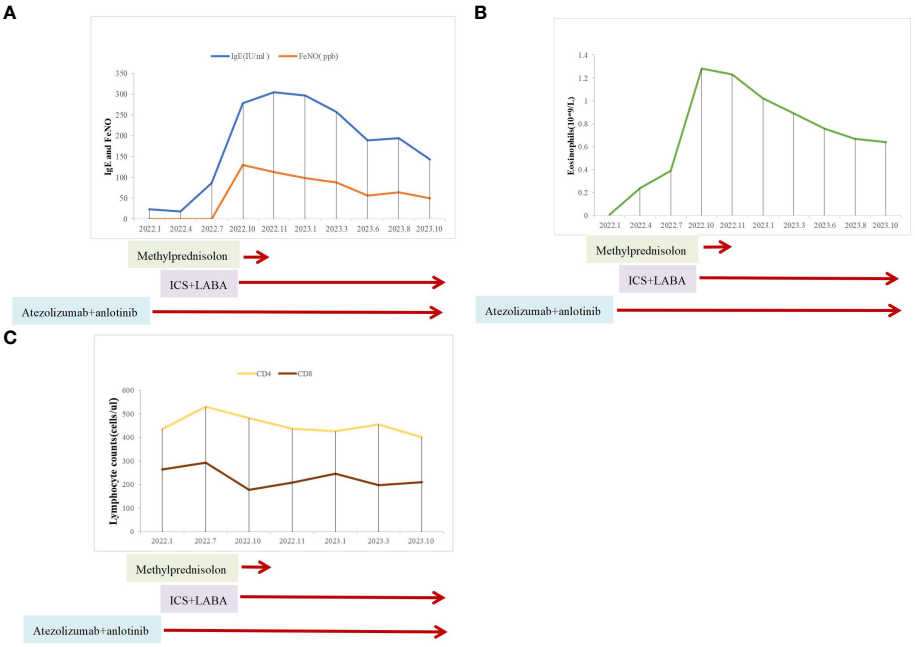

FIGURE 3
Clinical course of the patient after asthma diagnosis. **(A)** IgE and exhaled nitric oxide levels. **(B)** Eosinophil counts. **(C)** Lymphocyte counts.

anlotinib-related adverse reactions (20). However, the PFS or OS of anlotinib monotherapy or combination therapy is no more than 13 months, based on the current data.

Atezolizumab is a humanized anti-PD-L1-monoclonal antibody that regulates anticancer immunity by inhibiting PD-L1/PD-1 interactions (21). The IMpower133 trial concluded that atezolizumab plus chemotherapy significantly improved OS and PFS in ES-SCLS as first-line treatment (22). However, ICIs monotherapy did not demonstrate clinical benefits in terms of OS for ES-SCLC as a second-line or subsequent therapeutic option (23). Tumorigenesis can lead to a reduction in dendritic cells (DCs) by impairing antigen presentation and preventing T-cell activation, resulting in an immunosuppressive microenvironment (24). This enables tumor cells to evade immune surveillance via VEGF, a key mediator that reduces tumor infiltration by T cells, and increases the number and proliferation of immunosuppressive cells such as regulatory T cells, myeloid-derived suppressor cells, and M2-like tumor-associated macrophages (25, 26). In turn, PD-L1 expression can inhibit T-cell activation and prevent an innate cytotoxic T-cell response against tumors (27). In many solid tumors, these well-recognized events contribute to angiogenesis and growth. Atezolizumab, a PD-L1 inhibitor, can suppress immunosuppressive cells and indirectly downregulate the expression of angiogenic factors (28). However, due to the unstable expression of PD-L1 (29), insufficient lymphocyte infiltration in SCLC (30), and rapid disease progression, the efficacy of immunotherapy may be compromised.

Notably, anlotinib may boost the efficacy of immunotherapy by increasing the number of innate immune cells, preventing exhaustion of CD4+T cells, and reducing PD-L1expression via inactivation of the AKT pathway in vascular endothelial cells (31, 32). Thus, a combination of anlotinib with a PD-L1 inhibitor appears to transform the tumor microenvironment into an immune-permissive status, also enhancing the synergistic efficacy of the antitumor response by suppressing tumor neovascularization (33). Indeed, the results of a cohort study suggested that infiltration of immune cells, such as CD3+ T cells, CD4+ T cells, and monocytes, strongly influences long-term survival in SCLC (34). Supporting this hypothesis, the lymphocyte counts in the tumor immune microenvironment of the ES-SCLC patient in this case report remained stable throughout the course of treatment.

The most common treatment-related adverse events reported for the combination of anlotinib with ICIs in SCLC are hypertension, hepatic dysfunction, hypothyroidism, anorexia, fatigue, oral ulcers, hand-foot syndrome, diarrhea, and bleeding (10, 11). These adverse events are manageable and well-tolerated, with no treatment-related deaths reported. Asthma during immunotherapy is rare and has only been reported during treatment with the PD-1 inhibitor nivolumab in male patients with non-small-cell lung cancer (NSCLC) (35, 36). To our knowledge, this is the first report of an asthma-negative SCLC patient who developed asthma after treatment with atezolizumab plus anlotinib. The binding of PD-1 to its ligands, PD-L1 and PD-L2, is closely related to the increase in CD4+ T helper type 2 (Th2) lymphocytes and IgE-dependent activation in allergic diseases. CD4+ T cells are predominantly associated with allergic asthma and enhanced eosinophil activity, contributing to airway hyperreactivity (AHR) and cytokine secretion (37). Th2 cells are considered crucial in AHR because they produce IL-4 and IL-13 to induce an increase in IgE production (38). PD-L1, a negative regulator of T cells, strongly stimulates PD-1 expression after antigen presentation, leading to CD4+ T-cell exhaustion and tolerance (39, 40). In a murine model, PD-L1 favored Th2-driven inflammation by upregulating IL-4 and downregulating IFN-γ, which seems crucial for increasing AHR (41).

However, in a human asthma model, significantly downregulated PD-L1 expression was observed in dendritic cells (DCs) by circulating CD4 + T cells, along with high IgE concentrations detected in patients with allergic asthma (42). The basis of this discrepancy between humans and mice is unclear and may be related to species differences, model sensitization, or disease progression. Importantly, regulation of the PD-1/PD-L1 pathway by atezolizumab can lead to Th2-mediated eosinophil activation through a type-2 innate lymphoid cell-dependent mechanism (43). In this regard, eosinophilia has been proposed as a prognostic and potentially predictive biomarker for patients with lung cancer receiving immunotherapy and is significantly associated with an increased chance of achieving disease control and a higher probability of treatment toxicity (44). Current clinical data suggest that increased blood eosinophil counts may reflect favorable outcomes in patients treated with ICIs for advanced lung cancer. Nevertheless, more clinical trials are needed to further elucidate the value of eosinophilia as a prognostic biomarker and the correlation between treatment response and toxicity (45). Notably, a previous study indicated that AHR may be acquired by high-risk factors such as cigarette smoking, squamous cell lung cancer, and peripheral blood eosinophilia (46). Although the patient had no history of asthma, eosinophilia, or smoking before treatment, she experienced dyspnea with wheezing 10 months after starting the treatment for ES-SCLC. The eosinophil counts and IgE levels were significantly increased, possibly due to the administration of atezolizumab, which restored allergic inflammation and the tumor microenvironment. Based on these results and considering the above symptoms, radiographic findings, and lung function tests, ICI-related asthma was ultimately diagnosed. This would thus suggest a dual effect of PD-L1 blockade, involving therapeutic effects on SCLC, and potential activity as an asthmagen. Allergic inflammation usually occurs 2–12 months after ICI treatment (47). In this regard, ICI-related changes in immune tolerance in the tumor microenvironment might affect airway tolerance, leading to the occurrence of AHR. Hence, potential markers such as TGF-β, IL-10, and IL-17A need to be monitored in patients receiving ICIs (48). To develop preventive and control measures, further investigation of the specific mechanisms by which immunotherapeutic modulation of PD-L1 influences airway inflammation in SCLC is required.

In reviewing the treatment course of the ES-SCLC patient, we observed long and high-quality survival after a combined treatment with atezolizumab and anlotinib. Furthermore, this patient benefited from a PFS of 22 months with a currently stable disease status. ICI-related asthma after PD-L1 blockade in patients with SCLC has rarely been reported. This adverse event has not been reported during combination treatments in other SCLC cases.

Conclusion

The combination of atezolizumab and anlotinib appears to be a potentially effective therapy for ES-SCLC, possibly achieving long-lasting disease control and improved survival when closely monitored. The unusual occurrence of treatment-related adverse events should be carefully monitored and timely addressed to enable providing theoretical support. Clinical verification in the setting of adequately powered clinical trials and the assessment of adverse events are necessary to confirm the efficacy and safety of this combination therapy in ES-SCLC.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Second Affiliated Hospital of Chongqing Medical University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

WD: Conceptualization, Funding acquisition, Writing – original draft. JC: Data curation, Writing – review & editing. X-YD: Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Efficacy and safety of neoadjuvant immunotherapy plus chemotherapy followed by adjuvant immunotherapy in resectable non-small cell lung cancer: a meta-analysis of phase 3 clinical trials

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Objective: At present, several important trials have been published show that perioperative immunotherapy combined with chemotherapy can improve the prognosis of patients with resectable non-small cell lung cancer, which further optimizes treatment options. Therefore, we conducted a systematic review and meta-analysis to evaluate the efficacy and safety of perioperative immunotherapy combined with chemotherapy in resectable non-small cell lung cancer.

Methods: The following databases were searched for relevant studies: PubMed, EMBASE, Cochrane library (updated 12 October 2023). All randomized trials comparing perioperative immunotherapy combined with chemotherapy versus chemotherapy alone in resectable non-small cell lung cancer were eligible for inclusion. Data were analyzed using Review Manager 5.4.1 (Cochrane collaboration software). Primary outcomes and measures included overall survival (OS), event-free survival (EFS), pathological complete response (pCR), major pathological response (MPR), R0 resection rate, rate of underwent surgery and adverse events (AEs).

Results: A total of 2912 patients (1453 receiving perioperative immunotherapy plus chemotherapy and 1459 receiving chemotherapy alone) were included in this systematic review and meta-analysis. The result showed that compared with chemotherapy alone, combined therapy significantly improved OS (HR = 0.68;95% CI: 0.56-0.83), EFS (HR = 0.58;95% CI: 0.51-0.65), pCR (OR = 7.53;95% CI: 4.63-12.26), MPR (OR = 5.03;95% CI: 3.40-7.44), R0 resection (OR = 1.58;95% CI: 1.152.18) and rate of underwent surgery (OR = 1.25;95% CI: 1.04-1.49). However, combination therapy was associated with higher risk of severe adverse event (OR = 1.46;95% CI: 1.19-1.78; P=0.0002), grade 3 and higher treatment-related adverse event (TRAE) (OR = 1.25;95% CI: 1.06-1.49; P=0.010), TRAE that led to interruption (OR = 1.90;95% CI: 1.34-2.68; P=0.0003) and immune-related adverse event (OR = 2.78;95% CI: 2.18-3.55; P<0.00001).

Significant benefits were observed across most subgroups of EFS and pCR. However, no statistical differences were observed for EFS of never smoked (HR = 0.73;95% CI: 0.51-1.05) and EGFR-mutation positive (HR = 0.35;95% CI: 0.04-3.03).

Conclusion: This systematic review and meta-analysis found superior efficacy associated with perioperative immunotherapy plus chemotherapy compared with chemotherapy alone in both tumor regression and prolonged survival in resectable NSCLC, but increased the risk of TRAE, so monitoring for adverse events is warranted.

Systematic review registration: https://www.crd.york.ac.uk/prospero, identifier (CRD42023476786).

KEYWORDS

perioperative immunotherapy, immune checkpoint inhibitors, chemotherapy, resectable non-small cell lung cancer, meta-analysis

1 Introduction

Lung cancer is one of the most common malignancies in the world and one of the primary causes of death, among which non-small cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer diagnoses (1, 2). About 20% of patients with NSCLC are diagnosed at stage I or II, which are eligible for surgical resection (3). However, 80% of the patients with advanced NSCLC diagnosed at stage III or IV, meaning that they are not suitable for surgical resection (4). For resectable NSCLC, surgery is still the most common treatment option (5). Nonetheless, for unresectable NSCLC, the survival benefit of surgery is not ideal. In addition, the occurrence of local recurrence early after surgery poses great challenges to the long-term survival of patients. Resectable NSCLC is a refractory disease with a poor prognosis and a 5-year survival rate of just 36% (6). Currently, the progress of radiotherapy, chemotherapy, immunization and targeted therapy has improved the survival of patients with resectable NSCLC (7). However, results from an important phase III randomized trial showed that neoadjuvant chemoradiotherapy associated with superior OS, pCR, and R0 resection compared with chemotherapy alone. Nevertheless, neoadjuvant chemoradiotherapy did not result in longer EFS and OS, but pCR was still as high as 16% (8, 9). In addition, targeted therapy decreased the risk of postoperative recurrence, and the resection rate was higher than that traditional neoadjuvant chemotherapy containing platinum, but pCR had not been observed (10, 11). Therefore, how to optimize the treatment strategy has become a crucial topic to explore urgently. In recent years, neoadjuvant immunotherapy has increasingly become the focus of treatment for resectable NSCLC. Compared with chemoradiotherapy and targeted therapy, neoadjuvant immunotherapy can not only significantly reduce tumor size, but also bring greater survival benefit to patients (12). Previous studies have demonstrated the potential benefits of immunotherapy at different stages of NSCLC. For patients with high expression of PDL-1, PD-1 inhibitors significantly prolonged the median OS in first-line treatment, which showed better benefits than chemotherapy (13, 14). In second-line treatment, immunotherapy also demonstrated a significant survival benefit (15). Especially in recent years, abundant evidence-based medical evidence has been accumulated in many Exploratory research, such as neoadjuvant immunotherapy and chemotherapy, double-adjuvant immunotherapy and immune monotherapy. CheckMate-816 (16) was the first phase III clinical trial to evaluate the safety and efficacy of neoadjuvant immunotherapy combined with chemotherapy versus chemotherapy alone in resectable NSCLC. This study utilized neoadjuvant nivolumab in combination with chemotherapy without postoperative adjuvant immunotherapy. Analysis of OS showed that neoadjuvant immunotherapy plus chemotherapy decreased the risk of death and distant metastasis. Besides, there is a trend of OS benefits. Another study, IMpower010 (17), confirmed that adjuvant immunotherapy that perioperative immunotherapy significantly improved the pCR and OS in resectable NSCLC. However, the potential beneficiary population for perioperative immunotherapy plus chemotherapy is currently

Abbreviations: NSCLC, non-small-cell lung cancer; SAE, severe adverse event; TRAE, treatment-related adverse event; irAE, immune-related adverse event; ECOG, the Eastern Cooperative Oncology Group; HR, hazard ratio; OR, odds ratios; ICIs, immune checkpoint inhibitors; OS, overall survival; pCR, pathological complete response; EFS, event-free survival; MPR, major pathological response; AE, adverse event; 95% CI, 95% confidence intervals; MRD, minimal residual disease.

not well defined, and the safety and efficacy of this treatment still need to be evaluated by brought survival benefits to NSCLC which indicated that the combination of neoadjuvant immunotherapy and adjuvant immunotherapy is potentially beneficial. Based on CheckMate-816 and IMpower010, the combination of neoadjuvant immunotherapy and adjuvant immunotherapy may be a promising therapeutic method. NADIM II (18), a phase II clinical study, confirmed the sandwich cake scheme of neoadjuvant immunotherapy combined with chemotherapy followed by adjuvant therapy achieved a full range of therapeutic benefits in pCR, PFS and OS. This provides preliminary evidence for the potential value of neoadjuvant immunotherapy in the treatment of NSCLC. However, phase II clinical data are not yet mature. So more randomized controlled phase III trials are needed to ensure the efficacy of this treatment strategy (19). In addition, while neoadjuvant immunotherapy combined with chemotherapy has some advantages in patients with resectable small cell lung cancer, its safety still remains some uncertainty and requires further exploration (20). Based on this, we conducted a systematic review and meta-analysis to evaluate the efficacy and safety of perioperative immunotherapy plus chemotherapy versus chemotherapy alone in resectable NSCLC.

2 Methods

This study was registered in the PROSPERO database (CRD42023476786) and was conducted according to the preferred reporting project for systematic review and meta-analysis (PRISMA) statement (21). And this study aims to compare the efficacy and safety of perioperative immunotherapy plus chemotherapy with chemotherapy alone in resectable NSCLC.

The PICOS criteria of this meta-analysis are as follows:

Participants: patients with cytological or pathologic diagnoses of resectable non-small cell lung cancer (NSCLC).

Intervention: neoadjuvant immunotherapy combined with chemotherapy followed by adjuvant immunotherapy.

Control: neoadjuvant chemotherapy and placebo followed by placebo.

Outcomes: event-free survival (EFS) and overall survival (OS), which were reported in the form of hazard ratios. In addition, pathological complete response rate (PCR), major pathological response rate (MPR), R0 resection rate, and adverse events (AEs).

Study design: randomized controlled Phase III trial.

2.1 Search strategy

A comprehensive search of records through the PubMed, Embase and Cochrane Library databases was carried out (date of the last search: October 12, 2023). The keywords or corresponding grid terms used to search the database are: perioperative, immune checkpoint inhibitors, chemotherapy, resectable non-small cell lung cancer. The relevant bibliography of candidate articles was manually searched to identify additional studies. The proceedings of the American Society of Clinical Oncology (ASCO) and the European Society of Medical Oncology (ESMO)/European Cancer Congress (ECC) annual meetings were searched for abstract reports of relevant studies. If there was any overlapping data, the most complete and updated report was selected for inclusion in this meta-analysis. Additionally, the references from all eligible studies were manually reviewed to identify any other relevant studies.

2.2 Eligibility criteria

The inclusion criteria used to select studies in this meta-analysis were (1): patients with cytologic or pathological diagnosis of resectable NSCLC, (2) patients with an average age greater than 18 years, (3) Phase III prospective, randomized trials (RCTs) comparing perioperative immunotherapy plus chemotherapy with chemotherapy alone, (4) Studies reporting at least one of the following outcomes: overall survival (OS), pathological complete response (pCR), major pathological response (MPR), event-free survival (EFS), R0 resection rate, rate of underwent surgery and adverse events (AEs).

The exclusion criteria were listed below: (1) patients with inoperable non-small cell lung cancer; (2) phase II randomized trials, non-randomized controlled studies, basic research, retrospective studies, case reports, duplicate publications and studies for which no relevant data could be extracted; and (3) RCTs that were based on overlapping patients.

2.3 Study selection and data extraction

Two experienced investigators independently screened the records based on the established inclusion and exclusion criteria. Differences were resolved by consulting a third investigator. The investigators reviewed the literature by browsing titles and abstracts to complete an initial selection and following a full review of potentially eligible articles and the selection of eligible articles based on pre-established criteria.

Extracted data included baseline characteristics, sample size and interventions used, number of assessable patients. The primary and secondary outcome endpoints are OS, EFS, pCR, MPR, R0 resection rate, rate of underwent surgery and AEs. Two investigators independently extracted relevant data and resolved any differences by consulting a third investigator. When multiple articles contained overlapping patient series, we prioritized the extraction of outcome data from the primary articles with the largest sample size for early outcomes and the articles with the longest follow-up duration for the late outcomes.

2.4 Outcome

The results of this review include OS, EFS, pCR, MPR, R0 resection rate, rate of underwent surgery and AEs. OS is defined as

the time from randomization to death and pCR is defined as the absence of residual tumor cells after evaluation of removed tumor tissue and regional lymph, which was often used as an alternative marker for clinical trials of neoadjuvant therapy. MPR is defined as residual tumor cells which is less than or equal to 10% by pathological examination of postoperative specimens. However, the determination of MPR is susceptible to subjective factors. EFS is defined as the time from randomization to the occurrence of any event, including disease progression, discontinuation of treatment for any reason or death. R0 resection is defined as the successful removal of the tumor during surgery and the absence of residual cancer cells at the excision margin. Underwenting surgery is defined as patients receiving surgical treatment during the course of the trial. AEs, graded according to National Cancer Institute Common Terminology Criteria for Adverse Events version 4.03.

2.5 Risk of bias

Two investigators independently assessed the quality of the included trials using the Cochrane Collaboration tools with respect to randomized sequence generation, assignment concealment, blinding, incomplete outcome data, and selective outcome reporting (22). Any differences in quality assessment were resolved by consulting a third investigator.

2.6 Statistical analysis

Data were analyzed using Review Manager 5.4.1 (Cochrane Collaboration Software). These measures were either extracted directly from the articles or calculated. PCR, MPR, R0 resection rate, rate of underwent surgery and AEs were reported as odds ratios (OR) with corresponding 95% confidence intervals (95% CI). EFS and OS were reported as hazard ratio (HR) and had 95% CI. $p < 0.05$ was considered statistically significant. For effectiveness or side effects, HR > 1 or OR < 1 favored chemotherapy alone (control), while HR < 1 or OR > 1 favored combination therapy (experimental). The χ^2 (Cochran Q) and I^2 statistics will be used to assess heterogeneity between studies. A fixed-effects model is used for data synthesis unless heterogeneity is large (I^2>50%), in which case a random-effects model is used (23, 24). Funnel plots and an Egger test were adopted to investigate the potential for publication bias (25). Subgroup analysis was conducted for age, sex, smoking history, physical status, disease stage, pathological type, tumor cell PD-L1 expression level and region to assess the effect of combination therapy in resectable NSCLC.

3 Results

3.1 Study identification and quality assessment

A total of 230 articles were retrieved from the electronic databases: PubMed, EMBASE, the Cochrane Library. After excluding 32 duplicate articles, 194 articles were initially excluded based on the review of titles and abstracts. Full texts of 25 articles were reviewed, resulting in the inclusion of 5 articles in the final analysis. This meta-analysis comprised five randomized controlled trials (26–30), involving 2855 patients. Among these, one study was fully published (26), while four trials were published only in abstract form (27–30). The PRISMA flowchart illustrating the process of study identification and selection is provided in Figure 1. Since all studies included were randomized, selection and loss bias were minimized (Figures 2, 3).

3.2 Study and patient characteristics

All five studies reported detailed data on pCR and MPR. Three trials provided detailed data on OS. Five studies reported EFS. The characteristics of the five trials are reported in Table 1.

All five trials evaluated the prognostic effect of perioperative immunotherapy combined with chemotherapy versus chemotherapy alone in resectable non-small cell lung cancer. However, the five trials differed in the characteristics of patients included, immunosuppressant selection, dosing patterns and primary endpoints.

AEGEAN (26) enrolled 802 patients with IIA-IIIB (N2) NSCLC and no EGFR and ALK positive. The subjects were randomly divided into two groups and respectively received neoadjuvant durvalumab or placebo combined with platinum-containing chemotherapy for 4 cycles before surgery. And postoperative patients were treated with 12 cycles of durvalumab or placebo. The primary endpoints of the study were pCR and EFS.

CheckMate-77T (27) enrolled 461 patients with IIA to IIIB NSCLC and no EGFR or ALK mutations. Participants were randomly allocated into two groups that one received 4 cycles of neoadjuvant nivolumab combined with chemotherapy and the other received neoadjuvant chemotherapy plus placebo. Postoperatively, they were assigned to either 1 year of adjuvant

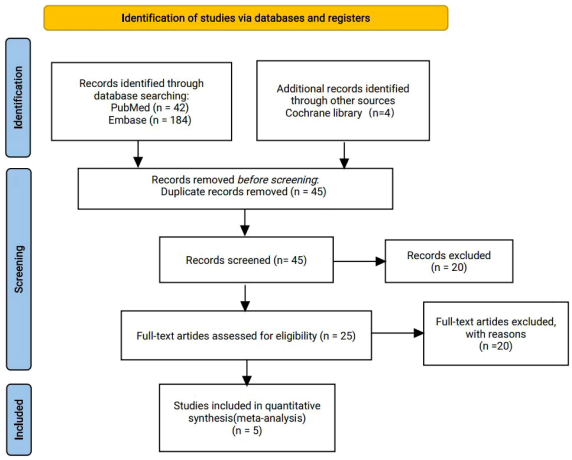

FIGURE 1
PRISMA flow diagram.

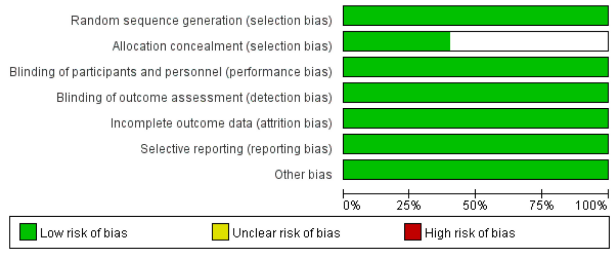

FIGURE 2
Risk of bias graph.

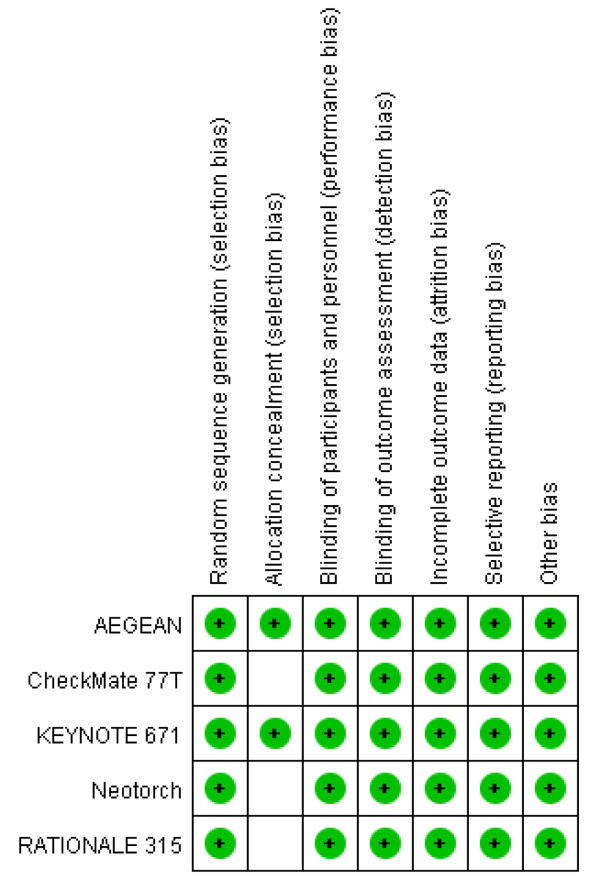

FIGURE 3
Risk of bias summary.

nivolumab treatment or adjuvant placebo treatment. The primary endpoint of this study was EFS.

KEYNOTE-671 (28) enrolled 795 patients with resectable II, IIIA, and IIIB (N2) NSCLC. Participants were randomized to receive either 4 cycles of pembrolizumab combined with chemotherapy as neoadjuvant therapy or chemotherapy combined with placebo as neoadjuvant therapy. Adjuvant immunotherapy or placebo-assisted therapy were given 13 weeks after surgery. The main endpoints of this study were EFS and OS.

Neotorch (29) enrolled 404 patients with stage III NSCLC. One group received preoperative treatment consisting of 3 cycles of toripalimab combined with chemotherapy as neoadjuvant therapy, while the other group received paclitaxel combined with carboplatin chemotherapy. After surgery, 1 cycle of adjuvant treatment with toripalimab plus chemotherapy and 13 cycles of toripalimab consolidation therapy were continued. The primary endpoints of this study include EFS in stage III patients and MPR in both stage III and II-III patients.

RATIONALE-315 (30) enrolled 453 patients with resectable II-IIIA NSCLC were included and randomly divided into two groups to receive preoperative 3-4 cycles of Tislelizumab combined with platinum double-drug chemotherapy neoadjuvant immunotherapy or platinum double-drug chemotherapy. Two to eight cycles of Tislelizumab immunoadjuvant therapy or platinum-containing chemotherapy were continued after surgery. The primary endpoints of the study were EFS and MPR.

3.3 The primary outcome

3.3.1 Overall survival

Results for OS came from three studies (28–30) involving a total of 1,652 patients. The results showed that perioperative immunotherapy plus chemotherapy further increased OS and reduced the risk of death by 32% (HR = 0.68; 95% CI: 0.56-0.83; P = 0.0002), with no heterogeneity (Chi^2 = 0.50; df = 2 [p = 0.78]; I^2 = 0%) (Figure 4).

3.3.2 Event-free survival

Results for EFS came from five studies (26–30) involving a total of 2,855 patients. Overall, patients receiving perioperative immunotherapy plus chemotherapy resulted in higher EFS (HR = 0.58; 95% CI: 0.51-0.65; P <0.00001). Additionally, moderate heterogeneity was found among the trials (Chi^2 = 5.87; df = 4 [p = 0.21]; I^2 = 32%) (Figure 5).

3.3.3 Pathological complete response

Results for pCR were extracted from 2,855 patients in three studies (28–30). The results showed that perioperative immunotherapy plus chemotherapy compared with chemotherapy alone was associated with higher pCR ((OR = 7.54;95% CI: 4.63-12.26; P <0.00001), with moderate heterogeneity (Chi^2 = 9.93; df = 4 [p= 0.04]; I^2 = 60%) (Figure 6).

3.3.4 Major pathological response

Detailed data of MPR were extracted from five studies (26–30) involving a total of 2,855 patients. Perioperative immunotherapy plus chemotherapy was associated with higher MPR (OR = 5.03; 95% CI: 3.40-7.44; P < 0.00001, Figure 3). A random-effect model was used because significant heterogeneity in the five studies was found (Chi^2 = 15.71; df = 4 [p = 0.003]; I^2 = 75%) (Figure 7).

3.3.5 R0 resection rate

Detailed data of R0 resection rate were extracted from four studies (26–29) involving a total 1,885 patients. The result indicated that perioperative immunotherapy plus chemotherapy was associated with significant benefit in R0 resection compared to

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TABLE 1 Characteristics of included studies.

	AEGEAN	CheckMate-77T	KEYNOTE-671	NEOTORCH	RATIONALE-315
Study design	randomized controlled Phase III trial				
Enrollment stage	TNM 8th II A-IIIB[N2]	TNM 8th II A-IIIB[N2]	TNM 8th II-IIIB[N2]	TNM 8th III	TNM 8th II-III A
Number of participants	802	461	795	404	453
Preoperative schedule	D 1500 mg IV +platinum-based CT Q3W for 4 cycles VS Placebo IV +platinum-based CT Q3W for 4 cycles	N 360mg Q3W + platinum-based CT Q3W for 4 cycles VS PBO Q3W +platinum-based CT Q3W for 4 cycles	P 200 mg IV Q3W + platinum-based CT for 4 cycles VS PBO Q3W+ GP or PP for 4 cycles	Tor 240mg IV + platinum-based CT Q3W for 3 cycles VS PBO + platinum-based CT Q3W for 3 cycles	Tis 200 mg IV Q3W + platinum-based CT Q3W for 3-4 cycles + VS PBO + platinum-based CT Q3W for 3-4 cycles
Postoperative schedule	D 1500 mg IV Q4W for 12 cycles VS PBO Q4W for 12 cycles	N 480mg Q4W for 1 year VS PBO Q4W for 1 year	P 200 mg IV Q3W for 13 cycles VS PBO Q3W for 13 cycles	Tor 240mg IV + platinum-based CT Q3W for 1 cycle followed by Tor 240mg IV Q3W for 13 cycles VS PBO + platinum-based CT Q3W for 1 followed by cycle PBO Q3W for 13 cycles	Tis 400mg IV Q6W for 8 cycles VS PBO IV Q6W
Primary endpoint	pCR, EFS	EFS	EFS, OS	EFS, MPR	EFS, MPR/pCR
Complete radical surgery	78% vs 77%	78% vs 77%	82% vs 79%	82% vs 73%	84% vs 76%
R0 resection	94.7% vs 91.3%	89% vs 90%	92% vs 84%	96% vs 93%	/
pCR	17.2% vs 4.3%	25.3% vs 4.7%	18.1% vs 4.0%	24.8% vs 1.0%	40.7% vs 5.7%
MPR	33.3% vs 12.3%	35.4% vs 12.1%	30.2% vs 11.0%	48.5% vs 8.4%	56.2% vs 15.0%
EFS	NR vs 25.9 m (HR=0.68)	NR vs 18.4 m (HR=0.58)	47.2 vs 18.3 m (HR=0.59)	NR vs 15.1 m (HR=0.40)	NR vs NR (HR=0.56)
grade 3 and higher AEs	42.3 vs 43.4%	32 vs 25%	45.2 vs 37.8%	63.4 vs 54.4%	69.5 vs 65.5%

pCR, pathological complete response; MPR, major pathological response (tumors with no more than 10% viable tumor cells); EFS, event-free survival; OS, overall survival; NR, not reach; CT, Computed Tomography; D, durvalumab; N, nivolumab; P, pembrolizumab; Tor, Toripalimab; Tis, Tislelizumab; NA, not available.

chemotherapy alone (OR = 1.58; 95% CI: 1.15-2.18; P = 0.005). Moderate heterogeneity was found among the trials (Chi^2 = 4.38; df = 3 [p = 0.22]; I^2 = 31%) (Figure 8).

3.3.6 Underwent surgery

Detailed data of underwent surgery were extracted from five studies (26–30) involving a total 2,854 patients. Perioperative immunotherapy plus chemotherapy was associated with higher surgical resection rate compared to chemotherapy alone (OR = 1.25; 95% CI: 1.04-1.49; P=0.02) (Figure 9) Low heterogeneity was found among the trials (Chi^2 = 4.41; df = 4 [p= 0.35]; I^2 = 9%).

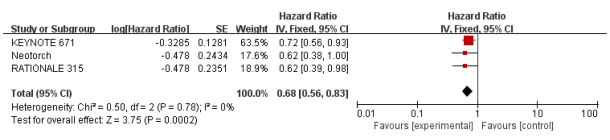

FIGURE 4
Assessment of overall survival. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); HR, hazard ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is no heterogeneity, a fixed-effects model is used.

3.3.7 Adverse events

Analysis of AEs showed that there was no statistical difference between perioperative immunotherapy plus chemotherapy and chemotherapy alone in the term of any treatment-related adverse event (TRAE) (OR = 1.52;95% CI: 0.95-2.45; P=0.08) and TRAE that led to dearth (OR = 1.12; 95% CI: 0.64-1.97; P=0.69). However, perioperative immunotherapy plus chemotherapy result in higher risk of grade 3+ TRAE (OR = 1.25;95% CI: 1.06-1.49; P=0.010), severe adverse event (SAE) (OR = 1.46;95% CI: 1.19-1.78; P=0.0002), TRAE that led to discontinuation of all trial treatment (OR = 1.90;95% CI: 1.34-2.68; P=0.0003), any iRAE (OR = 2.78;95% CI: 2.18-3.55; P<0.00001) and grade 3+ immune-related adverse event (iRAE) (OR = 2.89; 95% CI: 1.53-5.44; P=0.001) (Table 2; Supplementary Figure 1).

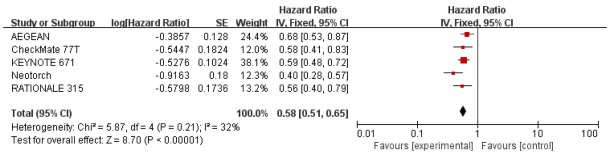

FIGURE 5
Assessment of event-free survival. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); HR, hazard ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is moderate heterogeneity, a fixed-effects model is used.

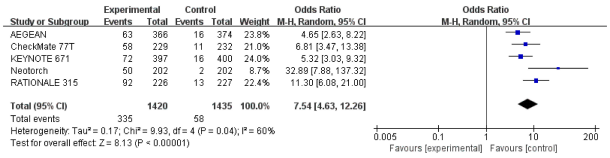

FIGURE 6
Assessment of pathological complete response. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); OR, odds ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is high heterogeneity, a random-effects model is used.

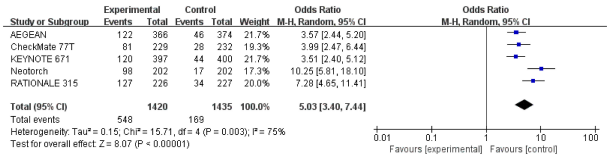

FIGURE 7
Assessment of major pathological response. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); OR, odds ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is high heterogeneity, a random-effects model is used.

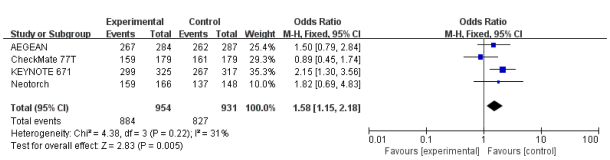

FIGURE 8
Assessment of R0 resection. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); OR, odds ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is moderate heterogeneity, a fixed-effects model is used.

3.4 Subgroup analysis

3.4.1 Subgroup analysis of EFS

Data for subgroup analysis of EFS came from five trials. Overall, no differences were observed in subgroup analyses of age, sex, ECOG performance–status score, Stage of disease, lymph node station, histologic features, PD-L1 tumor proportion score, region, planned neoadjuvant platinum agent, pCR status and MPR status. However, we found differences in subgroup analysis of smoking status, EGFR status and pathological stage. Subgroup analysis showed significant survival benefit in current smoker (HR = 0.52;95% CI: 0.38-0.70), former smoker (HR = 0.63;95% CI: 0.52-0.76) and EGFR-mutation negative (HR = 0.55;95% CI: 0.45-0.66), but no in never smoked (HR = 0.76;95% CI: 0.52-1.12) and EGFR-mutation positive (HR = 0.35;95% CI: 0.04-3.03). Both II stage (HR = 0.66;95% CI: 0.51-0.86) and III stage (HR = 0.54;95% CI: 0.43-0.63) cloud benefit from perioperative immunotherapy plus chemotherapy. Further stratified analysis of stage III patients showed significant benefit in III A stage (HR = 0.55;95% CI: 0.47-0.66) and III B stage (HR = 0.54;95% CI: 0.32-0.92) (Table 3; Supplementary Figures 2-4).

3.4.2 Subgroup analysis of pCR

Data for subgroup analysis of pCR came from three trials. In general, no differences were observed in subgroup analyses of sex, smoking status, ECOG performance–status score, pathological stage, histologic features, PD-L1 tumor proportion score, region and planned neoadjuvant platinum agent (Table 4; Supplementary Figures 5, 6).

3.5 Sensitivity analyses and publication bias

Sensitivity analysis via study-by-study removal showed that after removing Neotorch or RATIONALE-315, we found the stability of the results for R0 resection was compromised. Moreover, after removing, the stability of the results for rate of underwent surgery was also affected. However, the remaining efficacy endpoints remained stable. Qualitative assessment was performed by assessing various measures for each individual study using the Cochrane Risk of Bias Tool. In general, due to all trials were randomized, controlled, double-blind trials, they were considered to have low risk of bias. Funnel plot asymmetry was not obvious to any result (Supplementary Figures 7-12). Egger regression test results showed that EFS (0.322), MPR (0.068), R0 resection rate (0.327), rare of underwent surgery (0.220) had a low potential for publication bias, but pCR (0.008) and OS (0.039) had a significant publication bias.

4 Discussion

Resectable NSCLC mainly refers to stage I-II and some locally advanced (stage III) tumors (31). Surgery is the only radical treatment at present, but the recurrence rate is high, and

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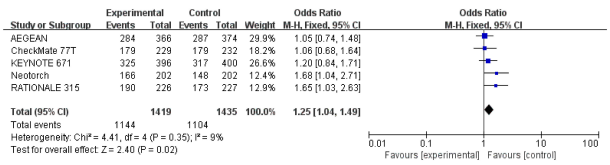

FIGURE 9
Assessment of rate of underwenting surgery. The diamond indicates best estimate of the true (pooled) outcome (with width indicating 95% CI); OR, odds ratio; experimental stands for perioperative immunotherapy combined with chemotherapy; control stands for chemotherapy alone. Since there is low heterogeneity, a fixed-effects model is used.

TABLE 2 Results of adverse events.

Toxicity	Odds Ratio	No. of trail	I^2 statistic (%)
Any TRAE	1.52 [0.95, 2.45]	4	0
Grade 3 and higher TRAE	1.24 [1.06, 1.49]	5	19
Serious TRAE	1.46 [1.19, 1.78]	4	5
Any irAE	2.78 [2.18, 3.55]	2	0
Grade 3 and higher irAE	2.89 [1.53, 5.44]	2	36
TRAE that led to death	1.12 [0.64, 1.97]	3	0
TRAE that led to discontinuation of all trial treatment	1.90 [1.34, 2.68]	3	29

TRAE, treatment-related adverse event; irAE, immune-related adverse event.

perioperative treatment cannot significantly improve the survival prognosis of patients. So even if the tumor is surgically removed, many patients may require further therapeutic interventions. Recent studies showed that perioperative immunotherapy combined with chemotherapy can improve survival benefits in patients with resectable NSCLC. For example, the previous NADIM study (18) and CheckMate-816 study (16) both confirmed that neoadjuvant immunotherapy plus chemotherapy can improve survival prognosis of patients with resectable NSCLC compared with chemotherapy alone. Particularly, in CheckMate-816 (16), three cycles of neoadjuvant nivolumab plus chemotherapy without postoperative adjuvant immunotherapy improved pCR and EFS in patients with resectable stage IB-IIIB NSCLC. This therapeutic regimen did not hind the feasibility of surgery or increase the incidence of adverse events, but showed significant survival benefit. This combination regimen will be given a brighter future. In addition, immune checkpoint inhibitors (ICI) have also shown durable response rates in NSCLC, especially in squamous cell NSCLC. A combination of neoadjuvant immunotherapy and

TABLE 3 Results of subgroup analysis for event-free survival.

Sub-Group	No. of trail	I^2 statistic (%)	Hazard ratio	P Value
Age				
<65 year old	4	0	0.54 [0.46, 0.65]	< 0.00001
≥65 year old	4	5	0.60 [0.50, 0.72]	< 0.00001
Sex				
male	4	29	0.56 [0.48, 0.64]	< 0.00001
female	4	28	0.64 [0.49, 0.83]	0.0010
Smoking status				
Current smoker	2	0	0.52 [0.38, 0.70]	< 0.0001
Former smoker	2	31	0.63 [0.52, 0.76]	< 0.00001
Never smoked	4	0	0.73 [0.51, 1.05]	0.09
ECOG performance-status score				
ECOG PS 0	3	0	0.58 [0.48, 0.71]	< 0.00001
ECOG PS 1	3	49	0.56 [0.44, 0.71]	< 0.00001
Patdological stage				
II	3	0	0.66 [0.51, 0.86]	0.002
III	4	15	0.54 [0.46, 0.63]	< 0.00001
IIIA	2	0	0.55 [0.47, 0.66]	< 0.00001
IIIB	2	84	0.54 [0.32, 0.92]	0.02
Lymph node station				
N2 single	2	0	0.56 [0.40, 0.78]	0.0007
N2 multi	2	0	0.54 [0.33, 0.90]	0.02
Histologic features				
Squamous	4	45	0.52 [0.40, 0.66]	< 0.00001
Non-squamous	4	0	0.64 [0.53, 0.77]	< 0.00001
PD-L1 expression at baseline				
PD-L1 TPS <1%	4	0	0.74 [0.61, 0.90]	0.003
PD-L1 TPS 1-49%	4	55	0.51 [0.37, 0.71]	< 0.0001

(Continued)

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TABLE 3 Continued

Sub-Group	No. of trail	I^2 statistic (%)	Hazard ratio	P Value
PD-L1 TPS ≥50%	4	37	0.45 [0.32, 0.62]	< 0.00001
Geographic region				
Asia	3	0	0.60 [0.46, 0.77]	< 0.0001
Non-Asia	3	21	0.62 [0.50, 0.76]	< 0.00001
Planned neoadjuvant platinum agent				
Cisplatin	3	0	0.59 [0.50, 0.71]	< 0.00001
Carboplatin	2	46	0.63 [0.46, 0.86]	0.004
pCR status				
pCR	2	0	0.33 [0.13, 0.86]	0.02
Non-pCR	2	0	0.72 [0.61, 0.86]	0.0003
MPR status				
MPR	2	0	0.48 [0.26, 0.86]	0.01
Non-MPR	2	0	0.77 [0.64, 0.93]	0.006
EGFR-mutation				
positive	2	72	0.35 [0.04, 3.03]	0.34
negative	4	37	0.55 [0.45, 0.66]	< 0.00001

ECOG, eastern cooperative oncology group; PS, performance-status score; PD-L1, programmed cell death-Ligand 1; TPS, tumor proportion score; pCR, pathological complete response; MPR, major pathological response; EGFR, epidermal growth factor receptor.

adjuvant immunotherapy could potentially be beneficial. Therefore, we pooled data on the efficacy and safety of perioperative immunotherapy combined with chemotherapy versus chemotherapy alone in resectable NSCLC, performing a meta-analysis to evaluate the efficacy and safety of perioperative immunotherapy combined with chemotherapy versus chemotherapy alone in the treatment of resectable NSCLC. The results indicated that perioperative immunotherapy plus chemotherapy versus chemotherapy alone significantly improved EFS, pCR, and OS in patients with resectable NSCLC. Previous meta-analysis by Marinelli et al. (32) showed that adding anti-PD (L)1 agents to neoadjuvant platinum-based chemotherapy led to improved prognosis in patients with resectable NSCLC. This meta-analysis also included patients who received neoadjuvant immunotherapy or perioperative immunotherapy. Moreover, phase II clinical studies are excluded in this meta-analysis. As we all know, the conclusions and data of phase II clinical studies are not mature and the level of evidence is not high. For example, Mobocertinib and Tiragolumab showed positive results in phase II clinical data (33, 34), but showed negative results in phase III clinical studies (35, 36). Our only included phase III clinical trials, excluding phase II clinical trials, to provide more direct and powerful evidence for the value of immunotherapy in perioperative treatment. Moreover, we also conducted subgroup analysis of EFS to further explore the influence of different factors, especially pCR status.

PCR is a predictor of long-term prognosis of neoadjuvant therapy (37, 38), which has been confirmed by some studies. Results from a research (39) showed that 5-year OS in patients who obtained pCR was 80% compared with those who did not obtain pCR, and the correlation between pCR and OS was statistically significant (P=0.0007). A retrospective study by Donington et al., which evaluated the relationship between EFS and OS in 221 patients with resectable stage II-III B (N2) NSCLC treated with neoadjuvant therapy, found a positive association between EFS and OS (0.68;95% CI: 0.52-0.79). Moreover, patients with recurrence were associated with a significantly shorter median OS (19.3 *vs.*116.9 months) and a 4.59-time increased risk of death (95%CI:2.56-8.26) compared with patients without recurrence (40). The results show that pCR and EFS can be used as alternative endpoints for survival benefit in patients with resectable NSCLC. It is worth noting that the five studies included in this meta-analysis all had EFS as their primary endpoint and all had positive results. In addition, different from the previous CheckMate-816, IMpower010 and KEYNOTE-091 studies, the OS of our meta-analysis showed statistical differences (HR = 0.68;95% CI: 0.56-0.83; P = 0.0002). So, the underlying trend in combination therapy in resectable non-small cell lung cancer is favorable, while data on overall survival require continued follow-up to mature. Besides, similar to the results of the Checkmate-816 trial, there is a higher proportion of patients achieving pCR after neoadjuvant ICI plus chemotherapy, and the patients have significantly longer EFS. Therefore, an important clinical question still remains whether adjuvant ICI monotherapy is necessary for patients who have not achieved pCR. However, it is worth noting that in the CheckMate-816 and CheckMate-77T trials, patients who did not achieve pCR all showed a trend of EFS benefit, but there was no statistical difference. A detailed study of the treatment regimen in CheckMate-816 revealed that it allowed patients to use adjuvant chemotherapy or radiotherapy, which confounded the accuracy of these results. Our meta-analysis also found significant EFS benefit for patients who did not achieve pCR after neoadjuvant immunotherapy. Additionally, although the data from CheckMate-816 showed a trend of benefit in OS, it was not statistically different. In conclusion, neoadjuvant immunotherapy plus chemotherapy alone may not achieve maximal benefit. In addition, the efficacy of adjuvant immunotherapy was explored in IMpower010 and KEYNOTE-091. The results showed the benefit of adjuvant immunotherapy in longer EFS, demonstrating the necessity of adjuvant immunotherapy. However, IMpower010 and KEYNOTE-091 did not show any OS benefit. Thus, it seems that adjuvant immunotherapy alone did not achieve maximum benefit. Based on the current data, there was no significant benefit in OS in CheckMate-816, Impower010, and KEYNOTE-091. Currently, KEYNOTE-671 study is the only one investigating neoadjuvant therapy for NSCLC with OS and EFS as primary endpoints, considering the significance of OS as the gold

standard and the follow-up time cost associated with OS as an endpoint. This study also confirms significant benefits in EFS and OS with pembrolizumab around the perioperative period, both achieving statistical differences. Similarly, Neotorch and RATIONALE-315 both confirmed significant benefits of perioperative immunotherapy in EFS and OS. Our meta-analysis also showed similar results. Compared with neoadjuvant immunochemotherapy alone, additional adjuvant immunotherapy may further eliminate residual lesions and micrometastasis. Taken together, these data may suggest that some patients could benefit from adjuvant ICI monotherapy following neoadjuvant ICI plus chemotherapy and surgical resection. In addition, from the results of a subgroup analysis of KEYNOTE-671, it appears to be a trend of greater benefit in the population with stage III. It is crucial to select appropriate candidates for perioperative immunotherapy. In addition, further exploration is needed to determine whether continuing adjuvant immunotherapy is warranted based on pathological response. DNA sequencing technology may give us an answer. With the progress of DNA sequencing technology, ctDNA (circulating tumor DNA, ctDNA) can serve as an early detection tool for cancer, to a certain extent, enabling improved treatment outcomes through early intervention. We found higher ctDNA clearance in the combination-therapy group (56%) than in the chemotherapy-alone group (35%). And patients with ctDNA clearance had longer median EFS than those without ctDNA clearance, regardless of whether they were treated with combination therapy or chemotherapy alone, the percentage of ctDNA-cleared patients achieving pCR was also higher in both treatment groups than in the ctDNA-uncleared patients (41). However, ctDNA is a common indicator of MRD. Based on the above conclusions, we consider whether the detection of MRD can further predict the risk of tumor recurrence (42). Minimal residual disease (MRD) may be helpful for the treatment decision after surgery. MRD refers to the molecular abnormalities of cancer origin that cannot be detected by traditional imaging (including PET/CT) or laboratory methods after treatment, but can be found by liquid biopsy, which represents the persistence and clinical progression of lung cancer. Zhang et al. examined peripheral blood MRD from 261 stage I to III NSCLC patients who underwent radical surgery. The result showed that adjuvant therapy can significantly improve disease-free survival in MRD+ patients, but not in MRD+ patients (43). This means that MRD - patients have a very low tumor burden and that these patients may not require adjuvant therapy. In the longer term, MRD to guide the choice of treatment mode may become a research direction Identifying and avoiding overtreatment of a potentially curable population is an important clinical issue. Further studies are still needed to stratifying patients so as to identify those who require postoperative adjuvant immunotherapy.

Our meta-analysis performed a subgroup analysis to explore the effect of baseline characteristics on EFS. The result showed differences in subgroup of smoking status and pathological stage. Notably, significant benefits were observed across most subgroups of EFS and pCR. However, no statistical differences were observed for EFS of never smoked patient. We also found that men benefited more from perioperative immunotherapy than women in the term of EFS ($P < 0.00001$ *vs* $P=0.00010$). Firstly, it (44) has been shown that sex differences in the immune system play a key role in cancer. Because men and women are born with sex chromosome differences, sensitivity to combination therapy is also different. The Y chromosome is rich in repair genes, which are consistent with anti-inflammatory M2-type tumor-associated macrophages. M2-type tumor-associated macrophages are also associated with tumor immunosuppression and poor prognosis. Therefore, this may be one reason why female benefit less from the combination therapy. In addition, data from previous studies may also explain this phenomenon. Estrogen may promote resistance to vascular endothelial growth factor targeted therapy by increasing myeloid recruitment (45). Female patients have more estrogen in their bodies, resulting in higher resistance to our combination therapy, which affects their benefit. Moreover, due to economic, social and cultural factors, the proportion of never-smoking females is larger compared to males. Although cross-study comparisons should be made with caution considering in different designs and chemotherapy regimens, there is more overlap between women and never smokers, which also provides an explanation for the lack of benefit among non-smokers. In addition, as a complex substance, tobacco can not only cause cancer, but also increase the mutation load of tumors and produce more neoantigens, which is conducive to further cancer treatment. Studies showed that carcinogens in cigarettes increased PD-L1 levels. PD-L1 helped tumor cells escape T-cell recognition and promoted tumor development. After the occurrence of tumor, the expression level of PD-L1 in tumor cells was positively correlated with the effect of immunotherapy to some extent (46). So patients who smoke will have better effects of immunotherapy than those who have never smoked. Subgroup analyses of stage IIIB suggest significant heterogeneity. In the AEGEAN study, stage IIIB patients did not benefit from perioperative immunotherapy. One possible explanation is that AEGEAN study applied PD-L1 inhibitors, and the Neotorch and KEYNOTE 671 studies applied PD-1 inhibitors. It is worth noting that Neotorch received a postoperative course of adjuvant immunotherapy plus chemotherapy followed by maintenance immunotherapy, whereas AEGEAN received only adjuvant immunotherapy. The Neotorch study designed innovatively adopted a new model of "3 + 1 + 13" perioperative immunotherapy, that is, "3 cycles of toripalimab plus chemotherapy" as neoadjuvant therapy, adjuvant therapy with 1 cycle of toripalimab plus chemotherapy and consolidation therapy with 13 cycles of toripalimab. After 1 cycle of immunotherapy plus chemotherapy, the patients were given adjuvant immunotherapy. For patients with higher tumor burden, the number and probability of residual lesions after surgery appear to be higher. Besides, immunotherapy plus chemotherapy also eliminated residual tumor cells better than immunization alone. Therefore, for patients with a higher tumor burden, the treatment mode of Neotorch may be more beneficial, which is worth further research to explore. Additional chemotherapy after surgery may allow better clearance of residual disease. Different types of immune checkpoint inhibitors may be another possible reason. AEGEAN trial and Neotorch trial included many N2 patients and the heterogeneity of patients with stage III N2 NSCLC was high. In stage III N2 NSCLC, toripalimab (a PD-1 inhibitor) plus chemotherapy resulted in longer EFS, but durvalumab (a PD-L1 inhibitor) plus

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TABLE 4 Results of subgroup analysis for pathological complete response.

Sub-Group	No. of trail	I^2 statistic (%)	Odds ratio	P Value
Sex				
male	2	56	7.70 [4.21, 14.06]	< 0.00001
female	2	0	4.38 [1.94, 9.91]	0.0004
Smoking status				
Current/ former smoker	2	59	7.10 [3.96, 12.74]	< 0.00001
Never smoked	2	0	6.19 [1.83, 21.00]	0.003
ECOG performance–status score				
ECOG PS 0	2	76	8.76 [1.04, 73.61]	0.05
ECOG PS 1	2	0	7.91 [4.36, 14.35]	< 0.00001
Patdological stage				
II	2	0	8.61 [4.65, 15.95]	< 0.00001
III	2	40	6.18 [3.51, 10.88]	< 0.00001
Histologic features				
Squamous	2	14	7.07 [4.43, 11.29]	< 0.00001
Non-squamous	2	0	6.52 [3.45, 12.34]	< 0.00001
PD-L1 expression at baseline				
PD-L1 TPS <1%	2	0	4.42 [2.41, 8.09]	< 0.00001
PD-L1 TPS 1-49%	2	16	5.10 [2.28, 11.38]	< 0.0001
PD-L1 TPS ≥50%	2	0	10.35 [4.70, 22.78]	< 0.00001
Geographic region				
Asia	2	61	9.45 [1.70, 52.53]	0.01
Non-Asia	2	0	4.76 [2.70, 8.40]	< 0.00001
Planned neoadjuvant platinum agent				
Cisplatin	2	0	7.25 [2.46, 21.37]	0.0003
Carboplatin	2	0	5.09 [3.15, 8.22]	< 0.00001

PD-L1, programmed cell death-Ligand 1; TPS, tumor proportion score.

chemotherapy did not. But there is no universal standard of treatment. According to the NCCN and CSCO guidelines, even if N2 is surgically resectable, the guidelines still primarily recommend concurrent chemoradiotherapy. Therefore, whether perioperative immunotherapy combined with chemotherapy can bring more survival benefits to more patients needs to be further explored. Moreover, patients with stage III B and patients with stage III A seem to be treated similarly, but the latter has a greater survival benefit. This is something that we need to explore. Our meta-analysis separately indicated that patients with higher PD-L1 expression had more significant benefit from perioperative immunotherapy. HR of EFS for PD-L1 TPS <1%, 1–49% and ≥50% was 0.74, 0.51 and 0.45 separately. This suggested that PD-L1 expression may be a biomarker for predicting the efficacy of perioperative immunotherapy. Subgroup analysis of EGFR status suggested that there was no clear evidence of clinical benefit with the use of perioperative immunotherapy plus chemotherapy in patient with EGFR-mutation positive.

Regarding AEs, our meta-analysis showed that perioperative immunotherapy combined with chemotherapy did not impede the feasibility of surgery. This combination did not lead to higher risk of death and any grade TRAE. However, compared with chemotherapy alone, it increased SAE, grade 3 +TRAEs and the TRAE that led to treatment interruption. This is mainly due to the fact that immunotherapy attacks tumor cells by activating the immune system, during which the immune system may attack normal tissues and result in autoimmune reactive adverse events such as rash, gastrointestinal reaction, hepatotoxicity, nephrotoxicity and so on (47). Overall, immunotherapy resulted in more severe adverse events, so monitoring for adverse events is warranted.

Neoadjuvant immunotherapy combined with chemotherapy is currently a hot treatment for resectable NSCLC. Especially, studies such as CheckMate-816 and NADIM have opened up an era of neoadjuvant immunotherapy. Perioperative immunotherapy is expected to become a better choice for patients with resectable NSCLC. Our meta-analysis also performed subgroup analyses to explore the effect of pCR status on EFS. The result showed that patients with or without pCR could benefit from Perioperative immunotherapy plus chemotherapy. Interestingly, an exploratory analysis of CheckMate-816 showed a similar pattern. But, for non-pCR population after neoadjuvant immunotherapy, the EFS HR in Check Mate-816 was 0.84, while that in KEYNOTE-671 and Check Mate-77T was 0.69 and 0.79, respectively. The NEOTORCH study showed a very good EFS benefit curve for non-pCR population, although the HR value of EFS benefit has not been calculated. These results indicated that continuing adjuvant immunotherapy is expected to further improve the prognosis of patients without pCR after neoadjuvant immunotherapy. Although the 2-year EFS rates of patients who reached pCR in CheckMate-816, KEYNOTE-671, and NEOTORCH studies were more than 90%. However, in CheckMate-816, the EFS curve of patients who reached pCR began to decline after 30-40 months of follow-up, suggesting that patients with pCR after neoadjuvant immunotherapy may need to continue adjuvant immunotherapy to further improve their survival benefits. Nonetheless, there is a lack of head-to-head study of perioperative immunotherapy and neoadjuvant immunotherapy. So, it is not clear which patients need adjuvant immunotherapy, how long immunotherapy is optimal in the adjuvant phase and can benefit from it.

This review has several advantages that it conducts subgroup analysis of resectable NSCLC to explore the effect of baseline features on perioperative immunotherapy combined with chemotherapy. Besides, five of the included studies mentioned blinding and these trials were considered to have a lower risk bias in addition to detection bias as assessed by the Cochrane Bias Risk Tool. In order to eliminate the limitation of follow-up time, we summarized the data of EFS and pCR to try to replace OS in evaluating the efficacy of perioperative immunotherapy combined with chemotherapy. Of course, our study also has limitations. First, only 5 studies were included in this meta-analysis. Second, there were some differences in the treatment protocols of the included studies. Third, the follow-up time of most studies is insufficient, which makes it difficult to evaluate the effect of perioperative immunotherapy combined with chemotherapy on OS more comprehensively, so it may lead to certain bias.

5 Conclusions

This meta-analysis found superior pCR, MPR and EFS associated with perioperative immunotherapy combined with chemotherapy in resectable stage II-IIIB NSCLC. Although the OS data is still immature, containing only three studies, it also shows a trend of benefit. Perioperative immunotherapy plus chemotherapy can also improve the R0 resection rate and the rate of surgery, but the results need to be interpreted with caution due to unstable results. The application of adjuvant immunotherapy after neoadjuvant immunotherapy plus chemotherapy remains inconclusive due to the lack of head-to-head studies. Additional studies are needed to identify patients who require adjuvant therapy. Patient, tumor, and treatment factors should be considered when using perioperative immunotherapy, as individualized therapy is the current trend. Further confirmation is still needed.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

WZ: Writing – original draft, Formal analysis, Methodology, Validation, Visualization, Conceptualization, Data curation. ZL: Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Conceptualization, Data curation. YZ: Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. YL: Software, Writing – review & editing. TC: Project administration, Writing – review & editing. WL: Software, Writing – review & editing. YC: Project administration, Validation, Writing – review & editing. PW: Project administration, Validation, Writing – review & editing. HZ: Funding acquisition, Supervision, Writing – review & editing. CF: Writing – review & editing, Supervision, Validation. LL: Funding acquisition, Supervision, Writing – review & editing, Project administration, Validation, Visualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1359302/full#supplementary-material

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Counterproductive effects of anti-CD38 and checkpoint inhibitor for the treatment of NK/T cell lymphoma

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Introduction: Natural killer/T cell lymphoma (NKTL) is an aggressive malignancy associated with poor prognosis. This is largely due to limited treatment options, especially for relapsed patients. Immunotherapies like immune checkpoint inhibitors (ICI) and anti-CD38 therapies have shown promising but variable clinical efficacies. Combining these therapies has been suggested to enhance efficacy.

Methods: We conducted a case study on a relapsed NKTL patient treated sequentially with anti-CD38 followed by ICI (anti-PD1) using cytometry analyses.

Results and Discussion: Our analysis showed an expected depletion of peripheral CD38+ B cells following anti-CD38 treatment. Further analysis indicated that circulating anti-CD38 retained their function for up to 13 weeks post-administration. Anti-PD1 treatment triggered re-activation and upregulation of CD38 on the T cells. Consequently, these anti-PD1-activated T cells were depleted by residual circulating anti-CD38, rendering the ICI treatment ineffective. Finally, a meta-analysis confirmed this counterproductive effect, showing a reduced efficacy in patients undergoing combination therapy. In conclusion, our findings demonstrate that sequential anti-CD38 followed by anti-PD1 therapy leads to a counterproductive outcome in NKTL patients. This suggests that the treatment sequence is antithetic and warrants re-evaluation for optimizing cancer immunotherapy strategies.

KEYWORDS

immunotherapy, lymphoma, T cell activation, checkpoint inhibition, combination therapy

1 Introduction

NK/T-cell lymphoma (NKTL) is a rare and aggressive form of an Epstein Barr Virus (EBV)-associated cancer with a predilection for Asian and South American populations (1). Early stage NKTL patients are typically treated with a combination of radiotherapy and chemotherapy, while L-asparaginase based regimens such as SMILE (steroid, methotrexate, ifosfamide, L-asparaginase and etoposide) are given at late stages or relapsed patients (2). However, these chemotherapy regimens typically give rise to adverse events such as high grade lymphopenia and increased infection risks, leaving SMILE refractory NKTL patients with a poor prognosis (3).

In recent years, advances in immunotherapy provide a new avenue for the treatment of cancer patients. By harnessing the body's natural defense to fight cancer cells, this approach has proven to be effective for the treatment of many cancers (4–8). One of these strategies is to destroy cancer cells by injecting depleting antibodies directed against tumor-specific surface markers. Daratumumab (anti-CD38) was found to effectively deplete multiple myeloma (MM) cells as these cells express high levels of CD38 (9). A case report showed that NKTL tumors also express CD38 and suggested that Daratumumab monotherapy may be efficacious in NKTL patients (10). A recent clinical trial however reported only limited clinical benefit for NKTL patients, casting some doubt on the usefulness of Daratumumab for this type of cancer (10, 11). More importantly, CD38 expression is not restricted to the transformed tissue. It is present on various immune subsets including B cells (12) and, as a common activation marker on NK cells (13, 14) and T cells (15–17). Thus, we hypothesize that the use of Daratumumab may thus also have some compromising effect on the immune system.

Another promising approach appears to be immune checkpoint inhibition (ICI). ICI therapies disrupt the sensing of inhibitory receptor signals delivered from the transformed tissue by tumor-specific T or NK cells to reinvigorate their cytotoxic capacity (18). Currently the most promising target is the PD1/PDL1 axis. PD1 is an immunoinhibitory molecule expressed on activated lymphocytes including CD8+ T cells (19). The ligand (PDL1) is commonly found on antigen-presenting cells and often expressed by tumor cells (20, 21). Ligation of PD1 with PDL1 inhibits T cell cytotoxicity (21, 22) and the use of anti-PD1 perturbing this receptor/ligand interaction can unleash the suppressed cytotoxicity of T cells to kill the tumor cells (23). In patients with relapsed/refractory NKTL the efficacy of ICI therapy was demonstrated with anti-PD1 antibodies such as Pembrolizumab or Nivolumab, which reportedly achieved complete response (CR) in around 30-70% of the patients (24–28).

In spite of the apparent effectiveness of ICI-based immunotherapies, they often work only in a subset of patients (24–27). A common strategy to increase treatment efficiency is through the combination with other (immune-) therapies to synergize their effects (29). For NKTL, it had been suggested that the combined use of anti-PD1 and anti-CD38 therapy may help to improve the low efficacy rates of these monotherapies (30). However, here we show that a combination of these two treatments is in fact counterproductive: the residual depleting anti-CD38 antibody (Daratumumab) effectively eliminates the effector CD8+ T cell populations that were (re-)activated by the anti-PD1 treatment and was associated with a strong upregulation of CD38. The counteracting effect of Daratumumab on checkpoint inhibition was evident more than 6 weeks after completing Daratumumab therapy. Residual levels of Daratumumab were still sufficient to deplete all effector memory CD8+ T cells, typically induced by the anti-PD1 treatment.

2 Materials and methods

2.1 Ethics approval and consent to participate

Fresh blood samples were obtained from healthy volunteers and patients in accordance to the Helsinki declaration. Healthy donor samples were collected under the SingHealth Centralised Institutional Review Board (CIRB Ref: 2017/2806) and HSA "Residual Blood Samples for Research" project titled "Blood biomarkers of immune-related diseases" (Ref: #201306-05). Patient samples were obtained under SingHealth CIRB Ref: 2004/407/F. Written informed consent was obtained from all donors prior to sample collection.

2.2 Search strategy and selection criteria for meta-analysis

The databases used to retrieve relevant articles include National Center for Biotechnology Information (NCBI) Pubmed and ClinicalTrials.gov. The keywords used are "anti-CD38 therapy, anti-PD1 therapy, anti-PDL1 therapy, combination therapy trials, cancer trials, Daratumumab, Pembrolizumab and Nivolumab". All studies with cancer patient receiving either anti-PDL1/anti-PD1 alone or in combination with anti-CD38 were included. Subsequently, trials in which patients received anti-CD38 or ICI in combination with another agent were excluded. Incomplete trials or trials with insufficient information were also not included. Anti-CD38 monotherapy studies which consists of only multiple myeloma patients were excluded from analysis since CD38 acts directly on the myeloma cells and not within the scope of this study (9). The Preferred Reporting Items for Systemic Reviews and Meta-Analyses (PRISMA) flow diagram can be found in Supplementary Figure 6.

2.3 Quantitation of genomic EBV DNA levels in patient plasma samples

Patient plasma samples were split into two fractions and treated either with Buffer RDD (Qiagen) or DNAse I (Qiagen). DNA detected from the DNAse I-treated fraction represents only virion-encapsidated DNA, while mock-treated DNA (by Buffer RDD) comprises the total DNA in the patient's plasma (cell-free

host DNA, virion-encapsidated DNA and free-floating viral DNA). An equal volume of cold phenol-saturated Tris-EDTA (10 mM Tris, pH 8.0 with 1 mM EDTA) was added to each tube followed by vigorous shaking. Samples were then centrifuged at 13,000 relative centrifugal force (rcf) at 4°C for 15 mins. An additional 100 µL of Buffer EB (Qiagen) was gently added to facilitate aspiration. The aqueous phase of DNA was extracted and stored at -20°C until use. Standard ethanol precipitation using glycogen as carrier was performed to concentrate the DNA. Buffer EB (Qiagen) was used to resuspend the purified DNA. Virus DNA quantification was performed in accordance to published qPCR protocol using 1 µL template DNA (31). Standard curve was generated using DNA from the Namalwa EBV-positive Burkitt's lymphoma cell line (32).

2.4 Anti-EBV viral capsid antigen IgG and total IgG quantification

Quantification of total IgG was performed according to manufacturer's protocol using Human IgG ELISA quantification set (Bethyl Laboratories) with heat inactivated human plasma samples diluted 1:50,000. Quantification of anti-EBV VCA IgG was performed according to manufacturer's protocol using EBV-VCA IgG ELISA kit (Calbiotech) using heat inactivated human plasma samples.

2.5 Competitive anti-CD38 staining

NK92 cells were first stained using the Fixable Aqua Dead Cell Kit (Thermofisher), followed by incubation with either with titrated amounts of Darzalex (Daratumumab) (Johnson & Johnson), or plasma from patients or healthy control and incubated for 10 mins at room temperature. The cells were then washed using MACS buffer (0.5% BSA + 2mM EDTA in PBS) followed by staining with different clones of anti-CD38: LS198-4-2 (Beckman Coulter), JK36 (Beckman Coulter) and HIT2 (BD Biosciences) for 10 mins in the dark at room temperature. The cells were then washed and analyzed using BD LSRFortessa™ cell analyzer. Data analyses were performed using Flowjo V10.5.3 (BD).

2.6 Immunophenotyping by flow cytometry

PBMCs were isolated using Ficoll-Paque density gradient centrifugation, frozen in freezing medium [10% DMSO, 90% fetal bovine serum (FBS)] and stored at -80°C until use. For staining, PBMCs were thawed using complete RPMI (RPMI+10%FBS) and washed with PBS. The cells were first stained using Fixable Aqua Dead Cell Kit (Thermofisher), followed by staining with anti-CD3 (UCHT1) (Biolegend), anti-CD4 (RPA-T4) (BD Biosciences), anti-CD8 (SK1) (Biolegend), anti-CD19 (HIB19) (BD Biosciences), anti-CD27 (O323) (Biolegend), anti-CD56 (B159) (BD Biosciences), anti-CD45RA (2H4) (Beckman Coulter), anti-CD38 (HIT2) (BD Biosciences) and anti-CD38 (JK36) (Beckman Coulter). The cells were washed and analyzed using BD LSRFortessa™ cell analyzer. Data analyses were performed using Flowjo V10.5.3 (BD)

2.7 Immunophenotyping by mass cytometry

Frozen PBMCs were thawed using complete RPMI (RPMI+10% FBS) and washed with PBS. The cells were then treated with cisplatin for 5 mins, followed by incubation with metal-conjugated surface antibodies cocktail (Table 1) for 30 mins at 37°C. Cells were washed twice with CyFACS buffer (PBS with 4% FBS, 0.05% sodium azide), followed by primary antibody staining for 30 mins on ice. Subsequently, cells were washed twice with CyFACS buffer, followed by permeabilization and fixation with

TABLE 1 List of antibodies used for mass cytometry staining.

	Marker	Clone	Company
Surface antibodies	CD3	UCHT1	Biolegend
	CD4	SK3	Biolegend
	CD8	SK1	Biolegend
	CD11c	B-ly6	BD Biosciences
	CD14	TüK4	Invitrogen
	CD16	3G8	Fluidigm
	CD19	HIB19	Biolegend
	CD25	M-A251	Biolegend
	CD27	LG.7F9	eBioscience
	CD38	HIT2	Biolegend
	CD39	A1	Biolegend
	CD45	HI30	Fluidigm
	CD45RA	HI100	Biolegend
	CD45RO	UCHL1	Biolegend
	CD56	NCAM16.2	BD Biosciences
	CD57	HNK-1	Biolegend
	CD127	A029D5	Biolegend
	CD161	HP-3G10	Biolegend
	CD197 (CCR7)	150503	R&D System
	HLA-DR	L243	Biolegend
	ICOS	C398.4A	Biolegend
	PD1	J116	eBioscience
Intracellular antibodies	Foxp3	PCH101	Invitrogen
	Helios	22F6	Biolegend
	Ki-67	B56	BD Pharmingen

Foxp3 fix/perm solution (eBioscience) for 30 mins on ice. Following this, cells were washed with permeabilization buffer (Biolegend) and then stained with intracellular antibody cocktail (Table 1) for 30 mins on ice, wash with Biolegend permeabilization buffer then stained with metal-conjugated streptavidin for 10 mins on ice. Finally, cells were washed with PBS and fixed overnight using 2% PFA made in PBS. The next day, cells were barcoded and stained with Cell-ID Intercalator-Ir (Fluidigm) in PBS for 20 mins at room temperature. Cells were washed twice with CyFACS buffer followed by a final wash using MiliQ water and passed through size filter. Filtered cells were analyzed using Helios mass cytometer (Fluidigm) with CyTOF software version 7.0.8493. Data analyses were performed using Flowjo V10.5.3 (BD) and Cytofkit2 (33).

2.8 Complement dependent cytotoxicity

NK92 cells were incubated with either Daratumumab, plasma from healthy control (HC) or combination therapy patient for 10 mins at room temperature. The cells were then washed using MACS buffer followed by incubating with serum from HC for 3hours at 37°C. Heat inactivated serum was included as negative control. After incubation, the cells were stained with Fixable Aqua Dead Cell Kit (Thermofisher). The cells were then washed and analyzed using BD LSRFortessa™ cell analyzer. Data analyses were performed using Flowjo V10.5.3 (BD)

3 Results

3.1 Treatment regime of the NKTL patient receiving combination therapy

To study the potential outcome of combining anti-CD38 (Daratumumab) and ICI treatments, we analysed the blood samples from a relapsed/refractory patient with NKTL who was treated sequentially with Daratumumab followed by ICI (combination therapy patient). The patient did not respond to conventional SMILE (steroid, methotrexate, ifosfamide, L-asparaginase and etoposide) therapy. He was then enrolled in a Daratumumab trial (ClinicalTrials.gov ID: NCT02927925) where he received 2 cycles of 16mg/kg Daratumumab (Jassen) over a period of 4 weeks (Figure 1A). As NKTL cells release EBV DNA, the tumor burden could be directly associated with the amount of EBV DNA circulating in the blood (34). The increasing levels of circulating EBV-DNA levels indicated that the patient did not respond to the Daratumumab treatment (Figure 1B) and thus the treatment was stopped. Three weeks post Daratumumab treatment (3wpD), the patient was started on 3 cycles of off-label escalating dose of anti-PD1 (Nivolumab) (Bristol Myers Squibb). Patient was given 140mg (first cycle), 180mg (second cycle) and 200mg (third cycle), with 2 weeks interval between each cycle. However, the patient also responded poorly to this treatment (Figure 1B). He developed secondary leukocytosis around 7 wpD treatment before passing on at 14wpD

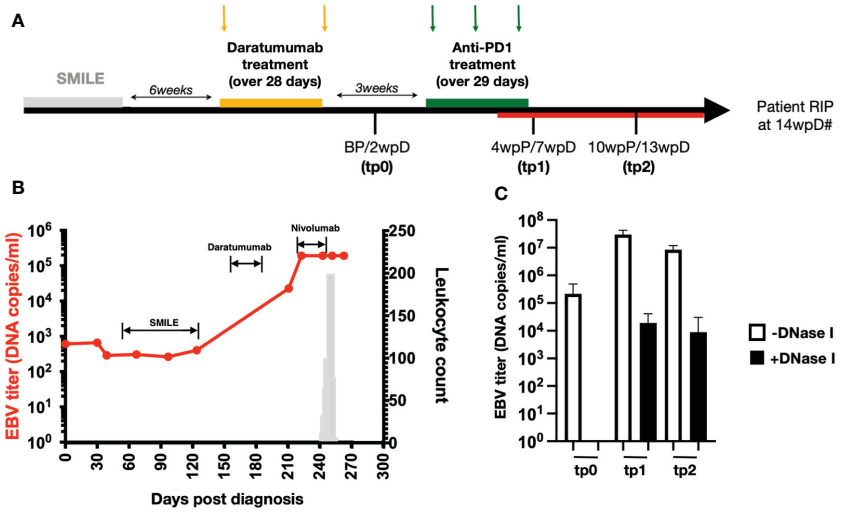

FIGURE 1
High levels of EBV load in NKTL patient who underwent combination therapy **(A)** Treatment timeline of the relapsing NKTL patient that underwent Daratumumab and anti-PD1 combination therapy (combination therapy patient). Patient was given SMILE therapy but did not respond to treatment. Six weeks after the end of SMILE therapy, patient was given 2 doses of Daratumumab over a period of 28 days. Two weeks after the last Daratumumab (2wpD) and before anti-PD1 (BP) treatment, plasma and PBMCs were collected (tp0). At 3wpD, patient was put on 3 doses of anti-PD1 (Nivolumab) treatment, spaced 15 days apart over a period of 29 days. Plasma and PBMCs were collected again at 7wpD (tp1) and 13wpD (tp2) respectively. Tp1 and tp2 also coincide with 4 weeks and 10 weeks post start of anti-PD1 (wpP) treatment. Combination therapy patient developed transient leukocytosis (indicated by red line on timeline) during anti-PD1 treatment. Hash (#) indicates demise of combination therapy patient at 14wpD. **(B)** Line graph (in red) showing plasma EBV load of the patient (left y-axis) over time after she was diagnosed with stage IVB NKTL. Labels on the chart indicate the treatment administered to the patient during that period as indicated in **(A)**. Bar chart (in grey) shows leukocyte count (right y-axis) in the patient as she develops secondary leukemia during the course of the disease. **(C)** Bar chart showing plasma EBV load in the patient at BP, tp1 and tp2 with and without DNaseI digestion prior to DNA extraction.

(Figures 1A, B). Blood samples were collected at 2wpD (tp0), 7wpD (tp1) and 13wpD (tp2), with the latter two being taken 4 and 10 weeks post anti-PD1 (wpP), respectively (Figure 1).

Both NKTL patient 1 and 2 were ICI (Pembrolizumab) (Merck) treatment responder and included as reference for ICI monotherapy.

3.2 Increased EBV viremia and reduced levels of EBV-specific IgG

Anti-PD1 treatment did not reduce the level of circulating EBV DNA, indicating that tumor burden did not decrease (Figures 1B, C). Resistance to DNase digestion indicated that a fraction of the measured viral DNA was derived from virions, suggesting re-activation of the associated-virus from the lymphoma (Figure 1C). Analysis of the plasma EBV virus capsid antigen (VCA)-specific IgG levels further showed that the patient undergoing combination therapy had only negligible levels of EBV-specific IgG in his plasma as compared to other NKTL patients who are not treated with Daratumumab, indicating a compromised humoral anti-EBV response (Supplementary Figure 1).

3.3 Effective *in vivo* depletion of CD38+ lymphocytes by Daratumumab

Daratumumab is a depleting antibody known to eliminate CD38-expressing B cells (35). This subset includes plasma cells, plasmablasts and memory B cells (36). As expected, we noted a complete absence of CD38+ B cells in the patient after the Daratumumab treatment (Figure 2A) (12). In order to confirm the observation is not due to epitope competition between anti-CD38 staining antibody (HIT2 clone) and Daratumumab bound on the cell surface, we repeated the staining using JK36, an anti-CD38 nanobody reported not to share a common epitope with Daratumumab (Supplementary Figure 2) (37, 38). Flow cytometry

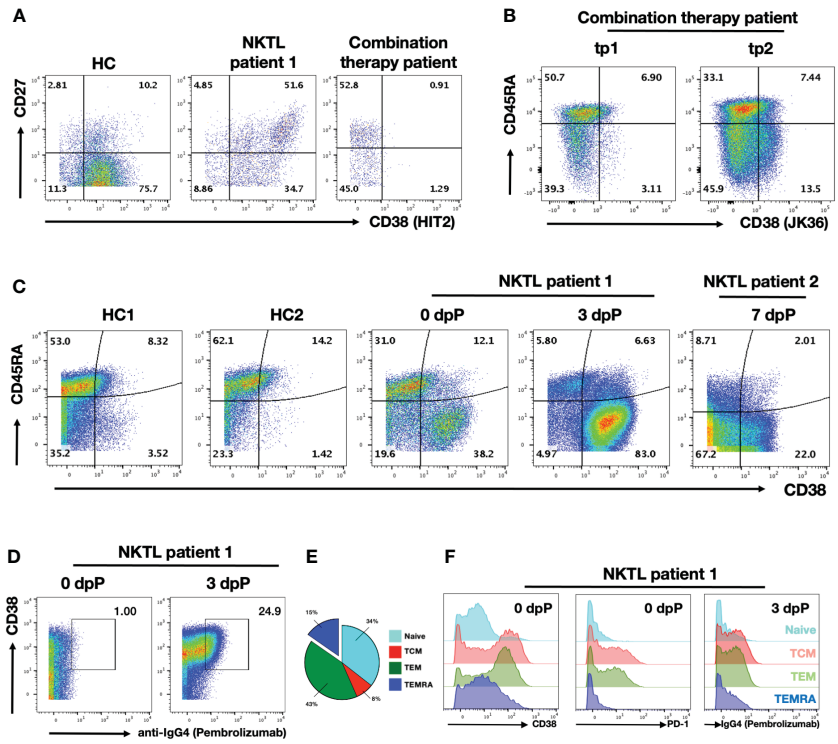

FIGURE 2
Daratumumab deplete B and activated T cells in NKTL patient undergoing combination therapy PBMC samples were obtained from healthy control (HC), NKTL patient control (NKTL patient 1) and combination therapy patient were analysed using flow cytometry. **(A)** Scatterplots showing CD27 and CD38 (HIT2 clone) expression on gated B cell subsets. Numbers in the scatterplot show the percentage of respective population within each quadrant of total B cells. **(B)** Scatterplots showing CD45RA and CD38 (JK36 clone) expressing CD8+ T cells in combination therapy patient from tp1 and tp2. Numbers in the scatterplot show the percentage of respective population within each quadrant of total CD8+ T cells. **(C)** Scatterplots showing CD45RA and CD38 (HIT2 clone) expressing CD8+ T cells in HCs and anti-PD1 treated NKTL patients (NKTL patient 1 samples obtained on 0dpP and 3dpP; NKTL patient 2 on 7dpP). Numbers in the scatterplot show the percentage of respective population within each quadrant of total CD8+ T cells. **(D)** Scatterplots showing CD38 and anti-IgG4 staining on CD8+ T cells in anti-PD1 treated NKTL patient 1 from 0dpP and 3dpP. Anti-IgG4 is used as a proxy to detect for binding of Pembrolizumab on the cells. Boxed up region shows proportion of IgG4+ cells. **(E)** Pie chart showing segregation of CD8+ T cell subsets from NKTL patient 1 into naïve (CCR7+CD45RA+), TCM (CCR7+CD45RA-), TEM (CCR7-CD45RA-) and TEMRA (CCR7-CD45RA+) based on their CCR7 and CD45RA expression prior to anti-PD1 treatment. **(F)** Histogram plots showing CD38 (left) and PD1 (middle) expression the respective on CD8+ T cells subsets in NKTL patient 1 prior to anti-PD1 treatment. Histogram plots (right) showing anti-IgG4 staining (targeting Pembrolizumab) on the respective CD8+ T cell subsets 3 days after anti-PD1 treatment.

analysis confirmed that the CD38+ B cell population is indeed missing (Supplementary Figure 2), which may explain the compromised humoral anti-viral response by the patient (Supplementary Figure 1).

While depletion of B cells and loss of antibodies is a reported side-effect of Daratumumab treatment, we also observed strong depletion of CD38+ CD8+ T cell subsets that was previously unreported (Figure 2B) (35). Interestingly, earlier report of Daratumumab depletion on T cell subsets was limited to only CD38+ Tregs (13). Anti-PD1 treatment aims to reinvigorate exhausted tumor-specific CD8+ T cells and this (re-)activation apparently triggers a substantial increase in the proportion of CD38+ CD8+ T cells (39, 40). We detected less than 10% of CD38+ CD8+ T cells at tp1 (7wpD/4wpP) (Figure 2B). The fraction of CD38+ CD8+ T cells started increasing at tp2 (10wpD/13wpP), indicating that the depleting capacity of the anti-CD38 antibody was diminishing (Figure 2B). Notably, the analysis of anti-PD1-treated NKTL patient revealed that the (re-) activation of T cells by anti-PD1 is associated with a strong upregulation of CD38 (Figure 2C). While a substantial fraction of CD38+ CD8+ T cells were already detected before the start of the therapy (day 0), the proportion of CD38+ CD8+ T cells increased substantially from 50% to 90% at day 3 after anti-PD1 treatment (Pembrolizumab) in NKTL patient 2 (Figure 2C). A similar activation was also observed in NKTL patient 1 at 7dpP (Figure 2C). Based on the CD45RA negative (CD45RA-) phenotype, these CD38+ activated cells mostly represented memory T cells (Figure 2C).

In order to confirm that these CD38+ T cells are re-activated by the binding of Pembrolizumab, we stained the cells using anti-IgG4. As Pembrolizumab is an IgG4 isotype, anti-IgG4 staining serves as a proxy for Pembrolizumab binding (41). Anti-IgG4 stained almost exclusively the CD38+ CD8+ T cells that were sampled 3 days after Pembrolizumab administration, confirming that anti-PD1 therapy is mediated through this subset (Figure 2D).

As it was previously noted that CD38+ CD8+ T cell subsets are mainly CD45RA-, we further segregated the CD8+ T cells into 4 major subsets to determine which subset Pembrolizumab is binding to. Separation of the CD8+ T cell populations into the 4 major subsets of naïve T cells (CCR7+CD45RA+), effector memory T cells (TEM, CCR7-CD45RA-), central memory T cells (TCM, CCR7+CD45RA-) and TEM expressing RA cells (TEMRA, CCR7-CD45RA+) subsets indicated that TEM and TCM comprise about half of the total CD8+ T cell population in NKTL patient 1 (Figure 2E) (42). Further characterization of these subsets confirmed that a large fraction TCM and TEM CD8+ T cells express CD38 (Figure 2F, left panel). The two subsets also expressed the highest levels of PD1 prior to the Pembrolizumab treatment indicating that these two subsets are the primary targets of ICI treatment (Figure 2F, middle panel). This was confirmed by the strong anti-IgG4 staining on both TCM and TEM 3 days after anti-PD1 treatment (Figure 2F, right panel). Notably, there were only 15% TEMRA CD8+ T cells in this patient.

3.4 Long-term persistence of active Daratumumab in circulation

In order to avoid potential side effects of the anti-CD38 therapy on ICI, the infusion of Daratumumab was stopped for 3 weeks prior to the start of the anti-PD1 treatment. However, it was unclear how long Daratumumab can remain functional in circulation. Earlier reports mentioned Daratumumab has a half-life of 21 days and around 11.5 ug/ml of circulating Daratumumab was detected at 6 weeks post infusion (38, 43). To determine the amount of circulating Daratumumab in the plasma of NKTL patient 1, we established an *in vitro* assay based on epitope competition. The anti-CD38 clone LS198-4-2 was found to be blocked by Daratumumab in a dose-dependent matter (Supplementary Figure 3). Using this antibody to stain the CD38-expressing cell line NK92 allowed us to quantify the amount of Daratumumab to concentrations as low as 500 ng/ml (Supplementary Figure 4). Using this assay to analyze plasma samples from NKTL patient 1, we estimated the amount of circulating Daratumumab in this patient to be 625, 25 and 2.5 ug/ml at 2 weeks (tp0), 7 weeks (tp1) and 13 wpD treatment (tp2) respectively. This is in line with what was previously reported (38, 43).

In order to determine whether circulating Daratumumab in the combination therapy patient is functional, we performed an *in vitro* complement dependent cytotoxicity (CDC) assay with NK92 target cells (44). Titration of Daratumumab showed effective CDC on NK92 at >400 ng/ml (Supplementary Figure 4B). The plasma samples collected at tp0, tp1 and tp2 were well above this threshold. Incubation of NK92 with these plasma samples resulted in CDC, confirming that circulating Daratumumab retains its lytic function up to 13 wpD (Figure 3B).

3.5 Anti-CD38 therapy enriches for (exhausted) PD1- TEMRA cells

Our earlier analysis on anti-PD1-treated NKTL patients suggests CD38+ TEM and TCM are most susceptible to depletion by Daratumumab treatment (Figure 2). In line with this, we found TCM and TEM to be strikingly underrepresented at tp0 (2wpD) in the combination therapy patient (Figure 4A). Almost three quarters of the CD8+ T cells from this patient were TEMRAs, which are senescent T cells with poor proliferative potential associated with poor survival in cancer patients (45).

A gradual re-emergence of TEM and proportionate reduction of TEMRA was evident at tp1 and tp2 (Figure 4A; Supplementary Figure 5). The re-emergence of TEM was also accompanied by recovery of CD38+ population at tp2 in the combination therapy patient, likely due to the waning of circulating Daratumumab at tp2 (Figures 2B, 3A). This was further confirmed by e*x vivo* staining with anti-IgG4 staining, indicating that the binding of the anti-PD1 antibody (Nivolumab) was restricted to TCM and TCM, while the bulk of TEMRA cells in this patient were not targeted by the treatment (Figures 4B, C).

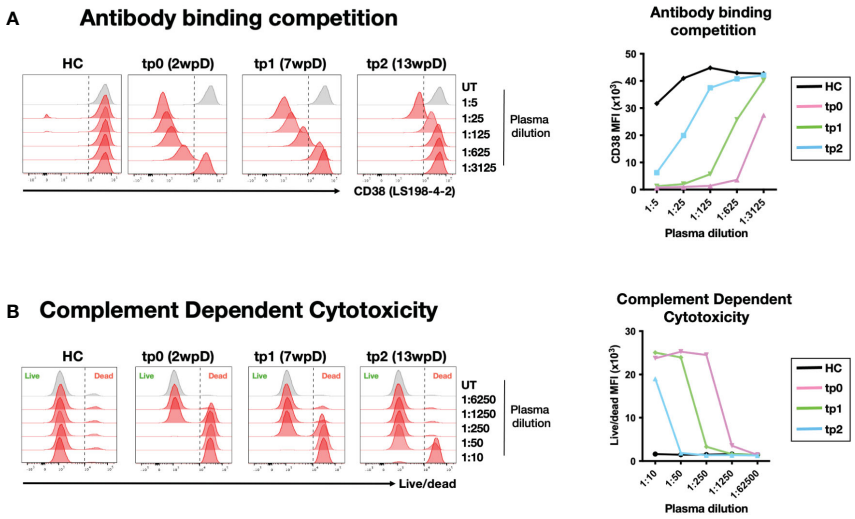

FIGURE 3
Circulating Daratumumab is functional up to 13 weeks post administration **(A)** Indirect measurement of circulating Daratumumab in combination therapy patient plasma samples. CD38-high NK92 cell line was pre-incubated with different dilution of combination therapy-treated plasma samples from tp0, tp1 and tp2 or HC. Histograms (left) and line graph showing interference of circulating Daratumumab on CD38 (clone LS198-4-2) binding using flow cytometry. Line graph (right) shows CD38 mean fluorescence intensity (MFI) on NK92 pre-incubated with combination therapy patient plasma samples. **(B)** NK92 cell line pre-treated with different dilutions of combination therapy patient plasma samples were incubated with pooled HC sera for 2-3 hours for complement dependent cytotoxicity (CDC). Cell viability was determined using live/dead staining and analysed using flow cytometry. Histogram plot (left) and line graph (right) shows live/dead MFI on NK92 after CDC was performed.

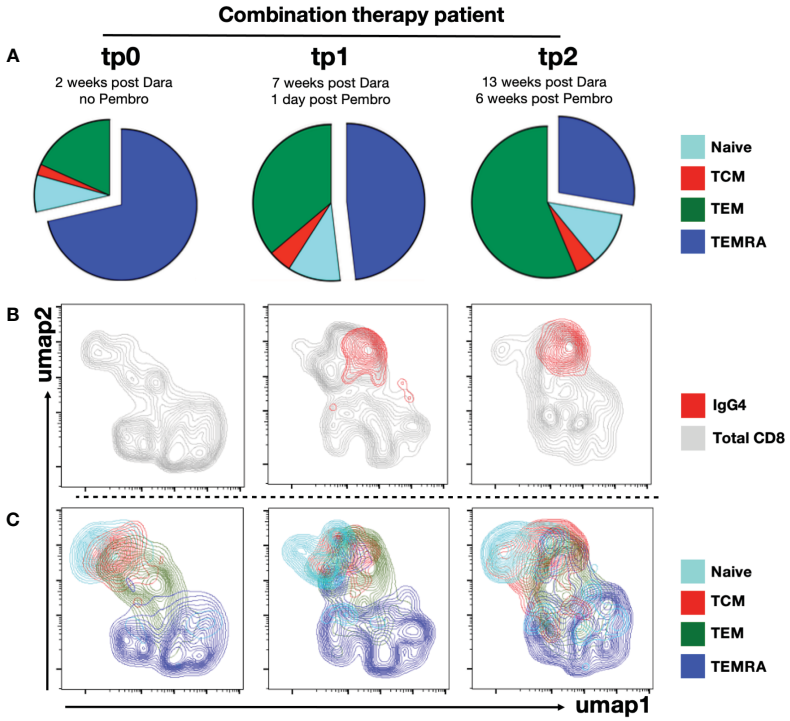

FIGURE 4
Anti-PD1 targets mainly effector and central memory CD8+ T cell subsets. PBMCs from combination therapy patient were immunophenotyped using CyTOF. CD8+ T cells were gated. **(A)** Piecharts showing distribution of into naïve, TCM, TEM and TEMRA CD8+ T cell subsets from combination therapy patient at tp0, tp1 and tp2. UMAP clustering of CD8+ T cells from combination therapy patient at tp0, tp1 and tp2 were performed. **(B)** Umap clusters of total CD8+ T cells (grey) were overlaid with anti-IgG4+ CD8+ T cells (red) or **(C)** segregated into naïve, TCM, TEM and TEMRA subsets.

3.6 Anti-CD38/ICI combination therapy is ineffective in a variety of cancer types

To assess the efficacy of combining anti-CD38 and PD1/PDL1-directed ICI in more generalizable scenario, we did a comprehensive literature search and compared the objective response rate (ORR) of small scale- and clinical trial-studies that administer either a combination of anti-CD38/ICI or ICI alone in various forms of cancer (Table 2). Taking into account all the published data of these studies, we noted a significant reduction in the ORR of patients undergoing combination (46, 62, 66) therapy as compared to ICI alone (24, 25, 27, 46–61, 63–65, 67–76) (Figure 5). This was independent of the type of PD1/PDL1 interference, as no significant difference in ORR was observed whether anti-PD1 or anti-PDL1 monotherapy was applied (Supplementary Figure 7). In our case study as well as these published combination therapies, anti-CD38 was given prior to the application of the PD1-based ICI. Thus, a combination of these two therapies in this order seem to be counter-productive (46, 62, 66).

4 Discussion

NKTL patients that failed L-asparaginase chemotherapy are usually faced with a dismal outcome (77). While ICI has shown to be a promising treatment for relapsing NKTL patients, the ORR ranges from 37-54% (11, 24, 25, 27, 61). As ICI and anti-CD38 therapy function through different mechanisms, the possibility of combining ICI and anti-CD38 to improve patient outcome was raised (30). Here we present data from a relapsed NKTL patient who

TABLE 2 List of anti-CD38 and/or checkpoint inhibitor clinical trials.

Authors	Cancer type	n	Treatment type	Anti-PDL1	Anti-PD1	Anti-CD38	ORR (%)	Phase	Age (Range)	Male (%)	Reference
Armand et al	B cell lymphoma	21	Checkpoint		Pembrolizumab	–	48	Ib	31 (22-62)	33	(47)
Armand et al	B cell lymphoma	53	Checkpoint	–	Pembrolizumab	–	45	II	33 (20-61)	43	(47)
Chung et al	Cervical cancer	98	Checkpoint	–	Pembrolizumab	–	12.2	II	46 (24-75)	33	(48)
Overman et al	Colorectal cancer	74	Checkpoint	–	Nivolumab	–	32.4	II	52·5 (44–64)	59	(49)
Fuchs et al	Gastroesophageal cancer	259	Checkpoint	–	Pembrolizumab	–	11.6	II	62.0 (24-89)	76.4	(50)
Simonelli et al	Glioblastoma	33	Combination	Atezolizumab		Isatuximab	0	I/II	55.0 (21-75)	69.7	(62)
Simonelli et al	Hepatocellular carcinoma	27	Combination	Atezolizumab		Isatuximab	7.4	I/II	62.8 (42-82)	74.1	(62)
Zhu et al	Hepatocellular carcinoma	104	Checkpoint	–	Pembrolizumab	–	17	II	68 (62–73)	83	(51)
Verset et al	Hepatocellular carcinoma	51	Checkpoint	–	Pembrolizumab	–	16	II	68 (41-91)	86	(52)
El-Khoueiry et al	Hepatocellular carcinoma	214	Checkpoint	–	Nivolumab	–	20	I/II	64 (55-70)	79	(53)
Simonelli et al	Head and neck cancer	29	Combination	Atezolizumab		Isatuximab	13.8	I/II	62.0 (40-76)	89.7	(62)
Ferris et al	Head and neck cancer	240	Checkpoint	–	Nivolumab	–	13.3	III	59 (29-83)	82.1	(54)
Guigay et al	Head and neck cancer	153	Checkpoint	Avelumab	–	–	9.2	I	63 (37–91)	81.7	(55)
Chen et al	Hodgkin Lymphoma	210	Checkpoint	–	Pembrolizumab	–	69	II	35 (18-76)	53.8	(56)
Wolchok et al	Melanoma	316	Checkpoint	–	Nivolumab	–	44	III	60 (25-90)	64	(57)
Robert et al	Melanoma	556	Checkpoint	–	Pembrolizumab	–	33	III	62 (51-71)	60.3	(58)
Nghiem et al	Merkel Cell carcinoma	50	Checkpoint	–	Pembrolizumab	–	56	II	70.5 (46-91)	68	(59)

(Continued)

TABLE 2 Continued

Authors	Cancer type	n	Treatment type	Anti-PDL1	Anti-PD1	Anti-CD38	ORR (%)	Phase	Age (Range)	Male (%)	Reference
Kaufman et al	Merkel Cell carcinoma	88	Checkpoint	Avelumab	–	–	33	II	72.5 (64.5-77)	74	(60)
Huang et al	NKTL	32	anti-CD38	–	–	Daratumumab	25	II	56.0 (22–78)	71.9	(63)
Kwong et al	NKTL	7	Checkpoint	–	Pembrolizumab	–	72	–	49 (31-68)	100	(25)
Li et al	NKTL	7	Checkpoint	–	Pembrolizumab	–	57	–	47 (17-61)	57	(24)
Tao et al	NKTL	28	Checkpoint	–	Sintilimab	–	75	II	37 (19-65)	67	(61)
Huang et al	NKTL	29	Checkpoint	CS1001	–	–	41	II	44 (30-74)	55.2	(63)
Kim et al	NKTL	21	Checkpoint	Avelumab	–	–	38	II	54 (24-78)	62	(27)
Kim et al	Non-Hodgkin lymphoma	30	Checkpoint	–	Pembrolizumab	–	23	–	49 (18-80)	70	(64)
Zucali et al	Non-small cell lung carcinoma	20	Combination	–	Cemiplimab	Isatuximab	0	I/II	65.5 (53–77)	0	(66)
Mok et al	Non-small cell lung carcinoma	637	Checkpoint	–	Pembrolizumab	–	39	III	63 (56-69)	71	(67)
Vokes et al	Non-small cell lung carcinoma	135	Checkpoint	–	Nivolumab	–	20	III	62 (39-85)	82	(68)
Vokes et al	Non-small cell lung carcinoma	292	Checkpoint	–	Nivolumab	–	19	III	61 (37-84)	52	(68)
Antonia et al	Non-small cell lung carcinoma	473	Checkpoint	Durvalumab	–	–	28.4	III	64 (31-84)	70.2	(76)
Pillai et al	Non-small cell lung carcinoma	46	Combination	Atezolizumab	–	Daratumumab	4.3	Ib/II	65.5 (38–85)	82.6	(46)
Pillai et al	Non-small cell lung carcinoma	46	Checkpoint	Atezolizumab	–	–	13	Ib/II	61.0 (30–81)	60.9	(46)
Simonelli et al	Ovarian cancer	18	Combination	Atezolizumab		Isatuximab	5.6	I/II	55.0 (35-80)	0	(62)
Zucali et al.	Prostate cancer	24	Combination	–	Cemiplimab	Isatuximab	4.2	I/II	69.5 (61–88)	100	(66)
Chung et al	Small cell lung carcinoma	83	Checkpoint	–	Pembrolizumab	–	19.3	Ib/II	62 (24-84)	63.9	(69)
Ready et al	Small cell lung carcinoma	147	Checkpoint	–	Nivolumab	–	12	I/II	63 (29-83)	58.5	(70)
Kojima et al	Squamous cell carcinoma	314	Checkpoint	–	Pembrolizumab	–	22	III	63 (23-84)	86.9	(71)
Bellmunt et al	Urothelial Carcinoma	542	Checkpoint	–	Pembrolizumab	–	21	III	67 (29-88)	74.1	(72)
Galsky et al	Urothelial Carcinoma	270	Checkpoint	–	Nivolumab	–	20	II	65.0 (38–90)	78.1	(73)
Rosenberg	Urothelial Carcinoma	310	Checkpoint	Atezolizumab	–	–	15	II	66 (32–91)	78	(74)
Balar et al	Urothelial Carcinoma	119	Checkpoint	Atezolizumab	–	–	23	II	73 (51-92)	81	(65)
Powles et al	Urothelial Carcinoma	191	Checkpoint	Durvalumab	–	–	17.8	I/II	67.0 (34-88)	71.2	(75)

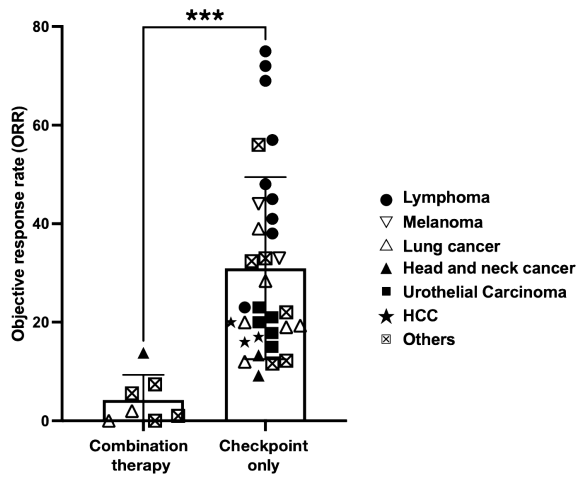

FIGURE 5
Cancer patients that received combination therapy fare significantly worse. Scatterplot comparing ORR of clinical trials performed in cancer patients that received combination therapy (anti-CD38 and immune checkpoint inhibitor (ICI) or ICI monotherapy only. ICI includes either anti-PD1 or anti-PDL1 therapy. The symbols indicate type of cancer that is being treated in the trial. Statistical analysis was performed using two-tailed Mann-Whitney U test. A p-value of less than 0.05 is considered statistically significant. (***p<0.0001*).

underwent combination therapy to demonstrate that the combined use of anti-CD38 and anti-PD1 produced antithetic effects. This was confirmed in a meta-study based on data from published cancer trials showing that ORR from combination therapies is significantly reduced as compared to ICI monotherapies.

ICI monotherapy is reportedly effective in a subset of refractory NKTL patients (24–27). As shown in the anti-PD1 treated NKTL patients, anti-PD1 treatment triggered a strong activation and expansion of the CD45RA- memory cells TCM and TEM. The two subsets expressed the highest levels of PD1 resulting in a strong binding of the anti-PD1 antibody (Pembrolizumab). As this reactivation is associated with an upregulation of CD38, these subsets were depleted by Daratumumab, leaving mostly TEMRA cells as the predominant CD8+ T cell subset in patient. TEMRAs are defined as terminally differentiated senescent memory cells with low replicative potential and typically are associated with poor anti-tumor response (42, 78).

Daratumumab is used for treating multiple myeloma (MM), a malignancy arising from CD38 high plasma cells (9, 12). It is thus unsurprising to find the patient to be severely depleted of CD38+ B cells such as plasma cells (35). Depletion of these cells likely accounted for the low anti-EBV IgG titer. The loss of antibody titer and B cell responses can also cause patients to be more susceptible to infections (79), which could be a contributing factor to the uncontrollable increase in EBV viremia and small burst of virion production as evidenced by the detection of encapsidated viral DNA.

On top of this, a more important factor associated with the failure of the combination therapy is likely the depletion of (reactivated) CD38+ T cells. Upregulation of CD38+ on activated CD8+ T cells has been widely used as a prognostic marker for various diseases, including HIV infection (15, 17) and chronic lymphocytic leukemia (80). The importance of these CD38+ leukocytes was observed in hepatocarcinoma patients, where patients with higher levels of infiltrating CD38+ cells were found to respond better to anti-PD1 treatment (81). A recent study in non-small cell lung carcinoma (NSCLC) patients also showed that a higher level of tumor-infiltrating CD38+ CD8+ T cells is correlated with better survival outcomes. In line with our own observation, their data further suggest that CD38+ CD8+ T cells are the prime target for ICI reinvigoration (82) and that CD38 was upregulated on CD8+ T cells after anti-PD1 therapy (40, 83). In agreement with these data, we also noted that Pembrolizumab was predominantly detected on CD38-expressing CD8+ T cells.

Daratumumab shows potent depleting effect. At 7 wpD treatment, we found only negligible level of CD38+ CD8+ T cells in the patient blood. High levels of circulating Daratumumab were shown to be fully functional up to 13wpD through *in vitro* CDC assay, suggesting that any activated CD38+ CD8+ T cells induced by the anti-PD1 treatment are likely being depleted by the circulating Daratumumab. This notion is supported by the re-emergence of CD38+ CD8+ T cells coinciding with waning levels of circulating Daratumumab at 13wpD. Further analysis of the re-emerging CD38+ CD8+ subsets revealed that these are CD45RA- TCM and TEM populations, the same subsets expressing PD1 and hence are targeted by anti-PD1 therapy. While the depletion of NK cells (13, 14) and B cells has been previously reported, activated CD8+ T cells are also apparently effectively removed by this antibody.

Taken together, our results show that Daratumumab can persist in the circulation for up to 13 weeks without losing its lytic function. As the activation of CD8+ T cells by the anti-PD1 treatment results in the upregulation of CD38, these reinvigorated cells become an immediate target for Daratumumab-mediated lysis, thus abrogating the effectiveness of anti-PD1 treatment. Notably, clinical trial involving the use of Daratumumab and Atezolizumab (anti-PDL1) (NCT03023423) was also halted due to the lack of clinical efficacy (46).

5 Conclusions

Despite the small sample size, this study has important clinical implications in the treatment of NKTL patients. Our results provided strong evidence to caution against the combined use of anti-PD1 and anti-CD38 agents, especially in the sequential order of anti-CD38 followed by anti-PD1.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

Healthy donor samples were collected under the SingHealth Centralised Institutional Review Board (CIRB Ref: 2017/2806). Patient samples were obtained under SingHealth CIRB Ref: 2004/407/F. Written informed consent was obtained from all donors prior to sample collection. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

WL: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal Analysis, Data curation, Conceptualization. JQL: Writing – review & editing, Writing – original draft, Data curation. TT: Writing – review & editing, Resources, Conceptualization. DT: Writing – review & editing, Resources, Methodology. SK: Writing – review & editing, Writing – original draft, Project administration, Investigation, Data curation. KP: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology. LW: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology. JL: Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology. KT: Writing – review & editing, Writing – original draft, Validation, Project administration, Methodology. WC: Writing – review & editing, Validation, Supervision. SL: Writing – review & editing, Resources, Funding acquisition. CO: Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Data curation. OR: Writing – review & editing, Writing – original draft, Supervision, Resources, Funding acquisition, Formal Analysis, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1346178/full#supplementary-material

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Treatment-related adverse events of immune checkpoint inhibitors in clinical trials: a systematic review and meta-analysis

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Aim: This study comprehensively assesses the incidence and profiles of treatment-related adverse events (trAEs) of immune checkpoint inhibitor (ICI)-based therapies across cancer at various sites.

Methods: We systematically searched the PubMed, Embase, and Cochrane databases for trials investigating ICI-based therapies published between their inception and August 2023.

Results: In total, 147 studies involving 45,855 patients met the inclusion criteria. Among them, patients treated with ICIs reported 39.8% and 14.9% of all-grade and grade ≥3 immune-related adverse events (irAEs), respectively. The most common all-grade irAEs were dermatological and gastrointestinal issues, diarrhea, and pruritus, whereas patients who received ICIs showed most common grade ≥3 irAEs, including gastrointestinal events, diarrhea, increased aspartate aminotransferase and alanine transaminase levels, and hepatic and dermatological events. The overall trAE incidence in patients treated with ICIs was 83.2% for all-grade trAEs and 38.2% for grade ≥3 trAEs. TrAE incidence was highest for patients treated with cytotoxic T lymphocyte antigen-4 inhibitors for all-grade and grade ≥3 trAEs, with incidences of 86.4% and 39.2%, respectively. ICIs combined with targeted therapy showed the highest all-grade and grade ≥3 trAEs, with incidences of 96.3% and 59.4%, respectively. The most common all-grade trAEs were anemia, decrease in white blood cell count, decrease in neutrophil count, nausea, fatigue, diarrhea, and alopecia; patients who received ICIs presented relatively high incidences of grade ≥3 trAEs.

Conclusion: This study provided comprehensive data regarding irAEs and trAEs in patients receiving ICIs. These results should be applied in clinical practice to provide an essential reference for safety profiles of ICIs.

Systematic review registration: INPLASY platform, identifier INPLASY202380119.

KEYWORDS

immune checkpoint inhibitor, immune-related adverse events, treatment-related adverse events, cancer, systematic review, meta-analysis

1 Introduction

Immune checkpoint molecules play a crucial role in the immune regulation of malignant tumors, and their biological significance is essential for the diagnosis, prognosis, and treatment of tumors (1). Checkpoints are located on various immune cells, including T lymphocytes, or on tumor cells, and they function like switch proteins by inducing various signals to control the excessive activation of T cells. T cell dysfunction may be attributed to continuous antigen exposure and the overexpression of multiple inhibitory receptors, ultimately leading to a decrease in the proliferation or function of T cells in cancer. Immune checkpoint blockade by immune checkpoint inhibitors (ICIs) primarily targets immune checkpoints expressed on the surface of immune cells, and it is a therapeutic approach that enhances the recognition and elimination of tumor cells by the immune system (2). Thus, use of ICIs is considered as a novel treatment strategy for cancer, which can inhibit tumor evasion and enhance the immune response via targeted silencing of cytotoxic T lymphocyte antigen-4 (CTLA-4) and programmed death-1/ligand-1 (PD-1/PD-L1) (3). Studies have demonstrated that targeted immune checkpoints have shown impressive antitumor activity across various types of cancer (4, 5). However, a certain proportion of patients do not respond to ICIs and show immune-related adverse events (irAEs); it is important to address irAEs in clinical practice (6, 7).

Although ICIs have significant benefits in cancer treatment, they can also cause various side effects because of checkpoints are heavily expressed in various organs other than the cancer (8–10). Although the prevalence of most serious adverse events (AEs) is low, they can still be fatal (11, 12). Moreover, considering the response rate to ICIs is important in clinical practice and ICIs combined with targeted therapies or chemotherapy are being widely used. However, there have been increasing concerns regarding the safety of ICI treatment. Furthermore, many patients do not benefit from therapy or even experience multiple irAEs; the side effects of ICIs can be devastating for the immune system and may accelerate disease progression. Thus, ICI safety profiles should be fully elucidated to achieve greater efficacy and minimize AEs.

Several systematic reviews and meta-analyses have investigated ICI adverse effects on cancer at specific sites and found that the use of ICIs could increase the risk of toxicity and treatment discontinuation (13–17). The increased risk of AEs is a challenge in the development of novel ICIs, especially for combined treatments in clinical practice (18). The safety profiles of ICI treatments should be summarized to guide clinicians in balancing the benefits and risks of therapy. Therefore, we performed this study to provide detailed toxicity profiles for ICIs and compare the incidence of AEs according to the types of cancer and ICI.

Abbreviations: AEs, adverse events; CI, confidence interval; CTLA-4, cytotoxic T lymphocyte antigen-4; ICIs, immune checkpoint inhibitors; irAE, immune-related adverse events; PD-1/PD-L1, programmed death-1/ligand-1; PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; RCTs, randomized controlled trials; trAEs, treatment-related adverse events.

2 Materials and methods

2.1 Search strategy and selection criteria

This study was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (19). Our study was registered in INPLASY platform (number: INPLASY202380119). Randomized controlled trials (RCTs) applying ICIs to cancer at various sites and reporting treatment-related adverse events (trAEs) were eligible for inclusion. PubMed, Embase, and the Cochrane library were systematically searched for eligible trials throughout August 2023, and the search terms included "immune checkpoint inhibitors" and "randomized controlled trial" (Supplementary 1). Trials that had already been completed but not yet published were searched on the https://clinicaltrials.gov website (US NIH). We manually searched the reference lists of relevant reviews and articles to avoid omitting eligible articles.

Two reviewers performed the literature search and selected the studies using a standardized approach, which refers to two authors independently conducting literature screening, followed by cross-checking the screening results. Disagreements were resolved by a third reviewer until a consensus was reached among all three reviewers. The following selection criteria were used: (1) studies designed as RCTs and published in English; (2) trials including patients who concurrently received two categories of treatments, at least one of which was an ICI (ipilimumab, pembrolizumab, nivolumab, tremelimumab, atezolizumab, durvalumab, avelumab, camrelizumab, cemiplimab, tislelizumab, toripalimab, sintilimab, adebrelimab, and sugemalimab); (3) trials reporting tabulated data of irAEs, trAEs, or specific AEs based on Medical Dictionary tor Regulatory Activities; and (4) sample size > 10. Trials that included patients treated with a combination of two classes of ICIs or patients who received sequential combination therapies were excluded. We selected the most recent trials or trials reporting a comprehensive AEs profile if the same population was published more than once.

2.2 Data collection and risk-of-bias assessment

A standardized flowchart was applied by two reviewers to extract all relevant information from the included studies, and any inconsistencies between the reviewers were resolved via discussion until a consensus was reached. The following data were collected: first author name, publication year, registered number, country, sample size, mean age, male proportion, cancer type, intervention, combined treatments, and outcomes. The primary endpoints of this meta-analysis were all-grade and grade ≥ 3 irAEs, whereas the secondary endpoints included all-grade and grade ≥ 3 trAEs and the profiles of all-grade and grade ≥ 3 specific AEs. The Cochrane risk-of-bias tool was used to assess methodological quality according to random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting, and other biases (biases associated with the

research design used, premature termination of the study, significant baseline feature imbalance, presence of deceptive behavior, and other factors) (20). Two reviewers independently assessed the quality of individual trials, and conflicts between the reviewers were resolved by an additional reviewer.

2.3 Statistical analysis

Random-effect models with a logit transformation were applied to pool the overall AE incidences and profiles, and restricted maximum likelihood estimation was used to fit all models via a classic continuity correction for zero cells and sample sizes (21). Effect estimates were calculated using incidence with a 95% confidence interval (CI), and a division method was used to calculate the incidence (22). I^2 and Q statistics were used to assess heterogeneity, and significant heterogeneity was defined as $I^2 >$ 50·0% or $P < 0{\cdot}10$ (23). Further exploratory analyses were performed to identify whether the incidence of all-grade and grade ≥ 3 irAEs and trAEs differed based on the type of ICI and combination therapy, and the differences between subgroups were compared using the interaction *t*-test (24). Publication bias was assessed using funnel plots and quantified using the Egger and Begg tests (25, 26). The *P* value for the pooled estimates was two-sided, and the inspection level was 0.05. All analyses were performed using the STATA software (version 12.0; Stata Corporation, College Station, TX, USA).

3 Results

3.1 Literature search and study selection

A total of 2,546 publications were identified from the literature searches, and 921 were excluded because of duplication. A further 1,186 articles were excluded because of irrelevant titles or abstracts. The remaining 439 studies were retrieved for full-text evaluation, and 292 were excluded for the following reasons: studies reporting the same populations (n = 156), combining two classes of ICIs (n = 65), single-arm trials (n = 43), and systematic reviews (n = 28). Manual reviews of the reference lists identified 23 articles, all of which were excluded because of duplication. Overall, 147 RCTs involving 45,855 patients were identified between 2010 and 2023, and 14 ICI types were compared in the final systematic review and meta-analysis (Figure 1).

3.2 Trial characteristics

The characteristics of the identified studies and their patients are summarized in Supplementary 2. The sample sizes of the included trials ranged from 13 to 906 participants, and the mean age ranged from 36.0 to 75.5 years. In total, 124 trials were multinational, whereas the remaining 23 were conducted in a single country. The safety profiles of ipilimumab, pembrolizumab, and nivolumab were investigated in 14, 39, and 37 trials, respectively, whereas four, 23, and 10 trials assessed the safety profiles of tremelimumab, atezolizumab, and durvalumab, respectively. Moreover, the safety profiles of avelumab, camrelizumab, cemiplimab, and tislelizumab were assessed in five, five, three, and four trials, respectively, whereas three, five, one, and one trials reported the safety profiles of toripalimab, sintilimab, adebrelimab, and sugemalimab, respectively. Supplementary 3 presents the quality of the included studies. Although 70 studies reported unclear risk of bias for allocation concealment, and 32 trials reported unclear other biases, the summary risk of bias in all trials were low.

3.3 IrAEs

The incidences of all-grade and grade ≥ 3 irAEs were 39.8% (95% CI: 24.3–55.4%) and 14.9% (95% CI: 10.5–19.3%), respectively. Moreover, we noted significant heterogeneity for all-grade (I^2 = 99.6%; $P < 0.001$) and grade ≥ 3 irAEs (I^2 = 96.3%; $P < 0.001$) in patients treated with ICIs. Exploratory analyses were performed to identify potential sources of heterogeneity, and we noted that the incidences of all-grade irAEs for patients treated with CTLA-4, PD-1, and PD-L1 inhibitors were 51.6% (95% CI: 7.3–95.9%), 32.7% (95% CI: 19.5–45.9%), and 43.9% (95% CI: 7.1–80.8%), respectively. For grade ≥ 3 irAEs, these respective percentages were 29.6% (95% CI: 10.4–48.8%), 8.8% (95% CI: 6.4–11.2%), and 16.8% (95% CI: 14.4–19.2%). When stratified by combined therapies, the incidences of all-grade irAEs for patients treated with ICIs alone, combined with singlet chemotherapy, and combined with doublet chemotherapy were 31.8% (95% CI: 7.3–56.2%), 77.7% (95% CI: 72.5–82.9%), and 46.0% (95% CI: 26.6–65.3%), respectively. For grade ≥ 3 irAEs, these incidences were 14.0% (95% CI: 6.7–21.4%), 41.7% (95% CI: 35.6–47.8%), and 12.0% (95% CI: 7.9–16.2%), respectively (Figure 2, Supplementary 4).

The incidences of specific all-grade and grade ≥ 3 irAEs are summarized in Figure 3. We noted that the incidences of all-grade dermatologic, gastrointestinal, diarrhea, and pruritus events in patients treated with ICIs were greater than 20%, as follows: 52.1% (95% CI: 30.2–74.0%), 38.8% (95% CI: 24.1–53.4%), 23.7% (95% CI: 11.2–36.3%), and 22.3% (95% CI: 13.4–31.3%), respectively. Moreover, the incidences of specific grade ≥ 3 irAEs for patients treated with ICIs were greater than 3.0%, including gastrointestinal events, diarrhea, increased aspartate aminotransferase and alanine transaminase levels, and hepatic and dermatological event as follows: 11.1% (95% CI: 1.4–20.8%), 6.3% (95% CI: 3.3–9.3%), 4.8% (95% CI: 1.1–8.6%), 4.5% (95% CI: 1.2–7.8%), 3.4% (95% CI: 0.5–6.3%), and 3.2% (95% CI: 0.9–5.4%).

3.4 TrAEs

After pooling the included trials, we noted that the incidences of any-grade and grade ≥ 3 trAEs were 83.2% (95% CI: 82.0–84.5%) and 38.2% (95% CI: 33.6–42.8%), respectively. Significant heterogeneity was observed for all-grade (I^2 = 98.5%, $P < 0.001$) and grade ≥ 3 trAEs (I^2 = 99.3%, $P < 0.001$). When stratified by ICI

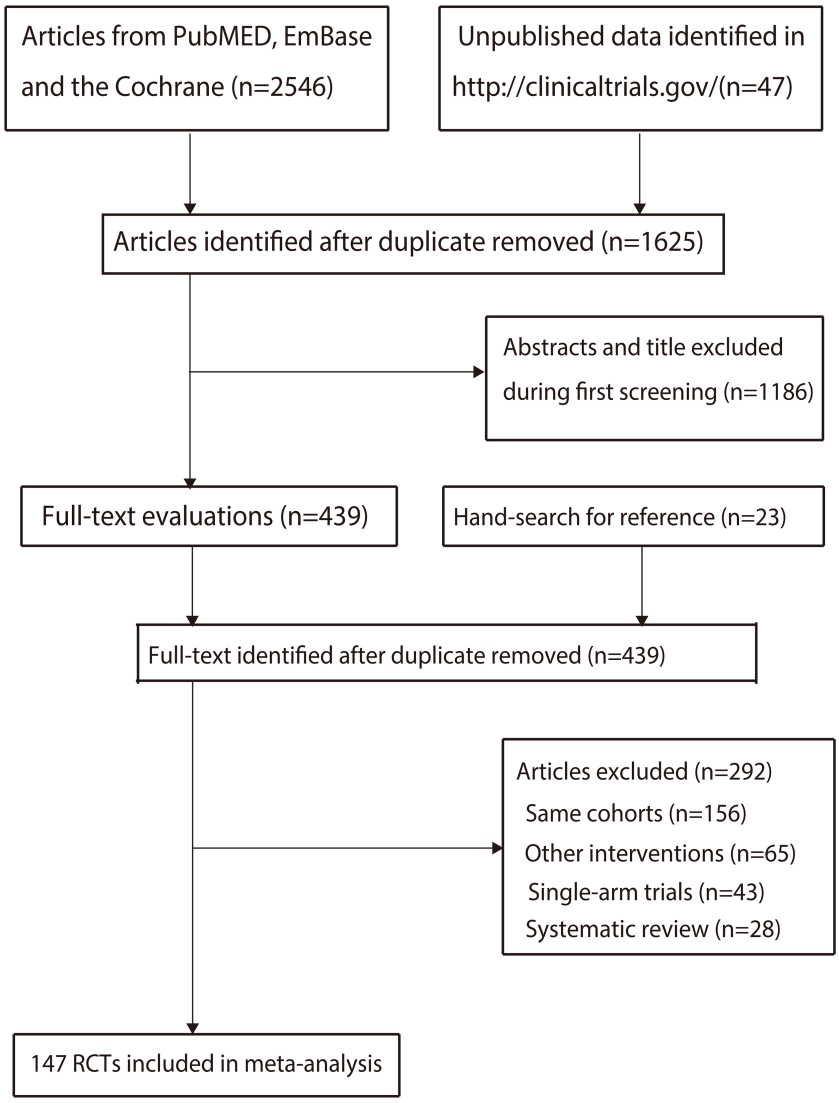

FIGURE 1
The PRISMA flowchart for trials selection process.

type, we noted that the incidences of all-grade trAEs for patients treated with CTLA-4, PD-1, and PD-L1 inhibitors were 86.4% (95% CI: 82.7–90.2%), 81.7% (95% CI: 80.0–83.4%), and 85.1% (95% CI: 82.8–87.4%), respectively; for grade ≥ 3 trAEs, these percentages were 39.2% (95% CI: 29.9–48.4%), 35.9% (95% CI: 29.9–41.9%), and 43.3% (95% CI: 34.6–51.9%). When stratified by combined therapy, the incidences of all-grade trAEs for patients treated with ICIs alone, combined with singlet chemotherapy, combined with doublet chemotherapy, combined with targeted therapy, and combined with radiotherapy were 74.4% (95% CI: 70.8–77.5%), 92.8% (95% CI: 86.4–99.2%), 93.4% (95% CI: 92.2–94.6%), 96.3% (95% CI: 94.9–97.7%), and 87.8% (95% CI: 77.8–97.8%), respectively. The respective incidences of grade ≥ 3 trAEs were 21.7% (95% CI: 18.9–24.5%), 41.8% (95% CI: 27.7–56.0%), 58.6% (95% CI: 52.2–65.0%), 59.4% (95% CI: 45.3–73.5%), and 24.4% (95% CI: 11.2–37.5%) (Figures 4, Supplementary 4).

3.5 Specific trAEs

The incidences of specific all-grade trAEs are summarized in Figure 5. We noted that the incidences of anemia, decreased WBC count, decreased neutrophil count, nausea, fatigue, diarrhea, and alopecia for patients treated with ICIs were greater than 20%, as follows: 27.3% (95% CI: 23.5–31.2%), 24.0% (95% CI: 19.9–28.0%), 23.9% (95% CI: 20.5–27.4%), 23.6% (95% CI: 21.0–26.2%), 23.0% (95% CI: 20.8–25.3%), 21.7% (95% CI: 19.4–24.0%), and 20.7% (95% CI: 18.4–22.9%), respectively. Moreover, the incidences of specific grade ≥ 3 trAEs, including decreased neutrophil count, neutropenia, decreased WBC count, anemia, hypertension, and decreased platelet count were greater than 5%, as follows: 15.5% (95% CI: 13.7–17.4%), 11.5% (95% CI: 10.2–12.8%), 8.6% (95% CI: 7.2–10.0%), 7.7% (95% CI: 6.8–8.5%), 7.5% (95% CI: 5.9–9.0%), and 5.9% (95% CI: 4.7–7.1%), respectively (Figure 6).

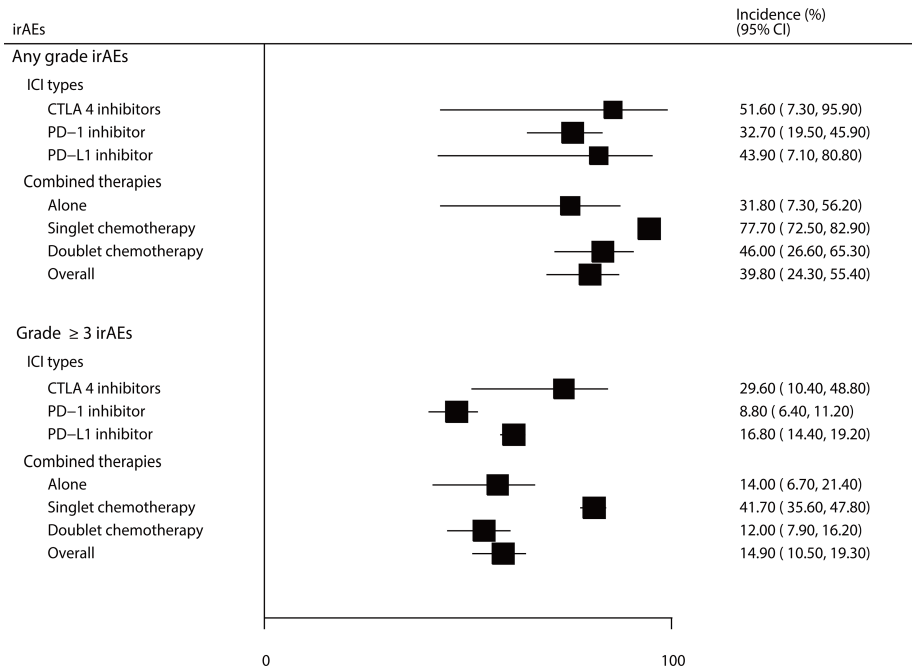

FIGURE 2
The summary incidences for all-grade and grade ≥ 3 irAEs.

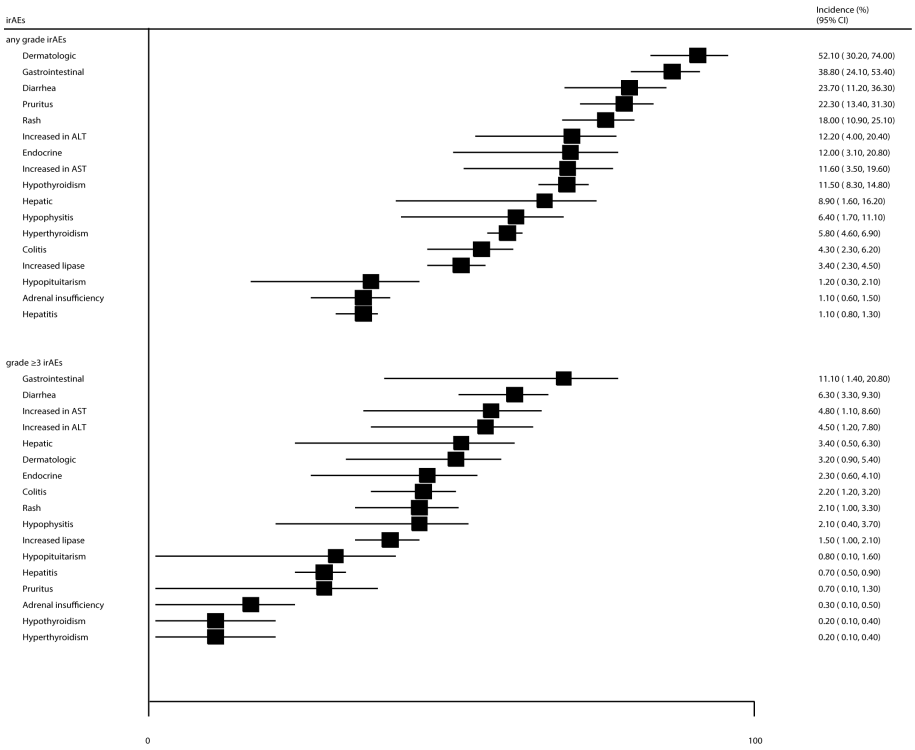

FIGURE 3
The summary incidences for all-grade and grade ≥ 3 specific irAEs.

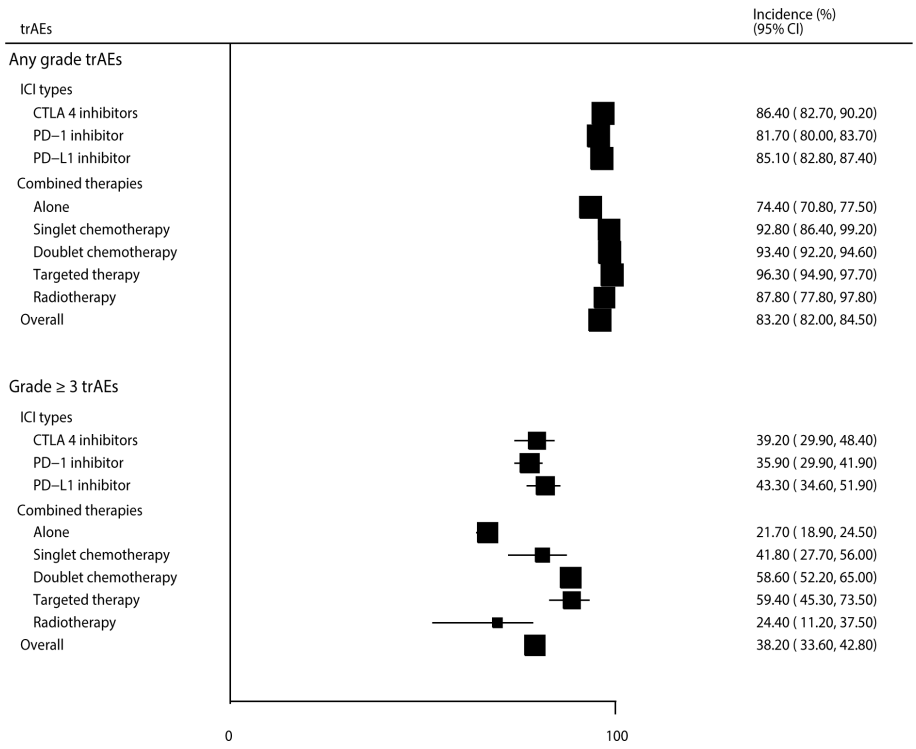

FIGURE 4
The summary incidences for all-grade and grade ≥ 3 trAEs.

3.6 Publication bias

There were significant publication biases for grade ≥ 3 irAEs and all-grade or grade ≥ 3 trAEs (Supplementary 5), and the conclusions were stabilized for all-grade trAEs and reduced for grade ≥ 3 irAEs and trAEs after adjusting the potential publication bias using the trim and fill method (27).

4 Discussion

This comprehensive, quantitative, systematic review and meta-analysis was based on 147 RCTs involving 45,855 patients with cancer at various sites who were randomly treated with 14 different ICIs. The present study is comprehensive as 14 different ICIs as well as all-grade and grade ≥ 3 irAEs and trAEs were included. After reviewing current published trials, we noted that ICIs were always combined with chemotherapy or targeted therapies, and more than half of the patients reported at least one AE. Grade ≥ 3 irAEs and trAEs were not rare, especially for patients receiving CTLA-4 inhibitors or combined targeted therapies. Moreover, the most common all-grade and grade ≥ 3 irAEs and trAEs should be monitored carefully to balance the benefits and adverse effects of ICI therapies.

Several systematic reviews have illustrated the safety profiles of ICIs for cancer treatment at various sites (17, 22, 28–32). Zhou et al. (22) comprehensively assessed the incidences and safety profiles of trAEs among various combination therapies based on 161 RCTs and found that all-grade and grade ≥ 3 trAEs were higher for patients receiving PD-1 or PD-L1 inhibitors combined with chemotherapy or targeted therapies. Inno et al. (28) identified 49 studies and found that the incidence of all-grade and grade 3–4 AEs was 52.2% and 21.5%, respectively, in patients treated with ICIs. Dolladille et al. (29) identified 63 RCTs and reported that ICI use was associated with myocarditis, pericardial disease, heart failure, dyslipidemia, myocardial infarction, and cerebral arterial ischemia. Gu et al. (30) identified 14 RCTs to assess the comprehensive safety profiles of ICIs in patients with advanced non-small cell lung cancer and showed that pembrolizumab caused severe dermatologic irAEs and colitis, nivolumab caused severe endocrine irAEs, and atezolizumab caused severe pneumonitis when combined with platinum-based chemotherapy. Xu et al. (17) investigated the safety profiles of ICIs for esophageal cancer and found that most AEs of combined therapies were tolerable, and all-grade pneumonitis differed between the PD-1 and PD-L1 inhibitor groups. Mei et al. (31) identified 33 RCTs and found that camrelizumab or avelumab combined with chemotherapy showed higher incidences of all-grade AEs, whereas durvalumab and sintilimab could be considered relatively safe PD-L1 and PD-1 inhibitors. Longo et al. (16) identified seven RCTs and found that ICI-based combined treatment was associated with a high risk of grade 3–5 trAEs in patients with small cell lung cancer. Hao et al. (32) showed that ICIs + nab-paclitaxel/paclitaxel were associated with a lower risk of irAEs than that seen with ICI monotherapy.

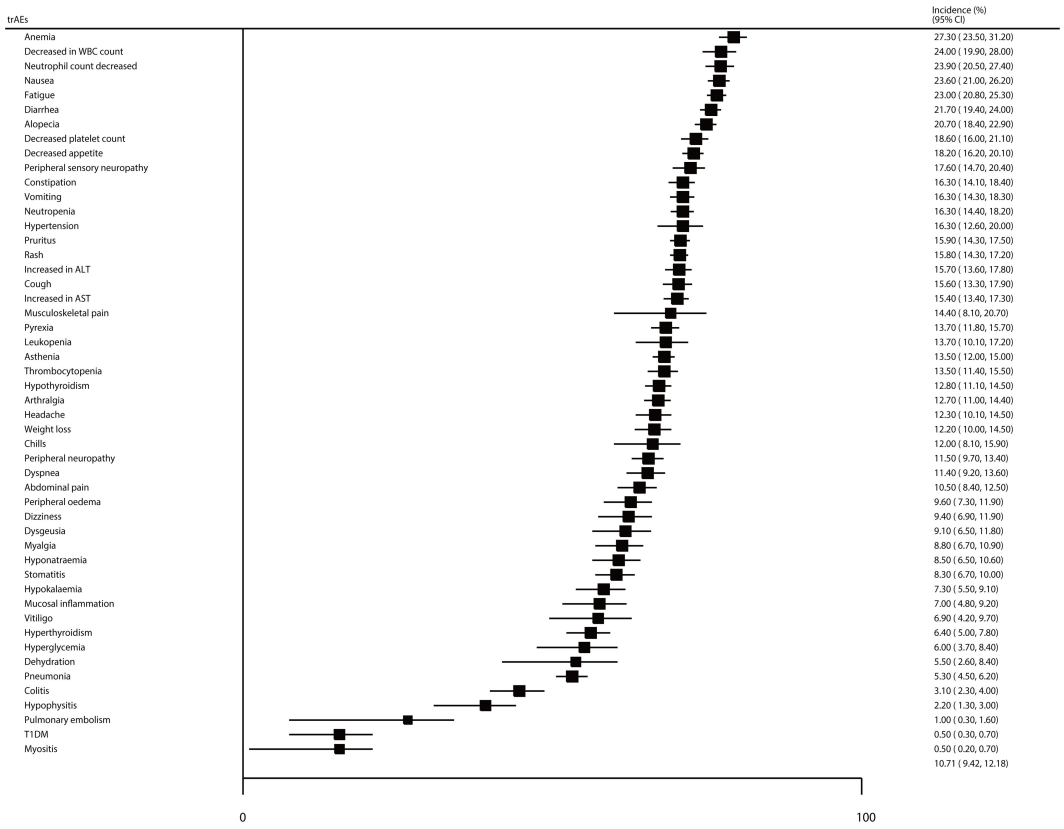

FIGURE 5
The summary incidences for all-grade specific AEs.

However, previous systematic reviews focused on the safety profiles of specific types of ICIs or in patients with specific cancers. Thus, the current study was performed to extend previous systematic reviews and comprehensively illustrate the safety profiles of ICIs in patients with cancer at various sites.

Our study found that the incidence of all-grade irAEs was higher in patients treated with CTLA-4 inhibitors or ICIs combined with singlet chemotherapy. The reason for the higher risk of irAEs in patients receiving CTLA-4 inhibitors could explained by T cell development at an earlier stage was blocked by CTLA-4 that could directly disrupt central tolerance (33). However, the high risk of irAEs related to the combination of ICIs and singlet chemotherapy might be because only one trial has reported such an outcome; this trial specifically reported 77.7% of any-grade irAEs and 41.7% of grade ≥ 3 irAEs (34). This apparent increase in the AE incidence related to ipilimumab could explain its combination with dacarbazine, which was associated with an increased risk of hepatotoxic events (35, 36). After removing this specific trial, we noted that the incidence of irAEs did not increase rapidly when combined with other antiangiogenic agents. Furthermore, we noticed decreased incidences of irAEs with PD-1 inhibitors compared to that seen with PD-L1 inhibitors, which was not consistent with the findings of previous meta-analyses (32). The binding of PD-1 to both PD-L1 and PD-L2 could be blocked by PD-1 antibody, presenting more comprehensive inhibition of the immune escape pathway (37). The combination regimens were also found to affect the incidence of irAEs, and further meta-analysis should be performed to compare the risk of irAEs between PD-1 and PD-L1 inhibitors. Moreover, the most common irAEs related to ICIs were dermatological and gastrointestinal, whereas the most severe ones were gastrointestinal and endocrine disorders, which should be carefully monitored in clinical practice. Finally, although the incidence of colitis was low, most cases were severe.

Similarly, the incidences of all-grade and grade ≥ 3 trAEs were higher for patients who received ICIs, and the most common trAEs were hematologic toxicity, including anemia, decreased WBC count, and decreased neutrophil count. As expected, these hematological toxicities could be explained by the use of ICIs combined with chemotherapy or radiotherapy (38). Moreover, combined treatments could explain the higher incidence of all-grade alopecia; most trAEs were tolerable, and only 0.4% of patients reported grade ≥ 3 alopecia. Several trAEs related to ICIs are noteworthy, including hypertension, hematological and gastrointestinal disorders, especially those associated with the concomitant use of CTLA-4 inhibitors or targeted therapies (33).

This study had several limitations. First, the incidence of irAEs and trAEs was obtained based on MeDRA in individual trials,

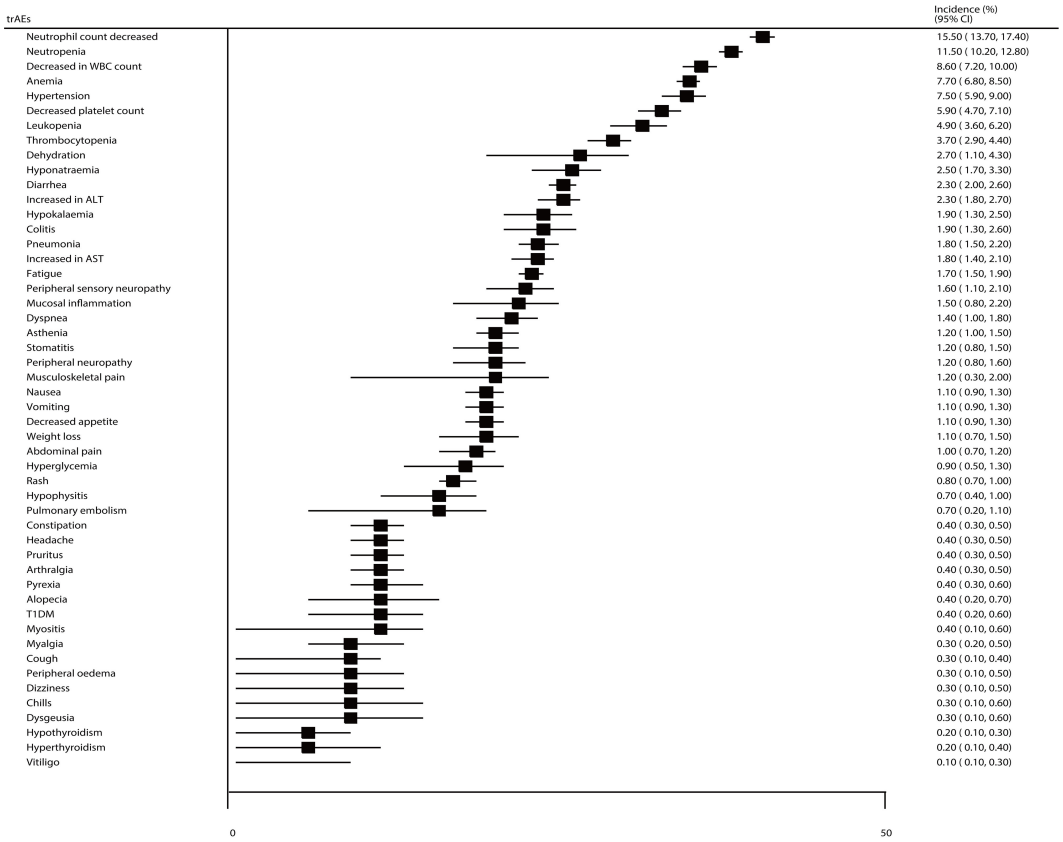

FIGURE 6
The summary incidences for grade ≥ 3 specific trAEs.

whereas some cases presented overlapping MeDRA definitions. Second, there was significant heterogeneity in irAEs, trAEs, and mostly specific AEs, which were not fully explained by stratified analyses based on ICI types and combined therapies. Third, the differences between various ICIs and combined therapies were compared indirectly, and further direct comparisons of results should be explored in large-scale real-world studies. Fourth, the combination treatments and cancer sites differed among included trials, which could affect the incidence of irAEs and trAEs. Further study should address the combination treatments for patients with specific cancer. Finally, the inevitable publication bias restricted the detailed meta-analysis of published data.

5 Conclusions

Our study systematically summarized the safety profiles of irAEs and trAEs associated with ICIs in patients with cancer at various sites. We noted that CTLA-4 inhibitors showed a higher risk of irAEs and trAEs than PD-1 or PD-L1 inhibitors. Moreover, the combination of ICIs and targeted therapies presented a higher risk of trAEs, whereas the risk of irAEs was not affected by combined therapies. The results of this study provide a clinical reference to balance the benefits and harms of ICIs treatment.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Author contributions

XS: Conceptualization, Formal Analysis, Investigation, Writing – original draft. JY: Conceptualization, Formal Analysis, Investigation, Writing – original draft. GQ: Data curation, Writing – review & editing. MS: Data curation, Writing – review & editing. YW: Data curation, Writing – review & editing. GL: Data curation, Writing – review & editing. JQY: Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2024.1391724/full#supplementary-material

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Case report: Regression after low-dose glucocorticoid therapy in a case of acute immune myocarditis induced by anti-PD-1 therapy for NSCLC

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Background: PD-1 inhibitors exhibit efficacy in managing unresectable/metastatic driver gene-negative NSCLC, albeit with potential immune-related adverse events (irAEs). Among these, immune checkpoint inhibitor-associated myocarditis (ICI-M) is rare yet lethal. This study presents the initial successful instance of ICI-M in a lung cancer patient, rescued by low-dose glucocorticoids post-deterioration during treatment.

Case summary: A 78-year-old male with a medical history of stage IV pT3N2M1 NSCLC underwent four cycles of palliative chemotherapy, resulting in stable disease (SD). Subsequent to declining further chemotherapy, the patient was transitioned to a targeted therapy regimen comprising Anlotinib in conjunction with PD-1 inhibitor immunotherapy. On the 26th day post-administration of the PD-1 inhibitor, the patient manifested Grade 2 immune-mediated myocarditis. Treatment encompassing 1mg/kg methylprednisolone combined with immunoglobulin shock therapy was initiated for 3 days, achieving symptomatic control. Nonetheless, upon tapering methylprednisolone dosage to 4–8mg/3–4d, the condition deteriorated, necessitating transfer to the intensive care unit. Methylprednisolone dosage was escalated to 80mg/day for 3 days, followed by gradual reduction by one-third to two-thirds weekly, culminating in the patient's safe discharge from the hospital.

Conclusion: Immune-related myocarditis linked to checkpoint inhibitors is often managed effectively with high-dose glucocorticoid therapy. However, in Asian populations, low-dose glucocorticoids are increasingly utilized for salvage therapy, yielding favorable outcomes and improving prognosis compared to European populations.

KEYWORDS

PD-1 inhibitor, immune myocarditis, non-small cell lung cancer, low-dose glucocorticoid, methylprednisolone

1 Introduction

Immune checkpoint inhibitors (ICIs) represent a significant advancement in cancer therapy in recent years. Primarily, their mechanism entails the inhibition of immune checkpoint activity via antibodies, thereby restoring and augmenting effector T lymphocyte function to selectively identify and eradicate tumor cells (1). PD-1 is a prototypical agent among ICIs. Nonetheless, treatment with PD-1 monoclonal antibodies targeting tumor cells may paradoxically exacerbate autoimmune responses, leading to immune-related adverse events including hypothyroidism, rash, myocarditis, pneumonitis, and enteritis.

ICI-M is classified as a rare immune-related adverse reaction, distinguished by early onset, nonspecific symptoms, rapid progression, and high mortality rates (2–5). The prevalence of ICI-M varies from 0.06% to 3.8%, with severe myocarditis occurring in approximately 0.09% of cases (3, 5, 6). Its clinical manifestations are varied, presenting typical symptoms including malaise, chest pain, anxiety, and dyspnea, which may progress to respiratory distress, and in severe cases, cardiogenic shock and sudden death.

This article reports a case of an elderly patient with advanced lung adenocarcinoma who developed ICI-M after treatment with the PD-1 inhibitor sintilimab. In terms of hormone dosage, there is a controversy between Chinese and European guidelines. 2021 CSCO related guidelines recommend an initial methylprednisolone dose of 1–4 mg/kg/d, and ESMO of the European Society of Medical Oncology believes that when myocarditis is suspected, high-dose shock therapy with methylprednisolone 0.5–1 g/d for 3–5 days should be given, which may be related to hormone sensitivity. This case demonstrates that early low-dose hormones are also effective benefits in salvage therapy for immune-related myocarditis in Asian populations compared to high-dose shock therapy in European Europe.

2 Case description

The patient, a 78-year-old male, underwent "Thoracoscopic radical treatment of right upper lung cancer with pleural adhesion branding and intercostal nerve closure" in March 2021. Postoperative pathology revealed poorly differentiated adenocarcinoma. Follow-up chest CT evaluation indicated pathological complete response (pCR). MRI of both hips suggested the possibility of left iliac metastasis. The AJCC staging was determined as pT3N2Mx, corresponding to stage IIIB. The patient received four cycles of postoperative palliative care, with evaluation indicating stable disease (SD). Subsequently, the patient declined further chemotherapy and was prescribed Anlotinib targeted therapy. In December 2021, PET-CT scan revealed left iliac metastasis, and the diagnosis was updated to pT3N2M1, corresponding to stage IVB. On December 20, 2021, the patient received the first cycle of sintilimab treatment at a dose of 200 mg/dose q3w. The baseline assessment of cardiac enzymes, thyroid function, cortisol, adrenocorticotropic hormone, and electrocardiogram were unremarkable. The patient received the second cycle of sintilimab at the same dosage on January 10, 2022. However, on January 16, 2022, the patient was admitted to the hospital with complaints of "chest tightness and dyspnea". The patient had a history of hypertension for more than 1 year, with a peak blood pressure of 150 mmHg/110 mmHg, which remained untreated. The patient denied any history of diabetes mellitus or coronary artery disease, and had no recent history of upper respiratory tract infections or exposure to toxins or radioactive substances. Chest CT revealed postoperative alterations in the right lung consistent with previous findings. The electrocardiogram demonstrated corresponding ST segment changes in select leads. Troponin I levels were elevated at three times the normal range, myoglobin levels were elevated at 29 times the normal range, creatine kinase levels were elevated at 19 times the normal range, creatine kinase isoenzyme levels were elevated at 8 times the normal range, lactate dehydrogenase levels were elevated at 3 times the normal range, while amino-terminal brain natriuretic peptide precursors pro-BNP and D-dimer levels were within normal limits. Echocardiogram combined with myocardial perfusion imaging revealed normal ejection fraction, with no significant abnormalities in left ventricular wall motion or ventricular diastolic function, and normal left ventricular myocardial perfusion. Coronary angiography demonstrated a right dominant coronary artery distribution pattern with a normal left main coronary artery and atherosclerosis in the left anterior descending artery (LAD) with TIMI grade 3 distal flow. Atherosclerosis was also noted in the left circumflex artery (LCX) with TIMI grade 3 distal flow, while the right coronary artery (RCA) exhibited normal anatomy with TIMI grade 3 distal flow. There was insufficient evidence of acute coronary syndrome (ACS) based on these findings (Figure 1).

In conjunction with the pertinent examinations, immune-related myocarditis G2 with class II cardiac function was considered. The patient's weight was 50 kg. Immediate administration of methylprednisolone 40 mg for 4 days in combination with immunoglobulin 20 mg for 4 days in accordance with the relevant 2021 CSCO guidelines (initial methylprednisolone dose 1–4 mg/kg/d). The patient's symptoms improved significantly, and creatine kinase decreased by 84%, creatine kinase isoenzyme decreased by 70%, lactate dehydrogenase decreased by 33%, and myoglobin decreased by 42%. The dosage was reduced to methylprednisolone 32mg/d for 4 days, and then reduced to 28mg/d after the symptoms stabilized. Subsequently, the patient manifested recurrent chest tightness and dyspnea, indicative of exacerbated symptoms, and was subsequently diagnosed with Type 2 respiratory failure accompanied by respiratory acidosis. Troponin levels exhibited an elevation compared to previous measurements, necessitating transfer to the intensive care unit for initiation of mechanical ventilation to address the respiratory insufficiency. Concurrently, hormonal therapy was intensified to a dosage of 40mg/d. However, clinical manifestations persisted unabated, with negligible alterations observed in certain cardiac enzymes, and troponin levels remained elevated. Transthoracic echocardiography revealed no notable

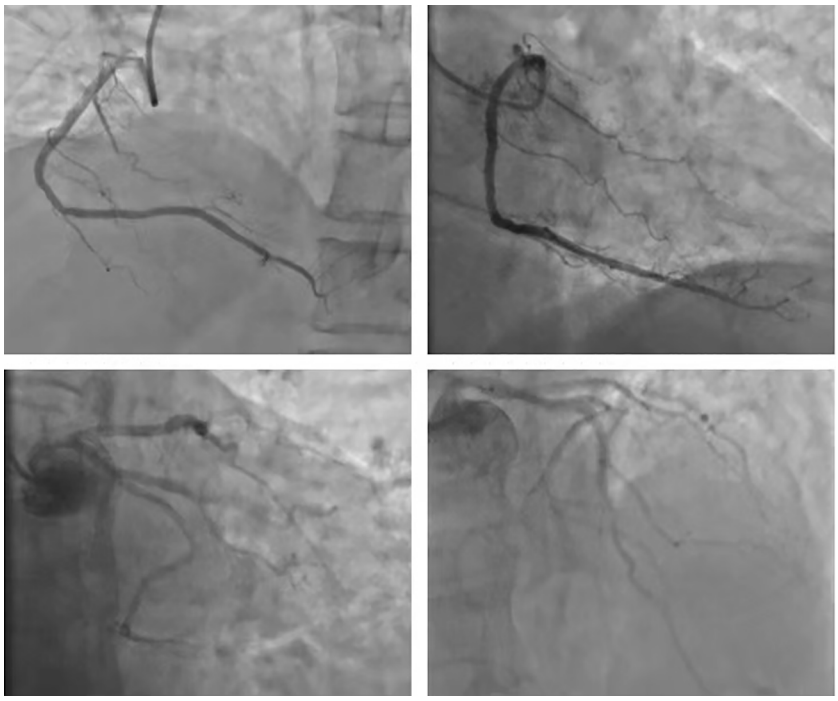

FIGURE 1
Coronary arterial photovation: coronary blood supply right advantage type; LM normal, LAD arteriosclerosis; remote blood flow TIMI 3; LCX arteriosclerosis, remote blood flow TIMI level 3; RCA normal arteriosclerosis, remote blood flow TIMI level 3 level 3 level 3, ACS evidence is not sufficient.

abnormalities in ventricular wall motion at rest and preserved left ventricular systolic function.

Based on the aforementioned information, progression of ICI-M from Grade 2 to Grade 3 was acknowledged, leading to adjustments in the treatment protocol. Methylprednisolone at a dose of 80mg/d was administered for a duration of 3 days alongside immunoglobulin at a dosage of 20mg/d, also for 3 days. Subsequently, the dosage was tapered to 40mg/d of methylprednisolone for 12 days, with subsequent reductions of 10mg/d every week. After two weeks, the dose was changed to 15 mg/d prednisone tablets for one week. Final monitoring demonstrated a 95% decrease in troponin I levels, a 66% decrease in myoglobin levels, a 98% decrease in creatine kinase levels, a 95% decrease in creatine kinase isoenzyme levels, and a 20% decrease in lactate dehydrogenase (Figures 2–4). Then the patient was safely stabilized and discharged to continue 5 mg/d prednisone tablets for a month (Figure 5). (5mg prednisone is equivalent to 4mg methylprednisolone).

Continued follow-up has been conducted via telephone, with the patient reporting no recurrence of symptoms such as chest tightness, dyspnea, or shortness of breath. Overall, the patient is capable of performing daily activities with minimal assistance.

3 Discussion

In 2020, there are projected to be an estimated 2.2 million incident cases of lung cancer and 1.8 million deaths attributable to this disease. Non-small cell lung cancer (NSCLC) represents over 85% of all lung cancer cases, with adenocarcinoma being the most prevalent histologic subtype, accounting for 40% of cases (7). In recent years, the advancement of immunotherapy in the field of lung cancer research has resulted in significant prolongation of patient survival. However, the associated adverse effects should not be underestimated. Among immune-related adverse events (irAEs), immune myocarditis is the rarest yet most deadly, with myocarditis carrying a mortality rate of 40% to 50% (5, 8, 9). A case report published in the New England Journal of Medicine in 2016 unveiled, for the first time, two instances of fulminant and fatal

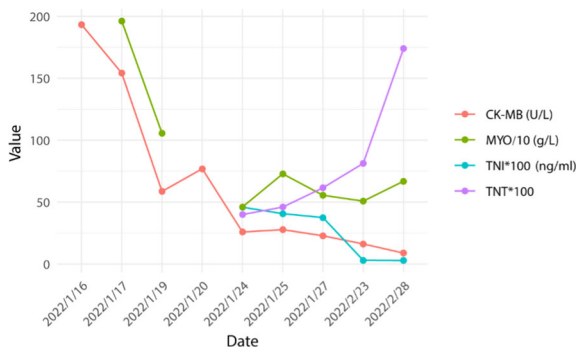

FIGURE 2
Trend of myocardial enzyme changes.

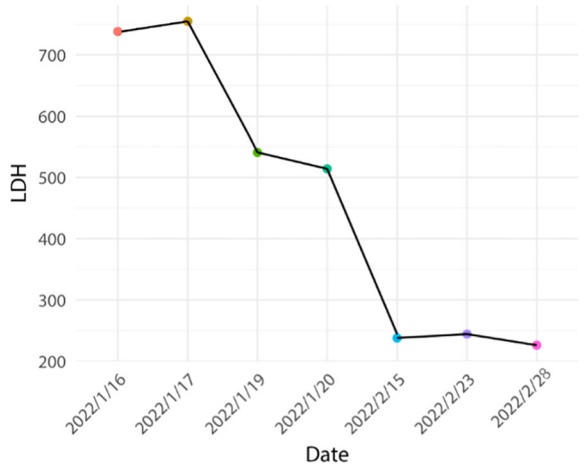

FIGURE 3
Trend of lactate dehydrogenase changes.

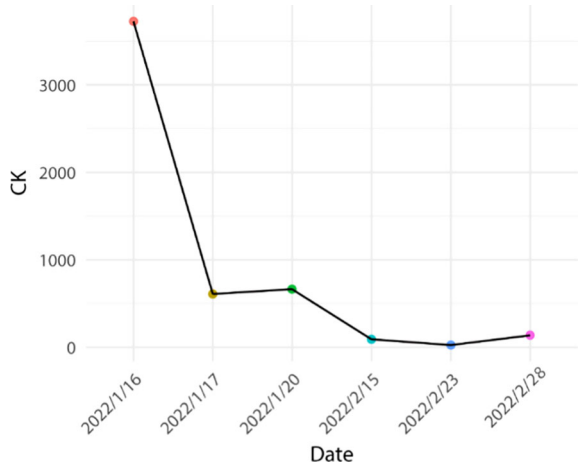

FIGURE 4
Trend of creatine kinase changes.

myocarditis induced by PD-1 monoclonal antibody (2). This discovery garnered global attention. The frequency of immune checkpoint inhibitors (ICIs)-associated myocarditis varies from 0.06% to 3.8%, with severe myocarditis occurring in approximately 0.09% of cases, and a median onset time of 17–34 days. Some patients experience severe myocarditis after receiving only 1–2 doses of ICIs, predominantly within 3 months of initiation of therapy (3, 5, 6). The elevated rates of morbidity and mortality associated with myocarditis present a significant challenge to both physicians and patients.

PD-1, an immunomodulatory checkpoint receptor expressed on activated T cells' surface, engages with its ligand, inducing T cell dysfunction. This mechanism serves as a crucial regulator of immune tolerance in peripheral tissues and areas of chronic inflammation (10, 11). Inhibiting PD-1 or its ligands with PD-1/PD-L1 inhibitors activates dormant T cells, enhancing tumor cell clearance while potentially triggering non-specific targeting of healthy cells by activated T cells, thereby predisposing to immune-related adverse events (12). The occurrence of prevalent T-cell receptor sequences with high frequency in cardiac, skeletal muscle, and tumor tissues of patients experiencing ICI-M (2, 13–15), along with the observed imbalance in PD-1 to PD-L1 expression induced by immune checkpoint inhibitors, thereby impeding the binding of PD-1/PD-L1 to the discordant ligand, may constitute precipitating elements in myocardial autoimmune reactions (16).

The varied clinical presentations of PD-1-induced ICI-M, often featuring asthenia, angina, anxiety, and dyspnea, alongside its nonspecific nature and the absence of systolic impairment in half of the cases (3), render it prone to underrecognition, contributing to a considerably elevated rate of misdiagnosis of PD-1-induced ICI-M in the initial phases of immunotherapeutic intervention. Furthermore, alternative forms of cardiomyopathy that bear resemblance to myocarditis have been linked to ICIs therapy, including Takotsubo syndrome (17).

There are currently no established standardized diagnostic criteria for PD-1-induced ICI-M. In this scenario, coronary angiography was employed to exclude acute coronary syndrome (ACS). Subsequently, endomyocardial biopsy is considered the most precise diagnostic modality for immune myocarditis (18), but its utility is limited due to invasiveness and potential complications. In order to optimize the management of patients with ICI-M, the CSCO Guidelines for the Management of Toxicity Associated with Immune Checkpoint Inhibitors classify myocarditis into 4 categories based on the following criteria: Grade 1 (G1): Elevated biomarkers of cardiac injury without accompanying cardiovascular symptoms, ECG alterations or echocardiographic abnormalities. Grade 2 (G2): Mild cardiovascular symptoms with concurrent abnormalities in cardiac injury biomarkers and ECG, but no severely deranged echocardiographic findings. Grade 3 (G3): Markedly abnormal cardiac biomarkers and severely disturbed ECG/UCG, accompanied by significant symptoms at rest or upon minimal exertion. Grade 4 (G4): Severe symptoms with hemodynamic instability, life-threatening presentation requiring urgent medical intervention.

Numerous studies have demonstrated that glucocorticoids represent the optimal therapeutic approach for immune checkpoint inhibitor-related cardiotoxicity (5, 19–21). Importantly, existing evidence indicates that the administration of hormonal treatment for immune-related adverse events associated with ICIs does not compromise the efficacy of ICIs. Alternative therapies such as anti-CD3 antibodies, CTLA-4 agonists, and anti-CD52 antibodies exist; however, there is a lack of pertinent clinical trials validating their efficacy (21). Prophylactic glucocorticoid administration in PD-1/PD-L1 patients may attenuate the antitumor effectiveness of immune checkpoint inhibitor drugs, thus prophylactic glucocorticoid use is discouraged. Furthermore, in cases of hormonal resistance development, intensive immunosuppression or second-line immunosuppressive protocols should be deliberated (21).

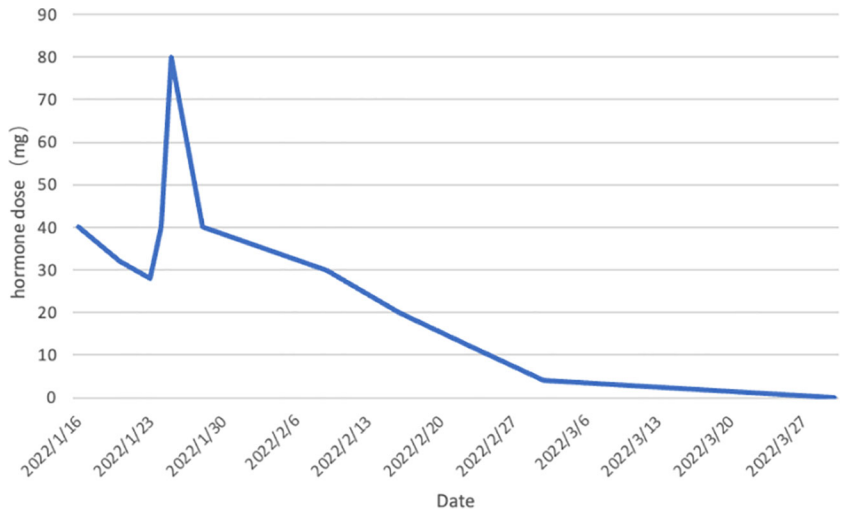

FIGURE 5
Trend of hormone dose changes. Prednisone was first administered on January 16, 2022 at 40mg.The dose was reduced to 32mg on January 20.The dose was reduced to 28mg on January 23.The patient's condition deteriorated and she was transferred to the ICU on January 24.The dose was adjusted upward to 40mg.The dose was re-adjusted to 80mg on January 25.The dose was reduced in steps until the patient's condition stabilized and she was transferred to the ICU on February 9.The dose was reduced to 5mg on March 2.The patient was discharged on March 30.The drug was discontinued on March 30.The patient's condition stabilized and he was discharged from the hospital. stabilized and was discharged from the hospital. The drug was discontinued on March 30th. (5mg prednisone is equivalent to 4mg methylprednisolone).

In this case, we postulate that the exacerbation of the patient's condition is correlated with inadequate maintenance duration during dose reduction, failing to impede further disease progression. As a recommendation, patients with ICI-M should be managed at the upper threshold of the dosing interval for maintenance duration (22, 23). The European Society of Medical Oncology (ESMO) suggests that when myocarditis is suspected, administration of a higher dose of methylprednisolone at 0.5–1 g/d for a period of 3–5 days can be considered (21). Variability in glucocorticoid sensitivity among distinct populations may exist(24), as demonstrated in this case where the patient achieved therapeutic efficacy comparable to that in the European population while following the dosage outlined in the CSCO guideline (significantly lower than the ESMO guideline). A systematic analysis of case reports of immune checkpoint inhibitor-associated myocarditis (24) showed that the most commonly used hormone dose for the treatment of immune myocarditis in China is 1–2 mg/kg/d, i.e., the low-dose hormones described in this article, and that similar use was made in two case reports of immune checkpoint inhibitor-associated hepatitis (25) and immune checkpoint inhibitor-associated rheumatic polymyalgia (26), both of which showed promising results. Concurrently, the patient was able to mitigate hormone-related adverse events such as femoral head necrosis, gastrointestinal bleeding, and secondary infections to some degree.

In individuals with previous immune-mediated myocarditis, there appears to be a modest advantage in reinitiating the same immunotherapy once markers indicating myocardial injury have normalized (27). However, the safety of this approach has not been definitively established, and there is presently a scarcity of data regarding this matter.

4 Conclusion

The onset of PD-1-induced ICI-M is exceedingly prompt, and once it is suspected that a patient is experiencing ICI-M, immunotherapies should be halted without delay, and a regimen of high-dose glucocorticoids should be initiated in accordance with the severity of symptoms and testing markers. Importantly, this instance illustrates that early implementation of low-dose glucocorticoid rescue therapy in the initial stages of immune myocarditis and gradual tapering can still achieve therapeutic efficacy that is not inferior to that of high-dose glucocorticoid rescue therapy. The utilization of low-dose glucocorticoids for the rescue of immune myocarditis represents a complex and relatively uncharted domain, potentially associated with variations in hormone regulation mechanisms among diverse populations. Further investigation, facilitated by ongoing technological advancements and research endeavors, is imperative to enhance our comprehension of the disease and optimize therapeutic strategies.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of Shenzhen People's Hospital. The studies were

conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

LC: Writing – original draft. SZ: Writing – original draft. LG: Writing – review & editing. YZ: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Multiple immune-related adverse events secondary to checkpoint inhibitor therapy in patients with advanced cancer: association with treatment effectiveness

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Introduction: Checkpoint inhibitors (CPI) are widely used in cancer treatment with a potential of causing immune-related adverse events (IRAEs). Several studies have reported a positive correlation between development of IRAEs and improved survival outcome. However, few studies have focused on the potential role of multiple IRAEs on treatment effectiveness. This study aimed at investigating the association between multiple IRAEs and treatment effectiveness in terms of progression-free survival (PFS) and overall survival (OS) in advanced cancer patients.

Methods: We performed a retrospective cohort study at three Swedish centers. All patients (n=600) treated with PD-L1 or PD-1 inhibitor, in monotherapy or in combination for advanced cancer between January 2017 and December 2021 were included. Multiple IRAEs were defined as IRAEs involving more than one organ system either simultaneously or sequentially. Time-depending Cox-regression model to mitigate the risk for immortal time bias (ITB) was applied.

Results: The major tumor types were non-small cell lung cancer (205 patients; 34.2%) and malignant melanoma (196 patients; 32.7%). Of all patients,32.8% developed single IRAE and 16.2% multiple IRAEs. Patients with multiple IRAEs showed significantly improved PFS (Hazard Ratio, HR=0.78 95% Confidence Interval, CI: 0.57–0.98) and OS (HR=0.65 95% CI: 0.44–0.95) compared to patients with single IRAE or no IRAE (HR=0.46 95% CI:0.34–0.62 for PFS vs HR=0.41 95% CI: 0.28-0.60 for OS).

Conclusion: In conclusion, our data supports a stronger association between development of multiple as opposed to single IRAEs and clinical effectiveness in advanced cancer patients treated with CPIs.

KEYWORDS

checkpoint inhibitors, multiple immune-related adverse events, immortal time bias, advanced cancer, cohort study

Introduction

Immunotherapy with checkpoint inhibitors (CPI), i.e. anti-PD-1, anti PD-L1 and CTLA-4 antibodies has dramatically improved survival rates in various cancer types during the last decade and they are frequently used in different treatment settings (1–3). Although the introduction of CPIs has improved outcomes in several cancer types, their use cause a considerable risk for immune-related adverse events (IRAEs) which may appear in nearly every organ system and at any point during treatment and even after treatment discontinuation (4). Potential mechanisms resulting in IRAEs include increased T-cell activity against antigens present in both tumors and healthy tissue, increased levels of cytokines, and preexisting autoantibodies, and enhanced complement-mediated inflammation (4). Despite lack of knowledge considering the exact underlying pathophysiological mechanisms, the occurrence of IRAEs reflects activation of the immune system (4, 5). Since the development of IRAEs depends on the mechanism of action of CPIs, it has been assumed that patients developing IRAEs might have a better response to treatment.

The potential association between development of IRAEs and survival outcome is extensively studied. Several studies have shown a positive association between development of IRAEs and clinical benefit for different cancer types including malignant melanoma (MM), non-small cell lung cancer (NSCLC), renal cell carcinoma, urothelial cancer, head and neck cancer, and gastrointestinal cancer (6–10). Immune-related adverse events involving more than one organ, called multiple IRAEs have not been studied to the same extent. The current evidence suggests a better prognosis in patients with multiple IRAEs with a stronger effect magnitude compared to patients with single IRAEs (11–16), even if conflicting results exists (17). However, the current evidence can be questioned due to the high risk for immortal-time bias (ITB) that either has not been considered in some of the studies (14, 15) or it was dealt with in landmark analysis (12, 13, 17) that can also lead to bias compared to the more robust time-dependent Cox model (18). Besides, most of the studies only included patients with NSCLC (11–13, 16, 17), thus impacting the generalizability of study results.

The aim of the present study was to investigate the patterns of multiple IRAE occurrence and their impact on CPI effectiveness in an unselected cohort of patients with advanced cancer using time-dependent Cox models to mitigate the ITB risk.

Patients and methods

Study design and setting

In this multicenter retrospective cohort study, we identified all patients treated with CPIs (PD-1 or PD-L1 inhibitors) for advanced solid tumors between January 1st 2017 until December 31st 2021 from three regions (Södermland county, Uppsala county, Örebro county) in Sweden. Patients treated with a combination of PD-1 and anti-CTLA4 inhibitor were identified from local electronic prescribing systems for oncological therapy, alongside patients treated with CPIs as part of a clinical trial, and all were included in the analyses. We excluded patients treated with CPIs in a curative setting.

The study was approved by the Swedish Ethical Review Authority (reference number 019–02469 and 020–06801) and the requirement for informed consent was waived. The study has been performed in accordance with the principles of the Declaration of Helsinki.

Data collection

Data were extracted from electronic medical records (EMR) by researchers (clinical oncologists) in a database with pre-specified variables of interest. The following data were collected: age at diagnosis (as years), sex, comorbidities expressed as Charlson Comorbidity Index, type of cancer, primary treatment at diagnosis, age at diagnosis of advanced cancer, metastatic sites, CPI initiation date, type of CPI, performance status (PS; WHO classification) at CPI initiation, number of previous lines of treatment, best treatment response on CPI, date of disease progression, IRAEs (date, type, grade, outcome), date of death and cause of death. IRAEs were collected before each treatment cycle or as acute events according to clinical practice.

Outcomes and definitions

Immune-related adverse events were categorized in grade according to CTCAE 5.0 grading system. If the grade was not included in the EMRs, an approximation of the grade was decided based on the description of adverse events in EMRs and the laboratory findings, whenever feasible.

Multiple IRAEs were defined as IRAEs involving more than one organ system either simultaneously or sequentially.

Progression-free survival (PFS) was defined as the time from initiation of treatment to the occurrence of disease progression (as stated in the EMRs) or death. Overall survival (OS) was defined as the time from treatment initiation to death, irrespective of cause of death.

Statistical methods

For descriptive statistics, numbers with percentages and median with range or interquartile range (IQR) were used for categorical and continuous variables, respectively. For bivariate analyses, either chi-square or Kruskal-Wallis test were used for comparisons among the different groups (no IRAE, single IRAE, or multiple IRAEs).

To identify factors associated with occurrence of IRAEs, logistic regression models were applied (no IRAE vs. multiple IRAEs or single IRAE vs. multiple IRAEs) to calculate Odds Ratios (OR) and their corresponding 95% Confidence Intervals (CI) using the following parameters as potential risk factors; age, sex, CCI, type of cancer, performance status, type of CPI, and treatment line.

To investigate the potential impact of IRAEs on time-to-event outcomes (PFS and OS), we performed time-dependent Cox

Abbreviations: CPI, checkpoint inhibitor; IRAE, immune-related adverse events; EMR, electronic medical records; ITB, immortal-time bias.

regression models as the main analyses to calculate Hazard Ratios (HR) and their corresponding 95% CIs. Occurrence of the IRAE was considered as a time varying covariate. The rest of the covariates included were age, sex, CCI, performance status, type of CPI, type of cancer, and treatment line. A sensitivity analysis was performed by excluding all patients treated with combined CPI (monotherapy-only cohort). In addition, two subgroup analyses were performed based on cancer type (MM, NSCLC). Within NSCLC cohort, the analyses were stratified by treatment line.

All adjusted analyses were based on complete case approach, namely only cases with complete information for all the covariates included in each analysis were used.

Kaplan-Meier curves were used to visualize the impact of IRAEs on time-to-event outcomes. For the visualization of the distribution of organ systems involved in multiple IRAEs, a chord diagram was constructed.

All reported p-values were two-tailed with a 0.05 cut-off for statistical significance. All analyses were performed using SPSS (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp).

Results

Characteristics of study cohort

Baseline characteristics of the study cohort are summarized in Table 1. In total, 600 patients were included in the study cohort with a median age of 66 years (range: 21 – 87). The most common underlying malignant disease was NSCLC (205 patients; 34.2%) followed by MM (196 patients; 32.7%) and renal cell carcinoma (87 patients; 14.6%). Monotherapy with nivolumab was the most used CPI treatment (283 patients; 47.2%) followed by single treatment with pembrolizumab (211 patients; 35.2%) whereas 41 patients (6.8%) were treated with the combination of nivolumab and ipilimumab. Furthermore, 57 patients (9.5%) were treated with atezolizumab, 4 patients (0.7%) with durvalumab and 4 patients (0.7%) with cemiplimab. Median follow-up time for the overall cohort was 15 months (IQR: 6 to 28 months), 23 months (IQR: 13 to 40 months) for patients with PS of 0, 12 months (IQR: 6 to 24 months) for patients with PS of 1, and 7 months (IQR: 1 to 16.5 months) for patients with PS of 2.

TABLE 1 Characteristics of study cohort based on the occurrence of IRAE.

Variable	Whole cohort (N = 600) n (%)	No IRAE (N = 306) n (%)	Single IRAE (N = 197) n (%)	Multiple IRAEs (N = 97) n (%)	p-value
Age, median (range), in years	66 (21 – 87)	66 (21 – 87)	67 (24 – 87)	63 (24 – 84)	0.684
Sex					
Female	252 (42.0)	126 (41.2)	86 (43.7)	40 (41.2)	0.848
Male	348 (58.0)	180 (58.8)	111 (56.3)	57 (58.8)	
Charlson comorbidity index, median (range)	3 (0 – 11)	3 (0 – 11)	3 (0 – 9)	3 (0 – 11)	0.541
Type of cancer					
NSCLC	205 (34.2)	118 (38.6)	64 (32.5)	23 (23.7)	0.005
Melanoma	196 (32.7)	87 (28.4)	65 (33.0)	44 (45.4)	
Renal cell carcinoma	87 (14.5)	42 (13.7)	28 (14.2)	17 (17.5)	
Urothelial carcinoma	35 (5.8)	25 (8.2)	6 (3.0)	4 (4.1)	
HNSCC	23 (3.8)	13 (4.2)	9 (4.6)	1 (1.0)	
Other	54 (9.0)	21 (6.9)	25 (12.7)	8 (8.2)	
de novo metastatic disease	281 (46.9)	149 (48.7)	90 (45.7)	42 (43.3)	0.538
Visceral metastases	401 (66.8)	205 (67.0)	136 (69.0)	60 (61.9)	0.468
Central nervous system metastases	50 (8.3)	29 (9.5)	16 (8.1)	5 (5.2)	0.403
Performance status according to ECOG					
0	213 (35.7)	79 (26.0)	81 (41.1)	53 (55.2)	< 0.001
1	279 (46.7)	153 (50.3)	90 (45.7)	36 (37.5)	
≥ 2	105 (17.6)	72 (23.7)	26 (13.2)	7 (7.3)	
Type of checkpoint inhibitors					
Nivolumab	283 (47.2)	139 (45.4)	103 (52.3)	41 (42.3)	< 0.001
Pembrolizumab	211 (35.2)	116 (37.9)	63 (32.0)	32 (32.0)	
Atezolizumab	57 (9.5)	38 (12.4)	15 (7.6)	4 (4.1)	
Nivolumab + Ipilimumab	41 (6.8)	9 (2.9)	14 (7.1)	18 (18.6)	
Durvalumab	4 (0.7)	2 (0.7)	1 (0.5)	1 (1.0)	
Cemiplimab	4 (0.7)	2 (0.7)	1 (0.5)	1 (1.0)	
Line of treatment for checkpoint inhibitors					
1st	268 (45.0)	124 (40.5)	88 (44.7)	56 (57.7)	0.031
2nd	231 (38.8)	124 (40.5)	82 (41.6)	28 (28.9)	
3rd or later	97 (16.3)	58 (19.0)	27 (13.7)	13 (13.4)	

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Occurrence patterns, outcomes, and risk factors for multiple IRAEs

In total, 97 patients (16.2%) developed multiple IRAEs whereas 197 (32.8%) had a single IRAE during follow-up. Severity, management, and outcome of patients with IRAEs based on number of IRAEs is shown in Table 2. Patients with multiple IRAEs were more likely to develop grade ≥ 2 or grade ≥3 IRAEs that could lead to higher discontinuation rate compared to patients with single IRAEs. Patients with a single IRAE recovered without sequelae to a higher extent than patients with multiple IRAEs (60% vs. 44.3% p=0.027).

Risk factors for developing multiple IRAEs are shown in Table 3. Patients with PS ≥ 2 were less likely to develop multiple IRAEs (OR: 0.42 95% CI: 1.83–11.79) whereas combined CPI treatment was associated with development of multiple IRAEs (OR: 4.64 95% CI: 1.83–11.79) compared to no IRAE. We could not identify any risk factor associated with multiple IRAEs when compared to patients who developed a single IRAE.

Distribution of organ systems involved in multiple IRAEs is demonstrated in Figure 1. The most common dyads of organs with IRAE development within the same patient were skin-gastrointestinal, skin-rheumatologic, skin-endocrine, and rheumatologic-endocrine.

Impact of multiple IRAEs on PFS and OS

A summary of results from time-dependent Cox analyses regarding the occurrence of IRAE and prognosis in terms of PFS and OS is presented in Table 4.

TABLE 2 Severity, management, and outcome of patients with immune-related adverse events (IRAEs) based on number of IRAEs.

	Single IRAE (N = 197) n (%)	Multiple IRAEs (N = 97) n (%)	p-value
Time to onset of IRAE (months), median (range)	2 (0 – 36)	1 (0 – 29)	0.925
Maximum grade of IRAE severity			
Grade ≥ 2	128 (65.0)	91 (93.8)	**< 0.001**
Grade ≥ 3	62 (31.5)	42 (43.3)	**0.046**
Therapeutic management of IRAE*			
No treatment or supportive	82 (52.2)	40 (44.0)	0.351
Corticosteroids	69 (43.9)	45 (49.5)	
Corticosteroids + alternative immunosuppression	6 (3.8)	6 (6.6)	
Outcome of IRAEs			
Resolved without sequelae	118 (60.0)	43 (44.3)	**0.027**
Resolved with minor sequelae	61 (31.0)	45 (46.4)	
Resolve with major sequelae	14 (7.0)	8 (8.3)	
Death due to IRAE	4 (2.0)	1 (1.0)	0.533
Discontinuation due to IRAE	57 (28.9)	43 (44.3)	**0.009**

*Lack of information in 40 patients with single IRAE and 6 patients with multiple IRAEs. Statistically significant results are presented in bold.

TABLE 3 Risk factors for developing multiple immune-related adverse events.

Risk factors	Compared to no IRAE	Compared to single IRAE
	Odds ratio (95% Confidence Interval)	Odds ratio (95% Confidence Interval)
Age	0.99 (0.97 – 1.03)	0.99 (0.96 – 1.02)
Sex		
Female	1.13 (0.67 – 1.91)	0.96 (0.56 – 1.63)
Male	1	1
Charlson comorbidity index	1.05 (0.87 – 1.27)	1.05 (0.85 – 1.31)
Type of cancer		
NSCLC	0.87 (0.42 – 1.82)	0.91 (0.42 – 1.97)
Melanoma	1	1
Other	0.92 (0.47 – 1.80)	0.84 (0.41 – 1.70)
Performance status according to ECOG		
0	1	1
1	0.62 (0.34 – 1.14)	0.79 (0.44 – 1.44)
≥2	**0.42 (0.24 – 0.75)**	0.54 (0.21 – 1.40)
Type of checkpoint inhibitors		
anti-PD-1	1	1
anti-PD-L1	0.52 (0.18 – 1.47)	0.89 (0.29 – 2.74)
combination with anti-CTLA4	**4.64 (1.83 – 11.79)**	2.18 (0.96 – 4.95)
Line of treatment for checkpoint inhibitors		
1st	1	1
2nd	0.79 (0.42 – 1.47)	0.66 (0.35 – 1.23)
3rd or later	0.78 (0.35 – 1.75)	0.94 (0.38 – 2.36)

Statistically significant results are presented in bold.

The occurrence of multiple IRAEs was associated with statistically significant improvement in PFS (HR: 0.46; 95% CI: 0.34 – 0.63) compared to no IRAE in the whole study cohort as well as in the sensitivity analysis when only patients with CPI as monotherapy were included. An addition analysis with single IRAE as a reference demonstrated statistically significant improvement in PFS (HR: 0.78; 95% CI: 0.57–0.98) for patients developing multiple IRAEs compared to single IRAEs. A graphical visualization of PFS based on the occurrence or absence of IRAEs is shown in Figure 2A. In subgroup analyses, multiple IRAEs were associated with improved PFS in MM cohort but not in NSCLC cohort (Table 4).

In terms of OS, the occurrence of IRAEs resulted in statistically significant improvement in OS both in single IRAE (HR: 0.63; 95% CI: 0.43 - 0.92) and in multiple IRAEs (HR: 0.41; 95% CI: 0.28 - 0.60) cohorts (Figure 2B) compared to no IRAE. The association remained statistically significant in the sensitivity analysis with CPI monotherapy patients only for the occurrence of multiple IRAEs. In subgroup analyses, the occurrence of both single and multiple IRAEs was associated with improved OS in MM cohort whereas no similar association was observed in NSCLC cohort (Table 3). An addition analysis with single IRAE as a reference demonstrated statistically significant improvement in OS (HR: 0.65; 95% CI: 0.44–0.95) for patients developing multiple IRAEs compared to single IRAE.

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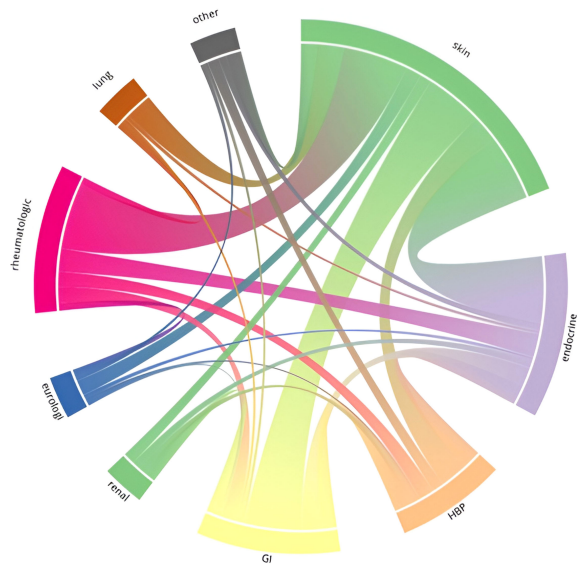

FIGURE 1
Chord diagram on the distribution of organ systems involved in multiple immune-related adverse events in a cohort of cancer patients with advanced disease treated with checkpoint inhibitors (n=600).

After applying stratification based on treatment line in NSCLC cohort, a numerically lower HR for both PFS (n=77; HR: 0.51 95% CI: 0.21–1.23) and OS (HR: 0.58 95% CI: 0.20–1.67) for patients with multiple IRAEs when treatment was given as 1st line was observed compared to patients receiving CPI as 2nd (n=137 PFS HR 0.85 95% CI: 0.39–1.87 OS HR 0.84 95% CI: 0.47–1.52) or later treatment line (n=57 PFS HR: 1.77 95% CI: 0.22–13.7 OS HR: 1.12 95% CI: 0.17–10.7).

In all main analyses, we could not reveal any difference in survival outcomes between PD-1 vs. PD-L1 treatment (HR for PFS: 0.84; 95% CI: 0.61 – 1.17; HR for OS: 0.94; 95% CI: 0.65 – 1.36). In an additional time-dependent Cox regression analysis of whether treatment discontinuation due to toxicity was associated with survival outcomes, we could not find a statistically significant association with either PFS (HR 0.78; 95% CI: 0.52 – 1.17) or OS (HR: 0.83; 95% CI: 0.53 – 1.29).

TABLE 4 Impact of multiple IRAEs on disease prognosis according to time-depending Cox regression models.

Models*	Variables	Hazard Ratio (95% Confidence Interval)
Progression-free survival		
Time-dependent Cox, whole cohort (main analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.78 (0.57 – 1.06) **0.46 (0.34 – 0.62)**
Time-dependent Cox, monotherapy only (sensitivity analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.82 (0.59 – 1.16) **0.46 (0.34 – 0.63)**
Time-dependent Cox, NSCLC only (subgroup analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.90 (0.52 – 1.56) 0.67 (0.39 – 1.15)
Time-dependent Cox, melanoma only (subgroup analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.67 (0.39 – 1.12) **0.36 (0.22 – 0.59)**
Overall survival		
Time-dependent Cox, whole cohort (main analysis)	No IRAE Single IRAE Multiple IRAEs	1 **0.63 (0.43 – 0.92)** **0.41 (0.28 – 0.60)**
Time-dependent Cox, monotherapy only (sensitivity analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.76 (0.52 – 1.13) **0.47 (0.32 – 0.68)**
Time-dependent Cox, NSCLC only (subgroup analysis)	No IRAE Single IRAE Multiple IRAEs	1 0.85 (0.45 – 1.60) 0.86 (0.47 – 1.59)
Time-dependent Cox, melanoma only (subgroup analysis)	No IRAE Single IRAE Multiple IRAEs	1 **0.46 (0.25 – 0.90)** **0.26 (0.14 – 0.50)**

*all analyses were adjusted for the following variables: age, gender, Charlson Comorbidity Index (CCI), performance status, type of checkpoint inhibitor, type of cancer (except from subgroup analyses), line of CPI treatment.
Statistically significant results are presented in bold.

Discussion

In our study cohort of 600 patients with advanced cancer treated with CPIs, we observed that approximately one-sixth developed multiple IRAEs. The occurrence of multiple IRAEs was associated with better treatment effectiveness as demonstrated by improvements in both PFS and OS with a magnitude of benefit significantly stronger compared to patients with single IRAEs.

Current evidence concerning development of multiple IRAEs for patients treated with CPIs and its impact on treatment effectiveness is scarce. There are only few previous studies (11–13, 16, 17), most of them indicating a stronger association between the development of multiple IRAEs, as opposed to single and survival (11, 12, 16). However, the cohorts in these studies included only patients with NSCLC and, therefore, the generalizability of study results can be questioned. Two earlier studies included patients with various malignant diseases, but smaller cohorts of approximately 200 patients (14, 15) demonstrated results in line with our larger cohort that provides more convincing evidence on this potential association in a broader patient population. A major methodological drawback of previous studies is how the risk for ITB was dealt. Immortal-time bias is a challenge to consider in the association between IRAEs and clinical outcome since patients responding to therapies continue treatment for a longer time and therefore increase their risk of developing IRAEs. To eliminate the risk of ITB in observational studies of survival outcomes established methodology such as landmark analysis, Cox model with time-varying variable or inverse-probability weighed models are routinely used (18–21). These methodological approaches cannot be considered as equal in terms of the validity of results since Cox model with time-varying variable seems to outperform landmark analysis (18). Considering the current evidence on potential association between multiple IRAEs and CPI effectiveness, some studies did not deal with ITB at all (14, 15) whereas others used landmark analysis only (12, 13, 17). We found two previous studies

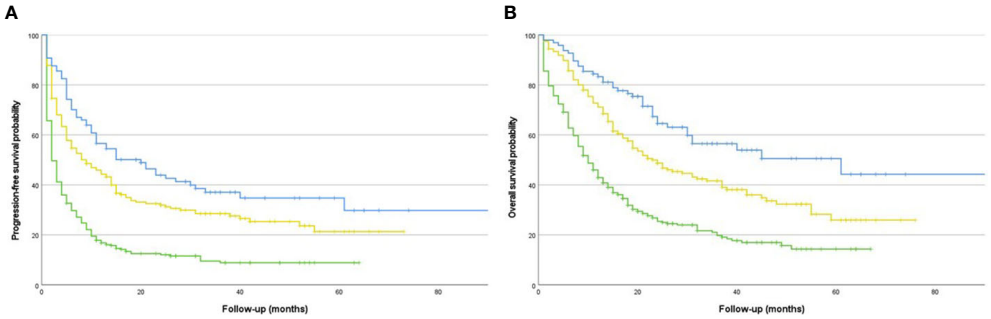

FIGURE 2
(A) Progression-free survival in a cohort of cancer patients with advanced disease treated with checkpoint inhibitors (n=600) based on the occurrence of immune-related adverse events. Blue line for multiple IRAEs; yellow line for single IRAE; green line for no IRAE. **(B)** Overall survival in a cohort of cancer patients with advanced disease treated with checkpoint inhibitors (n=600) based on the occurrence of immune-related adverse events. Blue line for multiple IRAEs; yellow line for single IRAE; green line for no IRAE.

with a proper analysis of ITB reporting positive correlation between multiple IRAEs and outcome. However, both face issues related to generalizability of results to the clinical setting since only patients with NSCLC were included (11, 16) and one of the studies only analyzed patients participating in clinical trials and not in real-world setting (16). Until today, the current study is the first investigating the association between multiple IRAEs and CPI effectiveness in a broader cohort of patients with various malignancies using the preferable Cox regression model with time-dependent covariate as methodological approach for mitigating the ITB.

Subgroup analysis in our cohort showed statistically significantly improved PFS and OS for patients with MM but, as opposed to *Shankar et al*, not for patients with NSCLC. The lower number of NSCLC patients in our cohort compared to the study by Shankar et al. which only included NSCLC patients (n=205 vs. n=623) may, in part, explain this discrepancy (11). Interestingly, a trend towards improved PFS and OS for patients given CPI as a first line treatment that was diminished in later lines was observed in our cohort of patients with NSCLC highlighting treatment line as a source of heterogeneity among studies that could impact the results. This information was lacking from Shankar et al. and could also contribute to the discrepancy of study results. At the same time, one could argue that the ability of IRAEs to predict CPI effectiveness might depend on cancer type, a notion that is supported by the observations on the substantial differences on tumor immunogenicity among different cancer types (22, 23). In fact, the treatment effect is not equal in patients with different cancer types and differences in adverse effects among different cancer types have also been observed. A systematic review of 48 trials reported higher risk of developing skin and gastrointestinal IRAEs and lower risk of experiencing lung IRAEs for patients with MM compared to those with NSCLC whereas higher incidence of arthralgia and hypothyroidism in MM patients compared to patients with renal cell carcinoma was observed (5). Furthermore, it has been demonstrated that specific types of IRAEs exhibit a more profound correlation to treatment effectiveness and the type of these IRAEs differ across various cancer types. For example, vitiligo has been linked to treatment response in melanoma patients and thyroid dysfunction in NSCLC and renal cell carcinoma patients (24–28).

Checkpoint inhibitor combination therapy with PD-1 and anti-CTLA4 inhibitor is, for subgroups of patients, known to have better clinical efficacy than monotherapy, but the incidence of IRAEs is higher (29, 30). In our study cohort, we confirmed that the combination was a risk factor for development of multiple IRAEs. However, subgroup analyses of monotherapy only showed significantly better PFS and OS for patients developing multiple IRAEs, indicating that the improved outcome for patients with MM is not only due to the well-known better effect of combination therapy. Performance status ≥2 was associated with lower risk for developing multiple IRAEs in our study. A conceivable explanation is that patients with worse PS are more vulnerable in general with higher risk for earlier treatment discontinuation after the first IRAE episode and thus are less likely to develop multiple IRAEs.

Immunosenescent, a term describing impaired function of the immune system that develops with aging, has been supposed to influence the effect of immunotherapies and the development of IRAEs. However, many studies did not find a higher risk for development of IRAEs in older patients (31–34), but conflicting results exist (35). In a previous study from our research group, we found that a simplified frailty score based on PS, age and comorbidity expressed as Charlson Comorbidity Index (CCI) could predict development of all grade and multiple IRAEs, whereas age, CCI or PS did not separately predict increased risk for development of IRAEs (36). Although not supported by our adjusted analyses, another potential explanation of the association between PS and the risk of IRAEs might be more related to the line of treatment rather than the PS per se. In fact, some evidence suggests a negative impact of prior chemotherapy to immune microenvironment (37) that might influence the risk for IRAEs as well. In this equation, PS could serve as a surrogate for later treatment lines rather than as an explanatory parameter for the risk of IRAEs.

In terms of occurrence patterns and outcomes of IRAEs, we found a higher frequency of IRAE grade ≥ 2 and ≥ 3 in patients with multiple IRAEs than in patients with a single IRAE which could

explain why a greater proportion of patients with multiple IRAEs than patients with a single IRAE discontinued treatment due to IRAE. At the same time, multiple IRAEs were not associated with higher risk for major sequelae from IRAEs. The organ systems involved in multiple IRAEs in this study consists of combinations of common single IRAEs without identifying any specific pattern. The number of patients within each pattern of distribution was too small and precludes any further analyses for potential associations of specific patterns with prognosis.

Besides from ITB potentially being the main bias in the association between IRAEs and clinical outcome, the present study has some additional limitations associated with its retrospective nature. These include the risk for misclassification bias regarding both IRAE grading but also classification between single and multiple IRAE as well as information bias where EMRs as data sources for identifying IRAEs might not include information on low grade IRAEs. The study is restricted to tree Swedish centers thus limiting its external validity whereas the relatively lower number of patients with tumor types other than melanoma and NSCLC limits the generalizability of the results. Finally, some variables of potential interest that could be associated with both the development of IRAEs and prognosis, as ethnicity, co-medication with immunosuppressive therapy, pre-existing autoimmune disease, were not available and were, therefore, not taken into account for the analyses. For some variables of interest as prior oncological treatment, the information was available, but the subgroups were too small for relevant analyses.

In conclusion, our study results suggest a statistically significant association between development of multiple IRAEs and CPI treatment effectiveness (measured as PFS and OS) that is mainly driven by patients with MM. These results support not discontinuing immunotherapy, even upon multiple but not severe IRAEs to increase the likelihood of treatment benefit. In addition, our findings suggest that multiple IRAEs may constitute a suitable surrogate marker for treatment efficacy that might be used in clinical trials. However, further studies with larger sample size and prospective design to overcome the inherent biases of retrospective studies is essential to further address the potential interplay between the development of IRAEs and treatment outcome.

Data availability statement

The raw data are not available due to ethical restrictions but anonymized data might be available upon request.

Ethics statement

The study was approved by the Swedish Ethical Review Authority (reference number 019-02469 and 020-06801). The studies were conducted in accordance with the local legislation and institutional requirements. Informed consent was waived from the Swedish Ethical Review Authority since the eligible patients were already treated with checkpoint inhibitors and the current study would not influence their subsequent treatment or prognosis.

Author contributions

CO: Data curation, Writing – original draft. AK: Data curation, Writing – review & editing. VR: Data curation, Writing – review & editing. AI: Data curation, Writing – review & editing. ED: Data curation, Writing – review & editing. AV: Conceptualization, Formal analysis, Methodology, Project administration, Software, Supervision, Writing – original draft. GU: Conceptualization, Funding acquisition, Methodology, Supervision, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Neoadjuvant immunochemotherapy improves clinical outcomes of patients with esophageal cancer by mediating anti-tumor immunity of CD8+ T (Tc1) and CD16+ NK cells

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Background: Esophageal cancer (ESCA) is one of the most common tumors in the world, and treatment using neoadjuvant therapy (NT) based on radiotherapy and/or chemotherapy has still unsatisfactory results. Neoadjuvant immunochemotherapy (NICT) has also become an effective treatment strategy nowadays. However, its impact on the tumor microenvironment (TME) and regulatory mechanisms on T cells and NK cells needs to be further elucidated.

Methods: A total of 279 cases of ESCA who underwent surgery alone [non-neoadjuvant therapy (NONE)], neoadjuvant chemotherapy (NCT), and NICT were collected, and their therapeutic effect and survival period were compared. Further, RNA sequencing combined with biological information was used to analyze the expression of immune-related genes. Immunohistochemistry, immunofluorescence, and quantitative real-time PCR (qRT-PCR) were used to verify the activation and infiltration status of CD8+ T and CD16+ NK cells, as well as the function and regulatory pathway of killing tumor cells.

Results: Patients with ESCA in the NICT group showed better clinical response, median survival, and 2-year survival rates ($p < 0.05$) compared with the NCT group. Our RNA sequencing data revealed that NICT could promote the expression of immune-related genes. The infiltration and activation of immune cells centered with CD8+ T cells were significantly enhanced. CD8+ T cells activated by PD-1 inhibitors secreted more IFN-γ and cytotoxic effector factor cells through the transcription factor of EOMES and TBX21. At the same time, activated CD8+ T cells mediated the CD16+ NK cell activation and secreted

more IFN-γ to kill ESCA cells. In addition, the immunofluorescence co-staining results showed that more CD276+ tumor cells and CD16+ NK cells were existed in pre-NCT and pre-NICT group. However, CD276+ tumor cells were reduced significantly in the post-NICT group, while they still appeared in the post-NCT group, which means that CD16+ NK cells can recognize and kill CD276+ tumor cells after immune checkpoint blocker (ICB) treatment.

Conclusion: NICT can improve the therapeutic effect and survival period of resectable ESCA patients. NICT could promote the expression of immune-related genes and activate CD8+ T and CD16+ NK cells to secrete more IFN-γ to kill ESCA cells. It provides a theoretical basis and clinical evidence for its potential as an NT strategy in ESCA.

KEYWORDS

esophageal cancer, neoadjuvant immunochemotherapy, tumor microenvironment, CD8+ T cells, CD16+ NK cells, CD276

Introduction

Esophageal cancer (ESCA) is still one of the leading causes of cancer-related deaths worldwide. If the incidence rate of ESCA remains stable, 957,000 new cases and 880,000 deaths will occur globally by 2040. Effective treatment is crucial in reducing ESCA-attributable mortality (1). Neoadjuvant therapy (NT) has become a first-line treatment option for many types of cancer in recent years, and its application in ESCA is also widespread (2, 3). However, commonly used neoadjuvant chemotherapy (NCT) and neoadjuvant radiochemotherapy (NRCT) still cannot meet people's expectations for therapeutic effects. Therefore, more optimized treatment strategies are still needed to address the current treatment challenges of esophageal cancer.

Immune checkpoint blockers (ICBs) have transformed the landscape of cancer therapy, providing substantial benefits to patients. Remarkable progress has been achieved in the field, particularly in the treatment of non-small cell lung cancer (NSCLC), gastric cancer, and liver cancer, among others (4–6). At present, the combination of ICBs and neoadjuvant chemotherapy, i.e., neoadjuvant immunochemotherapy (NICT), has been explored in therapy for ESCA. Several clinical trials have been initiated and improved survival in esophageal squamous cell carcinoma (ESCC) patients who received NICT (7, 8). Although some clinical evidence currently demonstrates the advantages of NICT, its specific mechanism is still unclear and has been a research hotspot (9). NICT can activate T cells in cancer patients and significantly improve disease remission rates by blocking signals that inhibit T-cell activation, which is one of the primary mechanisms of current clinical tumor immunosuppressive therapies (10). Some research has found that NICT could enhance anti-tumor immune responses by activating CD4+ and CD8+ T lymphocytes (11, 12). Activated CD8+ T cells subsequently kill tumor cells by mediating the release of lymphotoxin (LT) from a subset of cytotoxic T cells (Tc1) (13). Additionally, ICBs have the capability to activate NK cells, promoting the recognition of tumor cell surface antigens and the destruction of antibody-bound tumor cells (14). Moreover, activated NK cells could mediate apoptosis of tumor cells through the expression of transcription factors TBX21 and EOMES, factors that can regulate the high expression of cytokines (e.g., IFN-γ and TNF-α) and exert anti-tumor effects through immunomodulatory effects (15). Until now, NK cells have been used as feasible immunotherapy in several clinical trials (16–18). In the context of anti-tumor immunotherapy, ICBs enhance NK cell activation and cytotoxicity by increasing the frequency of CD16+ NK cells (19). Therefore, T cells and NK cells play complementary roles in tumor immunity, and their combination provides opportunities to deepen the impact of immunotherapy. However, further research is still required to elucidate the roles and effect details of CD8+ T and CD16+ NK cells in NICT.

Programmed cell death protein 1 (PDCD1, PD-1, CD279) and programmed cell death ligand 1 (PD-L1, CD274/B7-H1) are regarded as key targets for anti-tumor immunotherapy currently (20, 21). B7-H1 belongs to one of the members of the B7 immunoglobulin superfamily, which is closely related to tumor progression and plays a crucial role in tumor immunity (22, 23). However, given the fact that the response to treatment with ICBs does not always correlate with PD-L1 expression, some ESCA patients may not benefit from immunotherapy (24, 25). Therefore, it would be a meaningful subject in immunotherapy to find novel B7 immunoglobulin superfamily members serving as targets for ICBs. The B7 homolog 3 protein (B7-H3, CD276) of the B7 immunoglobulin superfamily, like its cognate member PD-L1, plays a crucial role in regulating tumor immune responses (26). CD276 has been found to be overexpressed in numerous solid tumors with very minimal expression in their corresponding normal tissues. In most tumors, high expression of CD276 is strongly associated with cancer progression and poor prognosis for cancer patients. Therefore, CD276

has been considered a promising target for immunotherapy research studies (27). However, the receptor for CD276 has remained unidentified, and the role of CD276 in immunotherapy remains controversial (28).

Herein, 279 cases of surgical ESCA patients were reviewed, and their efficacy and survival after undergoing NCT and NICT were compared. Further, the expressions of immune-related genes and the changes in the tumor immune microenvironment (TIME) were analyzed pre- and post-NT. The functions and regulatory pathways of CD8+ T and CD16+ NK cells were elucidated in EC tissues of the NICT group. Therefore, our research may lay a foundation for the mechanism research and serve as strong evidence of NICT in ESCA.

Materials and methods

Patients and samples

Clinical data were collected from 279 patients who underwent radical ESCA surgery from January 2017 to December 2022 in the same hospital. This study was approved by the ethics committee of the hospital (2020–50-IIT), and all the patients signed the informed consent form. The sample size was 120 patients for the NONE group, 64 patients for the NCT group, and 95 patients for the NICT group. The basic characteristics of the patients are listed in Supplementary Table S1, with no major differences across groups including sex, age, and smoking behavior. The patients from the NCT and NICT groups underwent one to four cycles of NT [i.e., paclitaxel for injection (albumin bound), platinum as chemotherapy drugs, and PD-1 inhibitors as ICBs] before surgery. A number of cancer and para-cancer tissue samples were collected from the included cases and stored in liquid nitrogen.

In accordance with the National Comprehensive Cancer Network (NCCN) guidelines and the patient's status, the follow-up appointments were scheduled, with the exception of those of four missing patients in the NCT group.

Acquisition of imaging data

With the assistance of imaging experts, imaging analyses, including CT, MRI, and PET/CT, were performed pre- and post-NT as well as pre- and post-surgery among all included cases. Attention was focused on the longitudinal diameter and the maximum pipe-wall thickness of the tumors, and the product of these two values was used as an auxiliary criterion for determining the changes in the tumor size pre- and post-NT. The imaging data came from the hospital's database.

RNA isolation, RNA sequencing, and data analyses

The clinical tissues of ESCA (50 mg) were sectioned using a cryostat microtome, and total RNA was exacted with TRIzol reagent (#15596018, Invitrogen, Carlsbad, CA, USA). After passing the quality control, the total RNA samples were sequenced, and the data analyses were performed. The differentially expressed genes were shown with fold change $|\log(FC)| > 1$ and $p < 0.01$. All statistical analyses were conducted using the R software (Version 4.0.2). Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment and Gene Ontology (GO) function annotation analyses were performed using the R package "clusterProfiler". KEGG or GO terms with BH-corrected $p < 0.05$ were considered significant. "Enrichplot" was used to visualize the significant results. Gene Set Enrichment Analysis (GSEA) function in R was applied to analyze the gene expression condition and identify the enrichment status of the gene set (i.e., NICT/NCT, NICT/NONE, and NCT/NONE). Specifically, the gene set of $|NES| > 1$ and false discovery rate (FDR) $q < 0.25$ were identified with the most remarkable changes.

The scoring of immune cell infiltration

Employing the "estimate" package of R, we scored the gene expression matrix of stromal and immune cells from different tumor tissues, as well as for the immune infiltration. The scoring of immune infiltration is positively associated with the proportion of immune cell infiltration within the tumor tissues.

The scoring of cell cytotoxicity

The scoring of cell cytotoxicity was applied to analyze the cytotoxicity of infiltrating immune effector cells including CD8+ T and CD16+ NK cells. The geometric mean of the expression quantity of GZMM and PRF1 was adopted to reflect the scoring of cell cytotoxicity (CYT) for the tumor tissue of included patients.

The correlation between T-cell surface marker and activity marker

The correlation between T-cell surface marker and activity marker was analyzed utilizing ESCA samples from the Gene Expression Profiling Interactive Analysis (GEPIA) database (http://gepia.cancer-pku.cn/index.html), and Pearson's correlation coefficient was employed to assess the correlation between T-cell surface marker (i.e., CD8A and CD8B) and activity marker (i.e., EOMES, TCIRG1, GZMA, GZMM, PRF1, and IFN-γ).

Quantitative real-time PCR

Total RNA from ESCA tissues was reverse-transcribed into cDNA using the PrimeScript RT-PCR Kit (#RR047A, Takara Biotechnology, Mountain View, CA, USA). Quantitative real-time PCR (qRT-PCR) was conducted using the FastStart Universal SYBR Green Master (#04913914001, Roche, Basel, Switzerland) on QuantStudio 3 (Applied Biosystems, Foster City, CA, USA). Primers for qRT-PCR are described in Supplementary Table S2. The mRNA level of β-actin was used as an internal control.

H&E staining and immunohistochemical staining

The paraffin sections (5 μm) of ESCA tissues were de-paraffinized, dehydrated by xylene and alcohol, and stained with hematoxylin and eosin (H&E) for each case. Other sections were used for immunohistochemical staining with specific antibodies. After antigen retrieval was performed with 10 mM Tris-EDTA (pH 9.0) in microwave for 10 minutes, the tissue slides were treated with 3% hydrogen peroxide for 15 minutes and blocked with 3% bovine serum albumin (BSA) for 1 hour at room temperature (RT). The slides were incubated with primary antibody at 4°C overnight. The CD8 (66868–1-lg), TCIRG1 (12649–1-AP), CD16 (66779–1-lg), and CD276 (14453–1-AP) antibodies were purchased from ProteinTech (Chicago, IL, USA) (Supplementary Table S3). Next, slides were incubated with a secondary antibody for 1 hour at RT and exposed to a diaminobenzidine (DAB) staining solution. Afterward, nuclei were stained with hematoxylin. Finally, the slides were mounted with neutral balsam and observed under a zoom stereo microscope (Axio Zoom.V16, Zeiss, Oberkochen, Germany).

Immunofluorescence staining

The ESCA tissue slides initially were blocked with 3% BSA for 1 hour at RT followed by overnight incubation with the primary antibody at 4°C. The IFN-γ (15365–1-AP), CD8 (66868–1-lg), TCIRG1 (12649–1-AP), CD16 (66779–1-lg and 16559–1-AP), and CD276 (14453–1-AP and 66481–1-lg) antibodies were purchased from ProteinTech (Supplementary Table S3). Then, they were incubated with a secondary antibody for 1 hour at RT after the slides were de-paraffinized and dehydrated with xylene and alcohol. The signals from specific antibodies were labeled with Fluorescein Isothiocyanate (green) and Rhodamine (red) and then re-stained with 4′,6-diamidino-2-phenylindole (DAPI) for 10 minutes at RT, and slides were mounted with 90% glycerin and kept in the dark. The slides were visualized under a confocal microscope (OLYMPUS, Tokyo, Japan; FV3000) and a fluorescence microscope (ECHO RVL2-K2).

Data source

Expression data for genes including PRF1, GZMA, and GZMM across various immune cells were obtained from "The Human Protein Atlas data analysis" (HPA: https://www.proteinatlas.org/). ESCA RNA sequencing (RNA-seq) data were obtained from The Cancer Genome Atlas (TCGA) database (https://cancergenome.nih.gov/) and ESCC data (GSE145370) from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/). The list of genes related to signaling pathways was obtained from the MSigDB database (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp).

Statistical analysis

In this study, data for continuous variables are presented as the mean ± standard of measurement in at least three independent experiments, and median [95% confidence interval (CI)] was used when sample distribution was non-normal. Statistical significance was determined using a two-tailed Student's t-test. For comparison of categorical variables between groups, chi-square tests in cross-tabulation were used. Overall survival (OS) was compared using the Kaplan–Meier curve analysis and log-rank tests. Median percentage reduction with a 95% CI was applied to reflect the reduction in the size of the tumor for each treatment group before and after NT. Statistical analysis was performed using IBM SPSS Statistics 27, and p-value <0.05 was considered statistically significant (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). For figures, statistical tests were justified as appropriate. Bars in the graphs represent mean ± SEMs. Analyses and graphical presentations were performed using the GraphPad Prism 9.5 software.

Results

NICT significantly reduces tumor stage and lesion size

As an important type of ICB, PD-1 inhibitors have been widely applied in clinical tumor treatment and NT for ESCA due to their effectiveness. To assess the clinical treatment effect of NT with PD-1 inhibitors, as well as to elucidate the immune activity and recognized target of PD-1 inhibitors in ESCA treatment, our study first compared the survival rates of patients who received NT using follow-up and survival analysis. As depicted in Figure 1A, the survival rates of the NICT (n = 95) and NCT groups (n = 64) were 70.9% and 51.5% (within 2 years), respectively. The NCT group exhibited a median survival period of 25 months, while no median survival period was determined for the NICT group due to its higher survival rates, which showed statistical significance [$p < 0.005$, hazard ratio (HR) = 0.44]. The survival analysis indicated that the treatment effect of the NICT group is superior to that of the NCT group. Then, the changes in tumor size pre- and post-NT were analyzed using the longitudinal diameters (mm) × maximum thickness (mm) of the tumor using the data of CT images. The NONE group had the minimum size of tumor (514.08 ± 439.86 mm^2), followed by the NCT group (819.17 ± 566.65 mm^2) and the NICT group (1,063.62 ± 683.50 mm^2) before treatment. The median size of the NICT group was significantly larger than that of the NONE group and the NCT group ($p < 0.05$) (Supplementary Figure S1A, Table 1). After NT, higher-level reduction was found in the NICT group (reduced 63.42%) as compared with the NCT group (reduced 43.52%), although the mean size of the tumor in both the NCT and NICT groups significantly reduced (Figure 1B, Supplementary Figure S1B). That is to say, the NICT group with larger tumors before treatment had smaller tumors after treatment. Subsequently, the differences in clinical staging among different

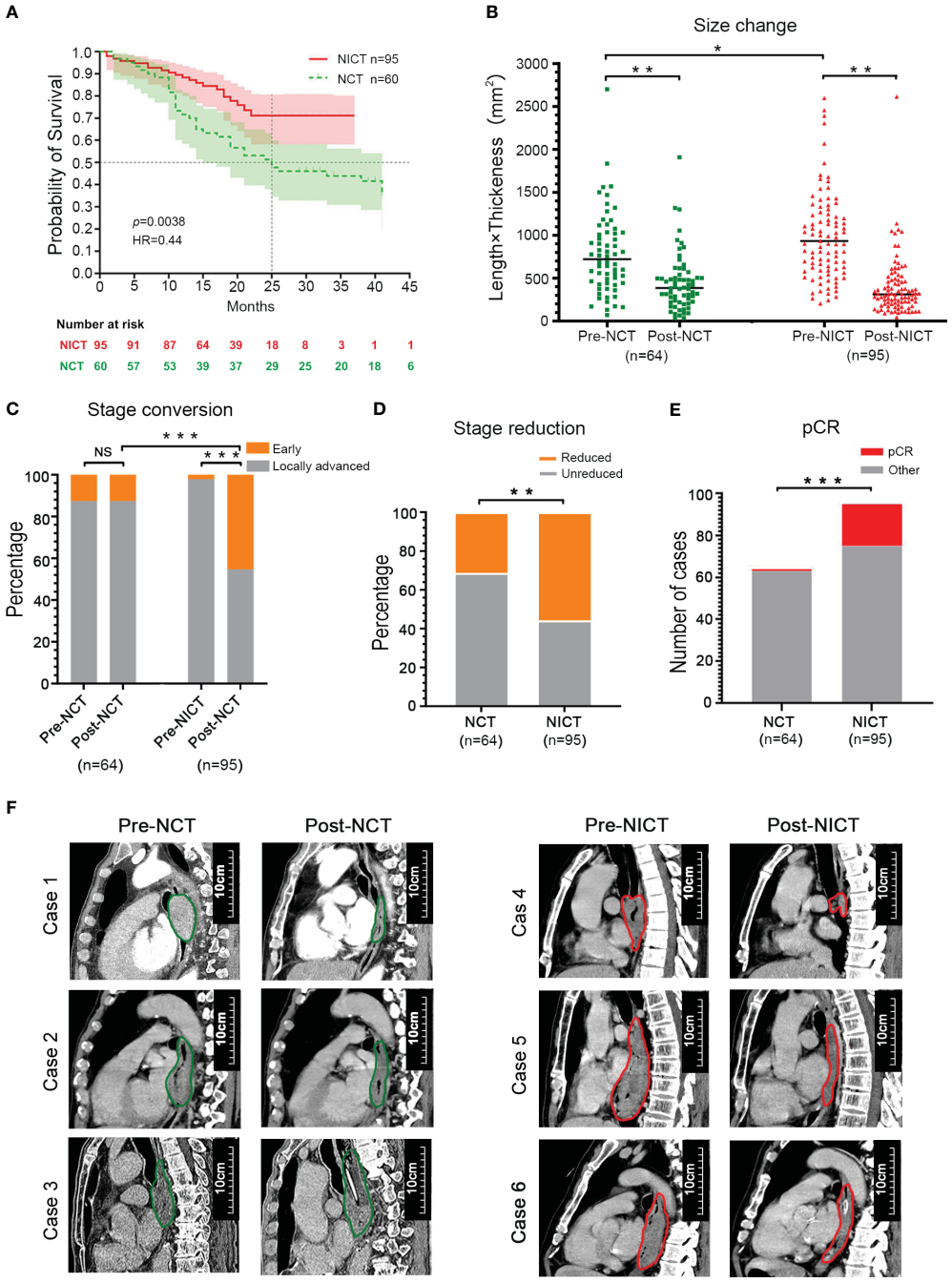

FIGURE 1
NICT significantly improved the therapeutic effect of esophageal cancer (ESCA). **(A)** Survival period. NICT, neoadjuvant immunochemotherapy; NCT, neoadjuvant chemotherapy; HR, hazard ratio. **(B)** Changes in tumor size. Pre-NCT, pre-neoadjuvant chemotherapy; Post-NCT, post-neoadjuvant chemotherapy; Pre-NICT, pre-neoadjuvant immunochemotherapy; Post-NICT, post-neoadjuvant immunochemotherapy. **(C)** Conversion rate of clinical stage. **(D)** The reduction rate of clinical stage. **(E)** pCR, pathologic complete response. **(F)** Clinical cases. The maximum longitudinal diameters of tumors in the sagittal plane of CT images. NCT group (left): Case 1 and Case 2 had partial response (PR), and Case 3 had progressive disease (PD). NICT group (right): Case 4 had pCR, and Case 5 and Case 6 had PR. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. NS, no significance.

groups were compared. Before NT, there were 56 (87.5%) and 93 (97.9%) cases classified as later staging in the NCT and NICT groups, respectively, along with eight (12.5%) and two (2.1%) cases classified as earlier staging ($p < 0.001$). After NT, apart from three cases who did not receive endoscopic examination or were without clear definitions of clinical staging in the NCT group, for the NCT and NICT groups, there were respectively 53 (82.8%) and 52 (54.7%) later clinical staging cases, along with eight (12.5%) and 43 (45.3%) earlier clinical staging cases ($p < 0.001$); see Table 2 and Figure 1C. Thus, in the NICT group, more cases were converted to an early stage, with a significant reduction in the number of locally advanced cases, while this conversion was not significant in the NCT group. Meanwhile, compared with the NCT group, more cases in the NICT group showed a reduction of clinical stage post-NT (n

TABLE 1 Comparison of changes in tumor size between groups.

Parameters	NONE (%) (n = 120)	NCT (%) (n = 64)	NICT (%) (n = 95)	*p*-Value
Pre-treatment				
Length[a] (mm)	40.47 ± 17.50	48.86 ± 18.68	60.89 ± 20.53	
Thickness[b] (mm)	12.10 ± 5.81	15.59 ± 6.06	16.78 ± 6.83	
Length × Thickness (mm^2)	514.08 ± 439.86	819.17 ± 566.65	1,063.62 ± 683.50	NONE vs. NICT <0.01 NCT vs. NICT 0.019
Post-NT				
Length (mm)		37.03 ± 14.92	39.22 ± 17.81	
Thickness (mm)		11.18 ± 5.16	9.74 ± 5.67	
Length × Thickness (mm^2)		449.05 ± 336.14	420.05 ± 438.05	0.655
Reduction cases No. (%)				
Length		48 (75%)	84 (88.4%)	0.027
Thickness		56 (87.5%)	92 (96.8%)	0.023
Length × Thickness		59 (92.2%)	95 (100%)	0.006

NONE, non-neoadjuvant therapy; NCT, neoadjuvant chemotherapy; NICT, neoadjuvant immunochemotherapy; NT, neoadjuvant therapy.
[a]Length, the maximum longitudinal diameter.
[b]Thickness, the maximum pipe-wall thickness.

= 53 55.8% vs. n = 20, 31.3%), which was significantly statistically different (Table 2 and Figure 1D, p = 0.005). Additionally, 10 (10.5%) cases showed clinical stage advancement in the NICT group, while there were 20 (31.3%) cases in the NCT group (Table 2 and Supplementary Figure S1C, $p < 0.001$). Furthermore, there were more cases of pathologic complete response (pCR) in NICT (NICT vs. NCT, n = 1 1.6% vs. n = 21 22.1%), as shown in Figure 1E.

The cross-sectional and longitudinal images of CT provided evidence of the changes in tumor size pre- and post-NT among the NCT and NICT groups, suggesting that the tumor size of the NICT group was mostly larger pre-NT, while it reduced dramatically post-NT, compared with the NCT group (Figure 1F, Supplementary Figure S1D).

These clinical findings indicated that the NICT group had a better therapeutic effect than the NCT group based on differences in survival period, tumor response, and changes in clinical stage.

NICT promoted the expression of immune-related genes in esophageal cancer tissues

To evaluate the characteristics of the immune response of the ESCA tissues after NICT and NCT, we applied the RNA sequencing technique to compare the differences in gene expression of ESCA tissues after NT, where the NONE group was treated as the control group. GO analysis revealed that the cytokine, chemokine, and their receptors in the ESCA tissues from the NICT group (as compared to the NCT group) were more active (Figure 2A). The NICT group also exhibited positive activity of the NK cells as compared to the NONE group (Supplementary Figure S2A). On the contrary, as compared to the NONE group, the enriched signal pathways in the NCT group involved non-coding RNA (ncRNA) processing, DNA, and mRNA metabolic process (Supplementary Figure S2B). Meanwhile, the function cluster of the differential genes from the groups suggested that lymphocyte-mediated immune response and activation immune of lymphocyte-mediated killer immunity in the NICT group were significantly enhanced (Figure 2B), compared to the NONE and NCT groups (Figure 2C, Supplementary Figure S2C). The results of the cycle net plot from the NICT group elucidated the signaling pathway and differential genes associated with positive regulation of NK cell activation and proliferation, and cellular response to chemokines (Figures 2D, E, Supplementary Figure S2D). Based on the differential genes, we constructed the network of regulating immune function. The network results further suggested that the increased expressions of genes in the NICT group promoted adaptive immune response based on somatic recombination of immune receptors built from the immunoglobulin superfamily domain, activation of immune response, lymphocyte and leukocyte proliferation, and positive regulation of immune cell–cell adhesion (Supplementary Figure S2E), as well as activation and positive regulation of NK cells (Supplementary Figure S2F).

To validate the relationship between these changes and the immunotherapy for ESCA, we first analyzed the therapeutic effects of each sample (i.e., the response of an individual to treatment, estimating the reduction rate of the length × thickness of the tumor, Figure 2F). Then, we compared the expression of immune-related genes in different post-treatment samples. The results of the immune-related gene qRT-PCR are shown in Figure 2G. In the NONE group, the detected genes of the case with a larger size of the tumor (i.e., Case 1) showed a low-level expression. In the

TABLE 2 Comparison of changes in clinical stage between NCT and NICT groups.

Parameters	NCT (%) (n = 64)	NICT (%) (n = 95)	*p*-Value
Pre-NT[a]			
I	8 (12.5)	2 (2.1)	0.008
II	18 (28.1)	28 (29.5)	
III	26 (40.6)	58 (61.1)	
IV	12 (18.8)	7 (7.4)	
Could not be determined	0 (0)	0 (0)	
Post-NT[b]			
I	8 (12.5)	43 (45.3)	<0.001
II	17 (26.6)	13 (13.7)	
III	26 (40.6)	38 (40.0)	
IV	10 (15.6)	1 (1.1)	
Could not be determined	3 (4.7)	0 (0)	
Stage reduction Stage progression	20 (31.3) 20 (31.3)	53 (55.8) 10 (10.5)	0.005 <0.001
pCR[c]	1 (1.6)	21 (22.1)	<0.001

NCT, neoadjuvant chemotherapy; NICT, neoadjuvant immunochemotherapy.
[a]Pre-NT, pre-neoadjuvant therapy.
[b]Post-NT, post-neoadjuvant therapy.
[c]pCR, pathologic complete response.

NICT group, these detected genes were highly expressed in Case 7 with better therapeutic effect (reduced size by 57.22%), while they showed a low-level expression in Case 6 with insignificant size reduction (by 19.32%) after NT.

Based on the findings demonstrated above, we analyzed the GSE145370 dataset from the GEO database according to the markers of T cells and NK cells (Supplementary Figure S2G). The analysis results suggested that the predominant immune-infiltrating cells in esophageal cancer tissue are T cells. Compared to the normal tissues, the presence of NK cells reduced dramatically ($p < 0.01$, Supplementary Figures S2H, I).

It can be seen that NICT could promote the expression of immune-related genes and activate T cells and NK cells in ESCA tissues.

NICT activated CD8+ T and NK cells of esophageal cancer tissues

In order to assess the regulating effect of NICT on the tumor microenvironment (TME), we applied RNA sequencing data to analyze the status of immune cell infiltration in ESCA tissues. We employed the ESTIMATE Score to acquire the scoring of immune cell infiltration and compared the number of immune cells and differences in activity among the NONE, NCT, and NICT groups (Figure 3A). The results showed the NICT group revealed a relatively stable high level of immunity. As compared to the NCT group, the function cluster of GO analysis showed that significant changes were observed in the cellular response to cytokine stimulus (Figure 3B), T-cell modulation (Figure 3C), protein phosphorylation, and the expression of kinases (Supplementary Figure S3A, $|\log(FC)| > 1$, $p < 0.05$). More important, compared with the NCT and NONE groups, differences in the expression of chemokines, interleukins, and killing effector factors had caught our attention in the NICT group (Figure 3D, Supplementary Figure S3B, fold change > 1, $p < 0.05$).

PRF1, GZMA, and GZMM (i.e., cytotoxic effector factors specifically expressed in T cells and NK cells; Supplementary Figures S3C, D) were significantly suppressed in the NCT group, while they increased in the NICT group (Figure 3D). Moreover, we evaluated the cytotoxic activity of immune cells in tumor tissues by the cytolytic activity score (CYT) or the marker molecules using PRF1 and GZMM (Figure 3F). This finding revealed that the NICT group can improve the capability for the immune cell infiltration of the ESCA tissues compared with the NCT group. Next, we used the HPA database to identify the types of cells expressing cytotoxic effector factors (e.g., PRF1, GZMA, and GZMM). As shown in Figure 3G, these factors were specially expressed in infiltrating CD8 + T and activated NK cells.

Furthermore, the expression of cytokine IFN-γ and its transcription factors EOMES and TBX21 showed an increased state in the NICT group (Figure 3D, $p < 0.05$), indicating the creation of the IFN-γ molecule regulating pathway, as well as the activation of T-cell subtype (i.e., Tc1) and NK cells. Given the findings above, we analyzed the IFN-γ response pathway by GSEA. The results showed that this pathway could be activated in the NICT group while remaining suppressed in the NCT group (Figure 3E). Furthermore, the correlation analysis further validated that CD8A and CD8B were correlated with transcription factors of IFN-γ (i.e., EOMES) and the regulatory factor of T cells (i.e., TCIRG1), which indicated that CD8+ T cells were strongly correlated with these effectors and the expression of IFN-γ (Supplementary Figure S3E).

The findings above suggested that NICT can significantly enhance the infiltration and activation of immune cells centered with CD8+ T and NK cells in ESCA tissues.

NICT boosted anti-tumor immunity of Tc1 cells

PD-1 inhibitors are the T cell-based immune checkpoint blockade, which can recognize the PDCD1 (PD-1, CD279) of CD8 + T cells. The immune response of CD8+ T cells is activated when PDCD1 is blocked (29, 30). First, the expression level of PDCD1 was analyzed in immune cells and esophageal tissues. The results showed that the expression level of PDCD1 was actually higher in some types of CD4 and CD8+ T cells (HPA database, Supplementary Figures S4A, S4B), as well as ESCA, but lower in normal esophageal tissues (GEPIA database, Supplementary Figure S4C). Therefore, in order to investigate the evidence that NICT increased the clinical remission rate and relied on enhancing the microenvironment of ESCA, we traced the quantity and activation status of CD8+ T cells in the NICT

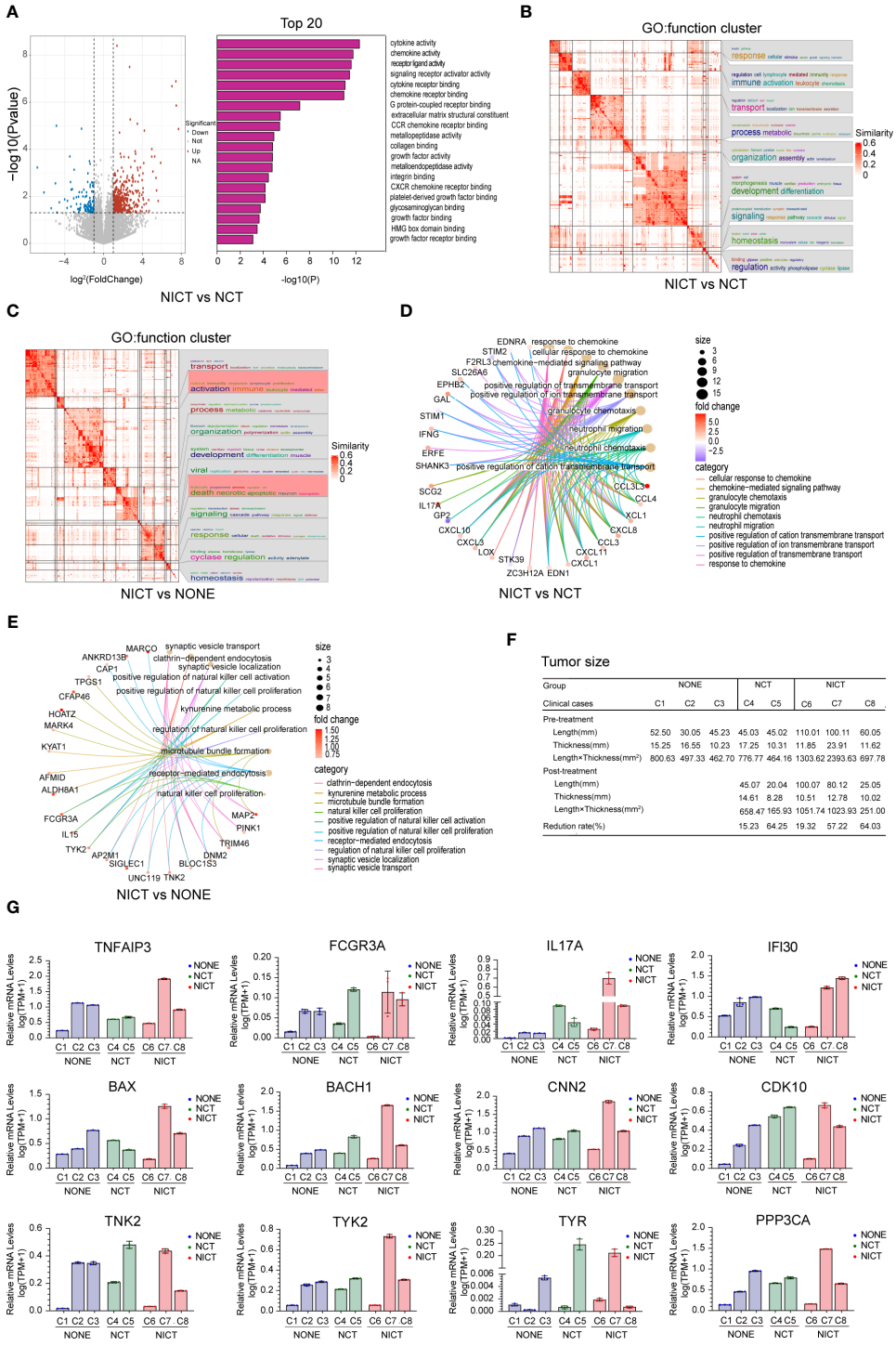

FIGURE 2
NICT promoted the expression of immune-related genes in ESCA tissues. **(A)** Pathway enrichment analysis of the cytokine, chemokine, and their receptors in NICT group compared to NCT group using RNA sequencing technique. **(B)** The function cluster analysis of Gene Ontology (GO) showing comparison of the differential genes between the NCT and NICT groups. **(C)** The function cluster analysis of GO showing comparison of function clusters of the differential genes between the NONE and NICT groups. **(D)** Comparison of cycle net plot between the NCT and NICT groups. **(E)** Comparison of cycle net plot between the NONE and NICT groups. **(F)** The table presents the tumor sizes pre- and post-treatment for eight clinical cases (from C1 to C8) from the NONE, NCT, and NICT groups. **(G)** qRT-PCR results of 12 immune-related genes and immune-regulated genes in clinical cases among the NONE, NCT, and NICT groups. NICT, neoadjuvant immunochemotherapy; ESCA, esophageal cancer; NCT, neoadjuvant chemotherapy; NONE, non-neoadjuvant therapy; qRT-PCR, quantitative real-time PCR.

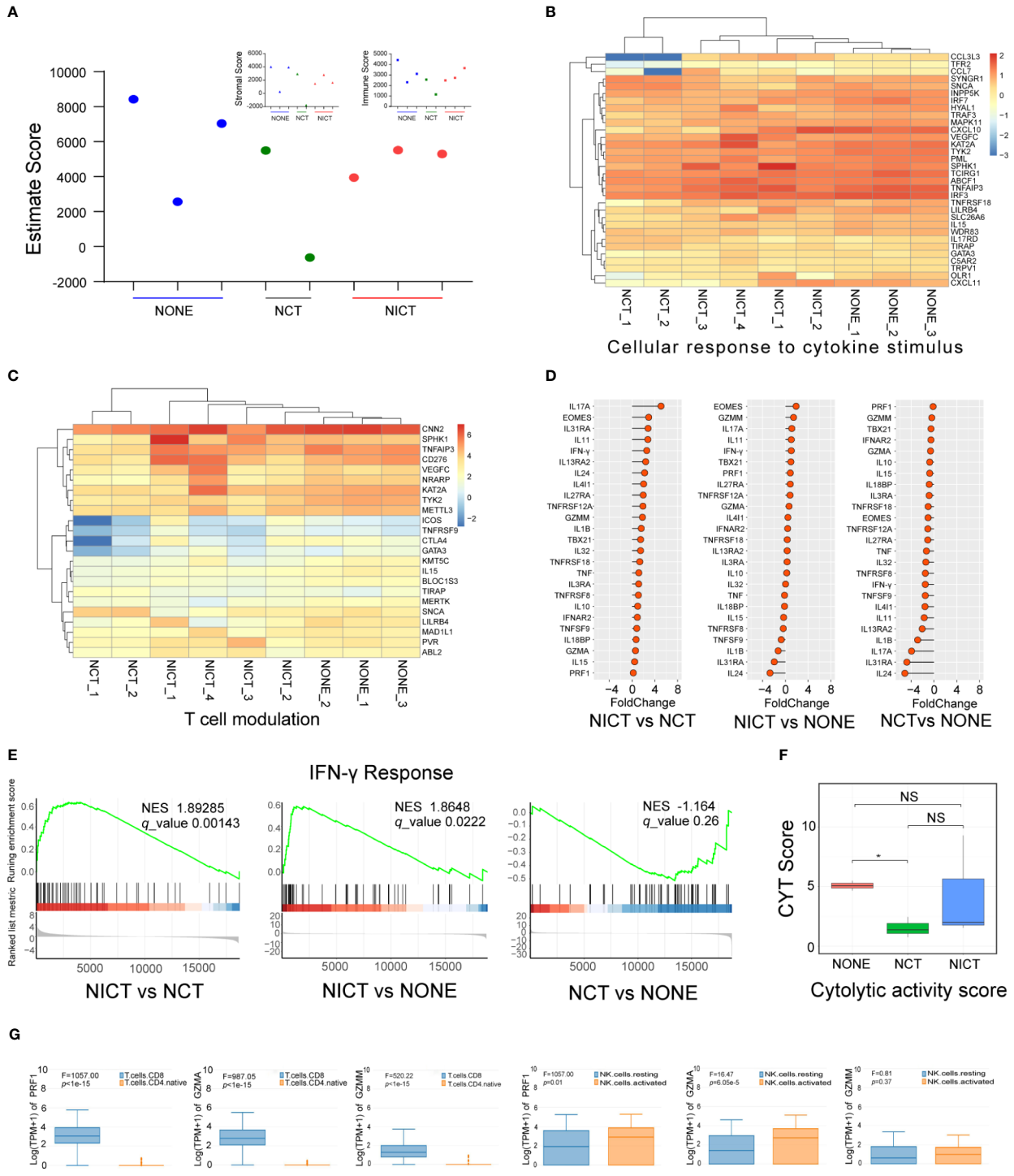

FIGURE 3
NICT activated the immune microenvironment of ESCA tissues. **(A)** Using ESTIMATE Score to analyze the scoring of immune cell infiltration among NONE, NCT, and NICT groups. **(B)** Heatmap showing the production of cellular response to cytokine stimulus in each group of clinical cases. **(C)** Heatmap showing the production of T-cell modulation in each group of clinical cases. **(D)** Comparison of the expression of cytokines in the NONE, NCT, and NICT groups, especially the cytotoxic effector factors specifically expressed in T cells and NK cells. **(E)** Comparison of IFN-γ response pathways among NONE, NCT, and NICT groups by Gene Set Enrichment Analysis (GSEA). **(F)** Evaluation of the cytotoxic activity of immune cells in tumor tissues by calculating cytolytic activity score (CYT) using PRF1 and GZMM. **(G)** Using HAP database to identify the types of T cells and NK cells expressing PRF1, GZMA, and GZMM in ESCA. NICT, neoadjuvant immunochemotherapy; ESCA, esophageal cancer; NONE, non-neoadjuvant therapy; NCT, neoadjuvant chemotherapy. *p < 0.05, NS, no significance.

group compared with the NCT group. In the NICT group, we found that the expression and quantity of CD8+ T cells were increased significantly post-NT compared with pre-NT (Figure 4A), while they decreased in the NCT group using immunofluorescence technique (Figure 4B, $p < 0.01$). The immunohistochemistry (IHC) results also showed that the quantity of CD8+ T cells in the NICT group was significantly higher than that in the NCT group (Supplementary Figure S4D, $p < 0.001$).

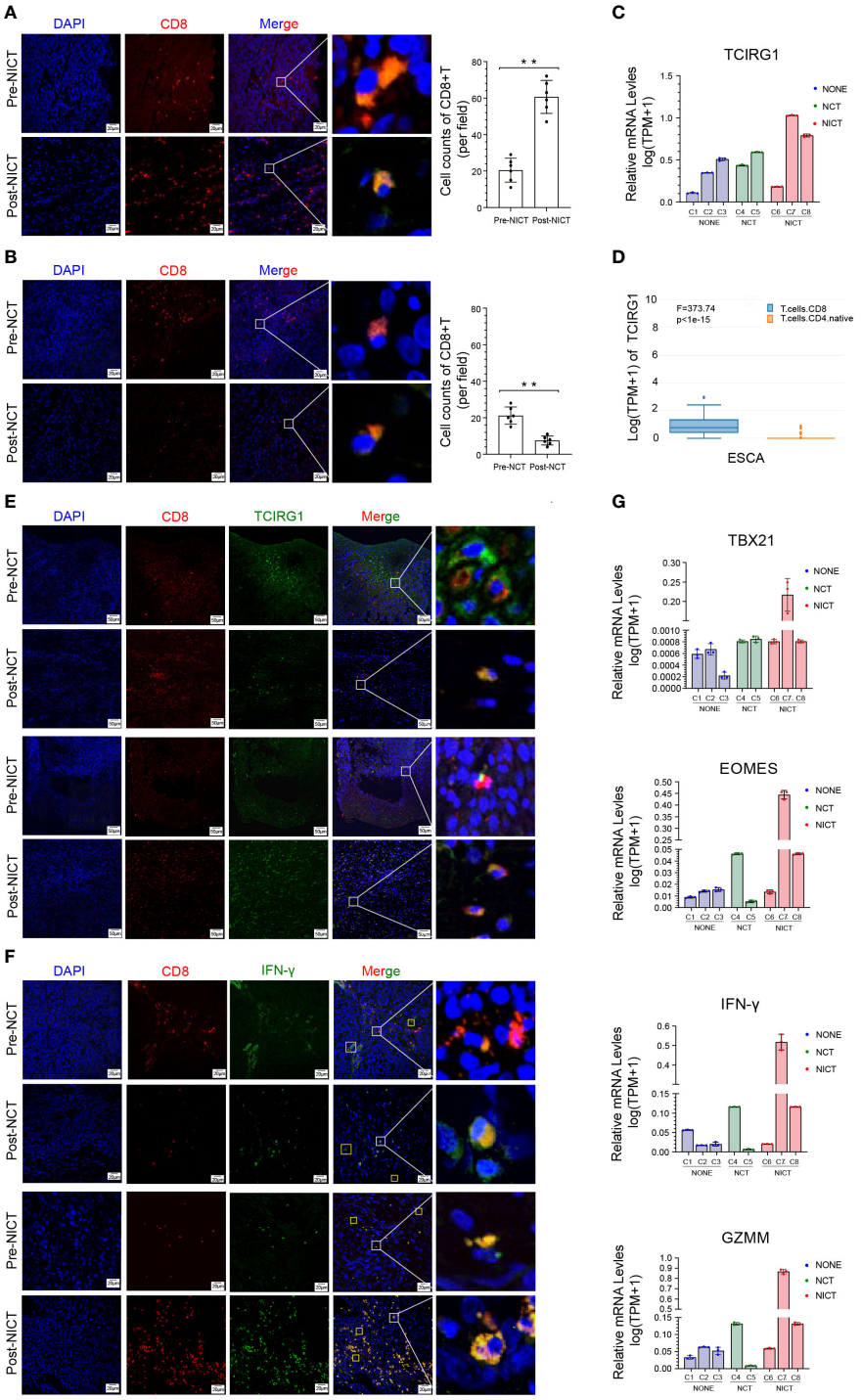

FIGURE 4
Anti-tumor immunity of CD8+ T cells was boosted by NICT. **(A)** Immunofluorescence showing the expression status and quantity changes in CD8+ T cells in the NICT group before and after PD-1 inhibitor treatment and cell count analysis. **(B)** Immunofluorescence showing the expression status and quantity changes in CD8+ T cells in the NCT group before and after chemotherapy and cell count analysis. **(C)** qRT-PCR showing the expression level of TCIRG1 in each group of clinical cases. **(D)** HAP database showing the types of T cells expressing TCIRG1 in ESCA. **(E)** Multiplex immunofluorescence showing the expression status and quantity changes in CD8+ T cells and TCIRG1 among the NICT and NCT groups pre- and post-NT. **(F)** Multiplex immunofluorescence showing the expression status and quantity changes in CD8+ T cells and IFN-γ among the NICT and NCT groups pre- and post-NT. **(G)** qRT-PCR showing the expression levels of TBX21, EOMES, IFN-γ, and GZMM in clinical cases among the NONE, NCT, and NICT groups. $^{**}p < 0.01$. NICT, neoadjuvant immunochemotherapy; qRT-PCR, quantitative real-time PCR; ESCA, esophageal cancer; NCT, neoadjuvant chemotherapy; NT, neoadjuvant therapy; NONE, non-neoadjuvant therapy.

To explore the regulatory mechanisms of the changes in CD8+ T cells, we further investigated the expression of TCIRG1 as a regulatory factor of T cells. The expression of TCIRG1 exhibited a low level in ESCA and no difference between normal esophageal tissues and ESCA in the GEPIA database (Supplementary Figure S4E). In the NICT group, the IHC results suggested that this protein had a certain-level expression in stromal cells (Supplementary Figure S4F). Using qRT-PCR for analysis, the expression of TCIRG1 showed clear differences in the pCR and partial response (PR) cases of the NICT group, and it was also related to changes in the tumor size of the NONE and NCT groups (Figure 4C). We also identified the expression of TCIRG1 in CD4+ and CD8+ T cells by applying the HAP database and found that it was specifically expressed in infiltrating CD8+ T cells (Figure 4D). Moreover, the immunofluorescent staining results suggested that in the NICT group, the expression of TCIRT1 occurred within CD8+ T cells, and the quantity of TCIRG1 was greater post- than pre-NT but decreased in the NCT group (Figure 4E).

Tc1 is a subtype of CT8+ T with tumor-killing function, regulated by transcription factors EOMES and TBX21. Because the expression level of IFN-γ can serve as a marker of Tc1 activation, we first analyzed the expression level of IFN-γ of the CD8+ T cells in the ESCA tumor tissues from the NICT and NCT groups. The HPA database showed that the expression was almost nonexistent in ESCA tissues but relatively high in naive CD4+, CD8+ T cells, and activated NK cells (Supplementary Figure S4G). Then, we compared the expression of TBX21, EOMES, IFN-γ, and GZMM in the ESCA tumor tissue among the NICT, NCT, and NONE groups by immunofluorescent staining and qRT-PCR. IFN-γ significantly boosted the expression in CD8+ T-cell infiltration in the NICT group (Figure 4F). The high-level expression of these four factors was correlated with better tumor remission of patients by qRT-PCR analysis (Figure 4G).

The above findings suggested that NICT enabled the activation of the Tc1 in ESCA tissues and expressed a high quantity of IFN-γ and cytotoxic effector factor (GZMM) through the regulation of transcription factors (EOMES and TBX21). Therefore, it constructed the regulatory and functional pathway of CD8+ T activation through the immune infiltration by NICT.

NICT promoted CD16+ NK cells killing tumor cells

Except for CD8+ T cells, activated NK cells are another important subtype of cytotoxic cells that play a crucial role in killing esophageal cancer cells. We utilized RNA-seq data (heatmap) to analyze the expression of NK cells' characteristic antigens and found that these NK cells' antigens showed different states among the NONE, NCT, and NICT groups (Supplementary Figure S5A). Further, we detected the infiltration status of the NK cells in the ESCA tissues by IHC using CD16 as a marker. The IHC results indicated widespread infiltration of CD16+ cells, with greater quantities of CD16+ cells observed in the remaining tumor tissues from the NICT group compared with the NCT group (Supplementary Figure S5B). Additionally, comparing the infiltration of CD16+ cells in tumor tissue before and after treatment, it was found that it significantly increased after the use of PD-1 inhibitors in the NICT group (Figure 5A, top), while it decreased in the NCT group (Figure 5A, bottom). At the same time, we analyzed the expression of IFN-γ in ESCA tissues and found that it significantly increased in the NICT group, but not in the NCT group. IFN-γ is also specially expressed in activated CD16+ NK cells in addition to being expressed in activated CD8+ T cells (Supplementary Figure S5C). The immunofluorescence analysis results showed that the detected IFN-γ amounts were mostly in CD16+ NK cells in the NICT group (Figure 5B, bottom).

ICBs often exert inhibitory effects on specific markers of immune cells or tumor cells and then activate the immune cells of the TME. However, it remains unclear whether the cytotoxicity of activated immune cells has selectivity in different ICBs. Therefore, applying RNA-seq data, we analyzed the expression of the ICB marker that has been utilized in clinical cancer treatment. We found that the expression of PD-1 (PDCD1/CD279) was lower in ESCA samples and that the differences in the corresponding ligand (tumor cell) CD274 (PD-L1) were non-significant across patients and groups (Supplementary Figure S5D). However, B7-H3 (i.e., CD276, a homolog of PD-L1), which is another member of the B7 immunoglobulin superfamily, drew our attention. As shown in Supplementary Figure S5D, this molecule exhibited the highest expression among ICB-related molecules in the expression profile data of each group of samples. Searching through the HPA database, we found that CD276 is highly expressed in many tumors and rarely expressed in immune cells (Supplementary Figures S5E, S5F). The data from GEPIA and UALCAN demonstrated a high-level expression of CD276 in ESCA tissues (Supplementary Figures S5G, S5H). We then utilized CD276 as a marker to detect the infiltration status of the ESCA tissues by IHC, and the results indicated that CD276+ cells also widely infiltrated the ESCA tissues (Supplementary Figure S5I). In order to explore the relationship between CD276+ cells and cytotoxicity cells, we further examined the pathology slides of ESCA tissues using CD16, CD8, and CD276 antibodies. Notably, CD276+ cells were found in eight out of nine (88.89%) ESCA lesions in the random testing of each group using immunofluorescence staining, and pre-NT, the results of immunofluorescence co-staining showed that CD16+ NK cells could recognize CD276+ tumor cells in the NCT and NICT groups (red fluorescence and green fluorescence exhibit close-range optical interference phenomenon), as shown in Figure 5C. However, post-NT, CD276+ tumor cells still appeared in the NCT group but were reduced significantly in the post-NICT group, while CD16+ NK cells could be observed in the remaining tumors of the NCT and NICT groups (Figure 5D). However, there were no such characteristics found with CD8+ T cells (Supplementary Figures S5J, S5K). It indicated that CD16+ NK cells that infiltrated tumor lesions could recognize CD276 + tumor cells but with no capability of cytotoxicity before the

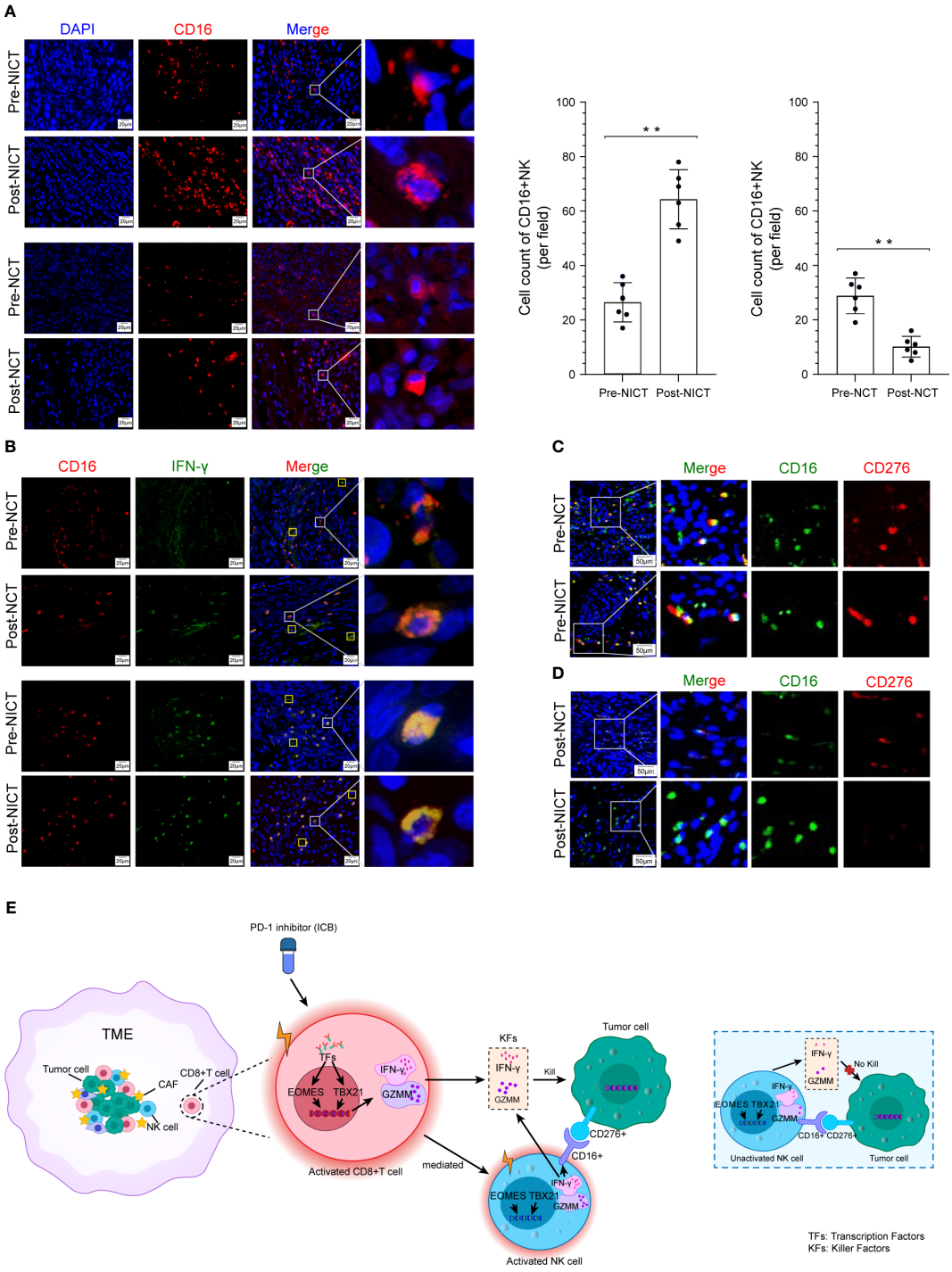

FIGURE 5
CD16+ NK cells killing tumor cells were promoted depending on NICT. **(A)** Immunofluorescence showing the expression status and quantity changes in CD16+ NK cells among the NICT and NCT groups pre- and post-NT and cell count analysis. **(B)** Multiplex immunofluorescence showing the expression status and quantity changes in CD16+ NK cells and IFN-γ among the NICT and NCT groups pre- and post-NT. **(C)** Multiplex immunofluorescence showing the expression status and quantity changes in CD16+ NK and CD276+ cells among the NICT and NCT groups pre-NT. **(D)** Multiplex immunofluorescence showing the expression status and quantity changes in CD16+ NK and CD276+ cells among the NICT and NCT groups post-NT. **(E)** Pattern diagram of NICT activated the immune cells to kill ESCA cells. $^{**}p < 0.01$. NICT, neoadjuvant immunochemotherapy; NCT, neoadjuvant chemotherapy; NT, neoadjuvant therapy; ESCA, esophageal cancer.

treatment of PD-1 inhibitors, while it can kill CD276+ tumor cells after the treatment of PD-1 inhibitors.

In summary, these findings showed that CD8+ T cells were activated by PD-1 inhibitors and secreted more IFN-γ to kill ESCA cells. At the same time, activated CD8+ T cells mediated the CD16+ NK cell activation and secreted more IFN-γ to execute the killing function, whereas CD16+ NK cells could not achieve the cytotoxicity of CD276+ tumor cells when the CD8+ T cells were inactive (Figure 5E).

Discussion

In recent years, NT has been widely adopted due to its capability in advancing the effectiveness of clinical cancer treatment, as well as improving the prognosis of cancer patients (29, 31, 32). However, there is still controversy over the options and models of NT for ESCA. Especially in the era of immunotherapy, many solid tumors have shown significant benefits in treatment with the participation of ICBs (30, 33). Similarly, traditional NT for ESCA is also facing challenges from immunotherapy.

ICBs resist tumor cells by engaging the immune system, utilizing the high specificity, monitoring capability, and long-term memory capability to achieve effective and sustained treatment effects. The combined treatment of NT and ICBs (i.e., NICT) is an important strategy in cancer treatment, leveraging the power of the immune system to enhance tumor control, increase the rate of surgical resection, and improve patient prognosis. Whether NICT can be used for the treatment of ESCA is currently one of the forefront research hotspots in clinical practice (34, 35). A study has found that NICT had promising clinical and safety outcomes for patients with resectable ESCA (36). Established evidence showed that patients with ESCA could gain survival benefits from NICT, especially for increasing the successful rates of surgery for locally advanced staged patients (37).

Our clinical data results, obtained by CT, esophagography, PET/CT, and other methods, also showed that ESCA patients exhibited a more effective response rate and clinical stage reduction when treated with NICT compared with NCT. The pCR rate and 2-year survival rate were 22.1% and 70.9%, respectively, in the NICT group, while they were 1.6% and 51.5% in the NCT group, respectively, consistent with recent research results (38–40), suggesting that NICT achieved remarkable benefits in patients with ESCA than NCT. Therefore, for the treatment of ESCA, NICT will have better efficacy and survival benefits and broader future clinical applications.

The efficacy discrepancy between NCT and NICT in ESCA is proposed to be associated with the ICBs, which can restore the monitoring capability of T cells on cancer cells in patients, thus effectively eliminating cancer cells (41). Therefore, we analyzed the clinical remission cases to compare the treatment response between NICT and NCT and discussed the changes in T cells in ESCA lesions. Our findings suggested that there was a significant difference in CD8+ T cells between NCT and NICT in ESCA samples. Post-NCT, the infiltration of CD8+ T cells in the TME significantly decreased, while post-NICT, it significantly increased, indicating that NICT can effectively enhance the anti-tumor immune response by increasing the number of CD8+ T cells, thereby killing tumor cells. It has been reported that the infiltration of CD8+ T within the TME is a key indicator to reflect the effectiveness of immune therapy (42). As the classical cytotoxicity CD8+ T cells, the Tc1 subtype is a unique cytotoxicity cell, which can effectively kill tumor cells (43). Functionally, the Tc1 cell is capable of producing high-level GZMM, IFN-γ, and TNF-α, and their activity is co-regulated by transcription factors TBX21 and EOMES coordinately (44). Classical IFN-γ+ Tc1 cells are the most common subtype in the TME and have been captured in tumor infiltration lymphocytes (TILs), including melanoma, ovarian cancer, breast cancer, and lung cancer. Originally from IFN-γ, prior evidence suggested that the potential of cytotoxicity could be the reason why Tc1 cells are related to better prognoses. Meanwhile, cytokine IFN-γ exerts a direct effect on tumor cells, enhancing its sensitivity to cytotoxicity dependent on CD8+ T cells (45). In fact, the direct effect of IFN-γ on tumor cells is highly related to the anti-tumor effect. Our results indicated that the expression of the IFN-γ and its transcription factors EOMES and TBX21 were increased post-NICT, suggesting that NICT could activate CD8+ T cells and enhance its cytotoxic effect in ESCA. In summary, CD8+ T cells were activated and produced stronger anti-tumor responses post-NICT, reflecting that NICT for ESCA may induce antigen exposure, thereby restoring the anti-tumor immune efficacy.

NK cells are another type of tumor-killing cells that can mediate tumor cell destruction through multiple mechanisms, including releasing perforin/granzyme pathways, secreting IFN-γ and TNF-α, and releasing antibody-dependent cell-mediated cytotoxicity (ADCC) pathways. The activated NK cells express TBX21 and EOMES, further regulating cell cytokines such as IFN-γ and TNF-α, which are the main source of cytokines (e.g., IFN-γ, TNF-α, and GM-CSF) and chemokines. The tumor-killing function could be driven by the release of GZMM to destroy the membrane of tumor cells to trigger apoptosis (46). The activated NK cells can effectively recognize the tumor cells' surface antigens by expressing the receptors of CD16 (FCγRIIIA), NKp30, and NKG2D. They achieve an anti-tumor effect by the pathway of ADCC and also play a role in ADCC and immune regulation. Results have suggested that NK cells induce an ADCC response in combination with anti-PD-L1 antibody, which promotes ADCC anti-tumor activity against PD-L1-positive tumors (47). Our data demonstrated a significant increase in the proportion of CD16+ NK cells following NICT, suggesting the potential for NK cells to be reactivated by CD8+ T cells and to exert anti-tumor effects after applying PD-1 inhibitors.

In addition to the activation of NK cells, we specifically focused on the antigen recognition and cell killing of NK cells to CD276+ tumor cells. Our results showed that NK cells could recognize CD276+ tumor cells through CD16+, but their killing function was limited in NCT. However, post-NICT, CD16+ NK cells were activated by the activated CD8+ T cells and secreted increased tumor killer factor IFN-γ, thereby effectively killing tumor cells. These results suggested that NICT played an indispensable role in the NT efficacy in ESCA by activating CD8+ T cells and mediating the activation of CD16+ NK cells, enhancing their tumor-killing capabilities.

NICT can sensitize the TIME and play a significant role in promoting therapeutic efficacy by affecting the TME. However, there are still some limitations that need to be addressed. First, the sample size of the mechanism study was limited, which needs further validation. Second, there were notable changes in chemokines among ESCA patients after NICT compared to NCT; for instance, the expression of factors such as IL17A that were secreted by CD4+ T cells also showed dynamic changes before and after different treatments. This suggests that CD4+ T cells also play

a role in the treatment process; however, their specific functions are still unknown (48).

In conclusion, NICT has been used in the treatment of ESCA, yet the mechanisms of the action are not clear. In this research, which included 279 clinical cases, we found that individuals in the NICT group showed significant clinical efficacy compared with the NCT group. Mechanism research revealed that NICT could promote the expression of immune-related genes and activate the immune microenvironment. The CD8+ T cells were activated post-NICT, then stimulated CD16+ NK cell activation, and achieved tumor cell killing by cytokines (e.g., IFN-γ and GZMM). In addition, we explored whether CD276 (B7-H3) may be regarded as a new ESCA cell marker that could be recognized by CD16+ NK cells. This study holds significant value in the development of NICT strategies for ESCA.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: Gene Expression Omnibus (GEO), accession number PRJNA1114802.

Ethics statement

The studies involving humans were approved by the Ethics Committee of Harbin Medical University Cancer Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

YH: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. DY: Data curation, Formal analysis, Methodology, Software, Validation, Writing – review & editing. XL: Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing – original draft. JFZ: Conceptualization, Data curation, Investigation, Resources, Supervision, Validation, Writing – original draft. RC: Conceptualization, Data curation, Formal analysis, Methodology, Software, Validation, Writing – original draft. LC: Data curation, Software, Writing – original draft. LY: Data curation, Writing – original draft. MZ: Data curation, Writing – original draft. XS: Data curation, Writing – original draft. XJ: Data curation, Writing – original draft. HDS: Formal analysis, Writing – original draft. HXS: Formal analysis, Writing – original draft. JYZ: Data curation, Writing – original draft. YL: Data curation, Formal analysis, Methodology, Supervision, Writing – original draft, Writing – review & editing. JM: Data curation, Methodology, Resources, Supervision, Writing – original draft. HN: Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1412693/full#supplementary-material

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Prognostic biomarkers for immunotherapy in esophageal cancer

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Esophageal cancer (EC), a common type of malignant tumor, ranks as the sixth highest contributor to cancer-related mortality worldwide. Due to the condition that most patients with EC are diagnosed at advanced or metastatic status, the efficacy of conventional treatments including surgery, chemotherapy and radiotherapy is limited, resulting in a dismal 5-year overall survival rate. In recent years, the application of immune checkpoint inhibitors (ICIs) has presented a novel therapeutic avenue for EC patients. Both ICIs monotherapy and immunotherapy combined with chemotherapy or chemoradiotherapy (CRT) have demonstrated marked benefits for patients with advanced EC. Adjuvant or neoadjuvant therapy incorporating immunotherapy has also demonstrated promising prospects in the context of perioperative treatment. Nonetheless, due to the variable response observed among patients undergoing immunotherapy, it is of vital importance to identify predictive biomarkers for patient stratification, to facilitate identification of subgroups who may derive greater benefits from immunotherapy. In this review, we summarize validated or potential biomarkers for immunotherapy in EC in three dimensions: tumor-cell-associated biomarkers, tumor-immune microenvironment (TIME)-associated factors, and host-associated biomarkers, so as to provide a theoretical foundation to inform tailored therapy for individuals diagnosed with EC.

KEYWORDS

esophageal cancer, immunotherapy, biomarkers, ICIs, TIME

1 Introduction

Esophageal cancer (EC), including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), ranks as the eighth most common malignancy worldwide, with a mortality rate ranking sixth (1, 2). In China, EC, predominantly consisting of ESCC cases, poses a substantial disease burden, accounting for approximately half of the global annual incidence and mortality rates (3). Anatomically, ESCC mainly occurs in the upper or middle segment of the esophagus, whereas EAC tends to manifest in the distal region (4). Geographically, ESCC is more commonly found in Eastern Asia, Eastern Europe, and Southern and Eastern Africa, while EAC has a higher prevalence in North America, Central America, and Central Africa (3, 4).

Conventional treatment strategies for EC include surgery, radiotherapy, chemotherapy and targeted therapy (5), but the five-year survival rate still remained less than 20% until 2021, due to delayed diagnoses and high recurrence rates (3, 4). In recent years, the administration of immune checkpoint inhibitors (ICIs) in EC has attained significant advances, as ICIs can both suppress cancer cells by modulating anti-tumor immunity, as well as exerting a synergistic effects when combined with chemotherapy or/and radiotherapy (6) (Table 1). Immune checkpoint genes and cellular interactions contributing to tumor immunity are illustrated in Figure 1 (7, 8) (Figure 1). The phase III KEYNOTE-181 trial demonstrated that pembrolizumab, compared with chemotherapy, contributed to superior overall survival (OS), objective response rate (ORR), and lower incidence of high-grade treatment-related adverse events (9). Moreover, other phase III trials, such as CheckMate648 (10) and KEYNOTE-590 (11), have confirmed that the combination of immunotherapy and chemotherapy as first-line treatment significantly improves OS and progression-free survival (PFS) compared with chemotherapy alone. However, the benefits of immunotherapy vary among patients with patients, highlighting

TABLE 1 The large-scale phase III clinical trials of immunotherapy for EC and efficacy outcomes in overall population regardless of PD-L1 expression.

Clinical trial	Treatment model	Cancer status	Region	Arm (No. of pts)	Treatment design	Efficacy outcomes (95% CI)	Ref.
KEYNOTE-590 (NCT03189719)	First-line	M/UA EC	global	1 (373)	PEM (200 mg) + chemotherapy	Improved OS: 12.4 (10.5 - 14.0) m; Improved PFS: 6.3 (6.2 - 6.9) m; Improved ORR: 45.0 (39.9 - 50.2) %	(11)
				2 (376)	placebo + chemotherapy	OS: 9.8 (8.8 - 10.8) m; PFS: 5.8 (5.0 - 6.0) m; ORR: 29.3 (24.7 - 34.1) %	
CheckMate 648 (NCT03143153)	First-line	M/UA ESCC	global	1 (321)	NIV (240 mg) + chemotherapy	Improved OS: 13.2 (11.1 - 15.7) m; PFS: 5.8 (5.6 - 7.0) m	(10)
				2 (325)	NIV (3 mg/kg) + IPI (1 mg/kg)	Improved OS: 12.7 (11.3 -15.5) m; PFS: NA	
				3 (324)	Chemotherapy	OS: 10.7 (9.4 - 11.9) m; PFS: 5.6 (4.3 - 5.9) m	
ESCORT-1st (NCT03691090)	First-line	A/M ESCC	China	1 (298)	CAM (200 mg) + chemotherapy	Improved OS: 15.3 (12.8 - 17.3) m; Improved PFS: 6.9 (5.8 - 7.4) m; Improved ORR: 72.1 (66.7 -77.2) %	(12)
				2 (298)	placebo + chemotherapy	OS: 12.0 (11.0 - 13.3) m; PFS: 5.6 (5.5 - 5.7) m; ORR: 62.1 (56.3 - 67.6) %	
ORIENT-15 (NCT03748134)	First-line	LA/M ESCC	global	1 (327)	SIN (3 mg/kg or 200 mg) + chemotherapy	Improved OS: 16.7 (14.8 - 21.7) m; Improved PFS: 7.2 (7.0 - 9.5) m; Improved ORR: 66 (61–71) %	(13)

(Continued)

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TABLE 1 Continued

Clinical trial	Treatment model	Cancer status	Region	Arm (No. of pts)	Treatment design	Efficacy outcomes (95% CI)	Ref.
				2 (332)	placebo + chemotherapy	OS: 12.5 (11.0 - 14.5) m; PFS: 5.7 (5.5 - 6.8) m; ORR: 45 (40 –51) %	
JUPITER-06 (NCT03829969)	First-line	LA/M ESCC	China	1 (257)	TOR (240 mg) + chemotherapy	Improved OS: 17.0 (14 - NA) m; Improved PFS: 5.7 (5.6 - 7.0) m; Improved ORR: 69.3 (63.2 - 74.8) %	(14)
				2 (257)	placebo + chemotherapy	OS: 11.0 (10.4 -12.6) m; PFS: 5.5 (5.2 - 5.6) m; ORR: 52.1 (45.8 - 58.4) %	
RATIONALE-306 (NCT03783442)	First-line	LA/M ESCC	global	1 (326)	TIS (200 mg) + chemotherapy	Improved OS: 17.2 (15.8 -20.1) m; Improved PFS: 7.3 (6.9 - 8.3) m; Improved ORR: 63 (58 – 69) %	(15)
				2 (323)	placebo + chemotherapy	OS: 10.6 (9.3 - 12.1) m; PFS: 5.6 (4.9 -6.0) m; ORR: 42 (37 – 48) %	
KEYNOTE-181 (NCT02564263)	Second-line	LA/M EC	global	1 (314)	PEM (200 mg)	OS: 7.1 (6.2 - 8.1) m; PFS: 2.1 (2.1 - 2.2) m; Improved ORR: 13.1 (9.5 - 17.3) %	(9)
				2 (314)	chemotherapy	OS: 7.1 (6.3 - 8.0) m; PFS: 3.4 (2.8 - 3.9) m; ORR: 6.7 (4.2 - 10.0) %	
ESCORT (NCT03099382)	Second-line	A/M ESCC	China	1 (228)	CAM (200 mg)	Improved OS: 8.3 (6.8 - 9.7) m; Improved PFS: 1.9 (1.9 - 2.4) m; Improved ORR: 20.2 (15.2 - 26.0) %	(16)
				2 (220)	chemotherapy	OS: 6.2 (5.7 - 6.9) m; PFS: 1.9 (1.9 - 2.1) m; ORR: 6.4 (3.5 - 10.5) %	
RATIONALE-302 (NCT03430843)	Second-line	LA/M ESCC	global	1 (256)	TIS (200 mg)	Improved OS: 8.6 (7.5 - 10.4) m; PFS: 1.6 (1.4 - 2.7) m; Improved ORR: 20.3 (15.6 - 25.8) %	(17)
				2 (256)	chemotherapy	OS: 6.3 (5.3 - 7.0) m; PFS: 2.1 (1.5 - 2.7) m; ORR: 9.8 (6.4 - 14.1) %	
ATTRATION-3 (NCT02569242)	Second-line	A/R ESCC	global	1 (210)	NIV (240 mg)	Improved OS: 10.9 (9.2 - 13.3) m; PFS: 1.7 (1.5 - 2.2) m; ORR: 19.3 (14 – 26) %	(18)
				2 (209)	chemotherapy	OS: 8.4 (7.2 - 9.9) m; PFS: 3.4 (3.0 - 4.2) m; ORR: 21.5 (15 – 29) %	

A, advanced; CAM, camrelizumab; IPI, ipilimumab; LA, locally advanced; M, metastatic; m, months; NA, not available; NIV, Nivolumab; ORR, objective response rate; OS, overall survival; PEM, pembrolizumab; PFS, progression-free survival; pts, patients; R, refractory; SIN, sintilimab; TIS, tislelizumab; TOR, toripalimab; UA, unresectable advanced.

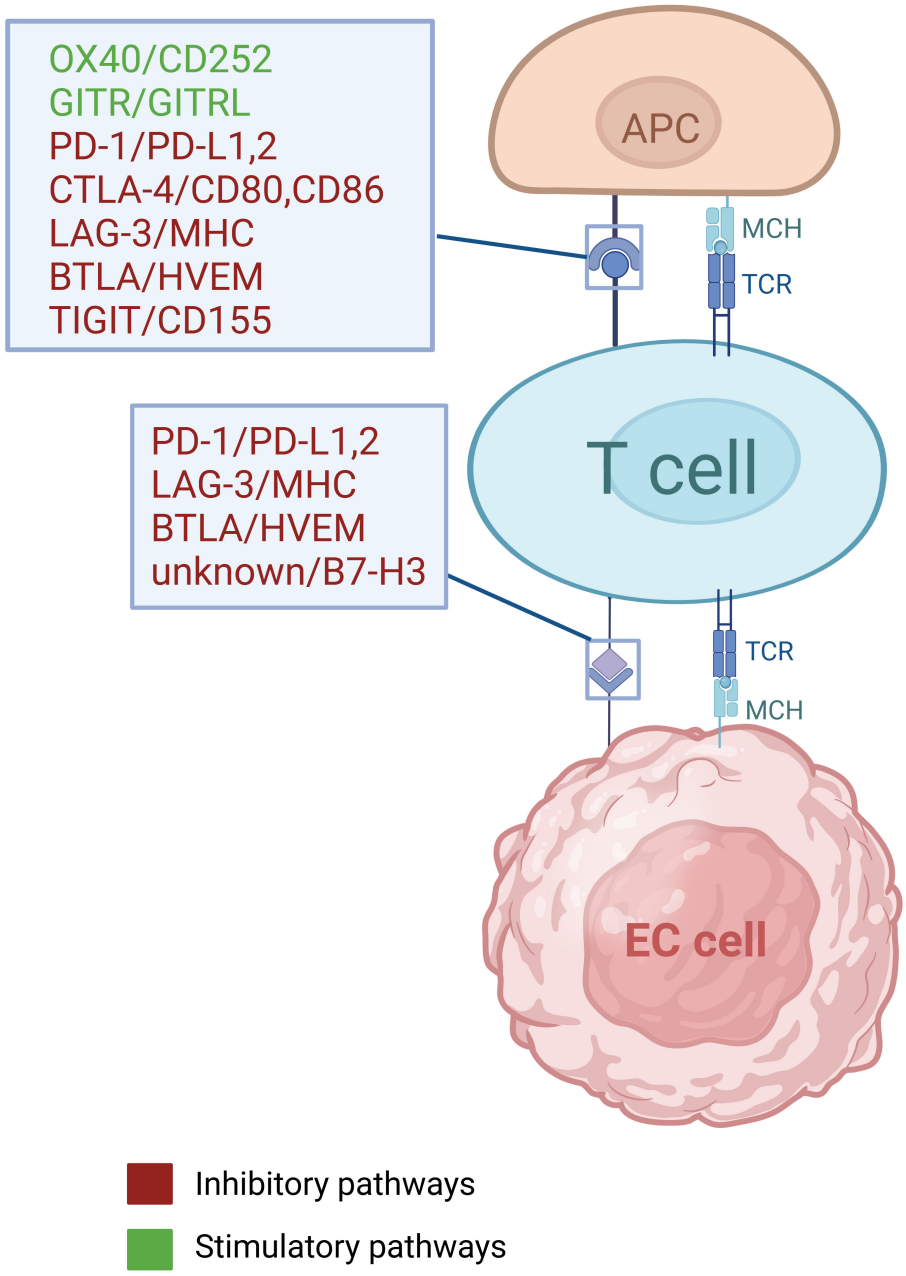

FIGURE 1
Immune checkpoint genes and cellular interactions contributing to tumor immunity.

the significance of identifying reliable biomarkers that can predict their response to immunotherapy.

Based on the latest advances in the field, this paper review categorizes prognostic biomarkers for patients with EC into three facets: tumor intrinsic factors, tumor-immune microenvironment (TIME)-associated factors, and host-related factors. Further research into these biomarkers, elucidating their mechanisms in mediating anti-tumor immunity and clarifying their clinical significance in EC immunotherapy, may aid in the identification of populations likely to benefit or in searching therapeutic targets that can enhance ICIs efficacy.

2 Tumor-cell-associated biomarkers

Tumor-cell-associated biomarkers, including tumor mutation burden (TMB) and high microsatellite instability (MSI-H), are established prognostic indicators for EC immunotherapy, while the evidence for programmed cell death ligand 1 (PD-L1) as a marker in this context has led to disputed conclusions (6). In this section, we summarize clinical findings relating both to these established biomarkers and to emerging markers, and explore the mechanisms potentially underlying related clinical phenomena.

2.1 PD-L1

The level of PD-L1 expression, generally assessed by combined positive score (CPS) or tumor proportion score (TPS), stands as a prognostic biomarker for predicting the efficacy of PD-1/PD-L1 inhibitors (19, 20). Many studies have reported correlations between elevated PD-L1 expression and adverse prognosis in patients with EC, including lymph node and distant metastasis, as well as poor OS (20). For example, Yagi et al. observed that high PD-L1 expression in patients with surgically resected EC indicated higher recurrence rate and shorter OS (21). The American Society of Clinical Oncology has issued a guideline pertaining to the use of immunotherapy for advanced gastroesophageal cancer, emphasizing the importance of PD-L1 testing and mismatch repair status assessment (22), which suggests conducting biomarker testing for individuals diagnosed with EC and gastroesophageal junction cancer, and formulating therapy models based on the results of CPS and TPS (22).

Although high PD-L1 expression tends to suggest the poor prognosis, some studies have shown that PD-1/PD-L1 inhibitors yield better therapeutic effects in patients with raised PD-L1 expression. In KEYNOTE-590 trial, pembrolizumab plus chemotherapy improved OS ($p < 0.001$) and PFS ($p < 0.001$) in ESCC participants with PD-L1 CPS ≥ 10 versus chemotherapy alone (11). CheckMate 648 study obtained similar results, showing that patients with elevated PD-L1 expression had superior OS and PFS after nivolumab treatment compared with the overall population (10). Nevertheless, clinical trials, such as ESCORT-1st (12), ORIENT-15 (13), and Jupiter-06 (14), have reported results indicating that PD-1 blockade agent efficacy is not correlated with PD-L1 level. In other words, immunotherapy plus chemotherapy was beneficial for patient OS regardless of PD-L1 status (Table 2). Therefore, whether PD-L1 can be considered a prognostic biomarker for immunotherapy in EC remains controversial.

Notably, some studies have reported that patients with heightened PD-L1 expression may exhibit favorable complete response rate (CRR), disease-free survival (DFS) and OS, but the statistical differences were not significant ($p > 0.05$) (23, 24). These contradicting findings regarding PD-L1 may be attributable to various factors including: 1) disease heterogeneity, such as variation in pathological types and stages; 2) differences in the ICIs administered; 3) variations in PD-L1 detection methods; 4) discrepancies in cut-off points; 5) inconsistency in the timing of PD-L1 detection (i.e., baseline versus post-treatment); 6) variations in sample sizes (25, 26). In the future, normalization and standardization of PD-L1 detection may contribute to clarifying its relationship with ICIs efficacy (27).

2.2 TMB

TMB, characterized by the frequency of somatic mutations in the coding regions of tumor genomes, has emerged as a prognostic biomarker for ICIs therapy in various types of cancer, including EC (33–35). After transcription and translation, mutations in tumors generate neoantigens, which increase the immunogenicity of tumor cells and thus elicits an intenser anti-tumor immune activation induced by ICIs (34). Huang et al. found that ESCC patients with higher TMB (more than 60 missense mutations) showed improved clinical benefits in response to camrelizumab remedy (36). The KEYNOTE-158 study confirmed that patients with elevated TMB status (more than 10 mutations per megabase) in advanced solid tumors who received pembrolizumab had a significantly higher ORR than other patients (33), which led FDA to grant accelerated endorsement for the use of pembrolizumab for managing unresectable or metastatic solid tumors with TMB above 10 Mut/Mb in 2020 (37).

Tumors with enhanced TMB tend to generate a greater density of neoantigens, thereby inducing anti-tumor immune response characterized by recognition and cytotoxicity (38), which can be enhanced when ICIs block immune checkpoints to facilitate anti-tumor immunity. A multiomics analysis revealed a correlation between TMB and mismatch repair (MMR) status, as well as the infiltration of immune cells such as regulatory T cells (Tregs), monocytes, and T helper cells (Ths) in EC (39). Another bioinformatics analysis identified a positive correlation between nonsynonymous TMB level and the infiltration of resting NK cells ($p = 0.028$), Tregs ($p = 0.064$), and $CD8^+$ T cells ($p = 0.12$) (40). Furthermore, elevated TMB also suggests a shorter distance between EC cells, especially $PD\text{-}L1^-$ tumor cells, to DCs and macrophages, which is associated with improved OS and PFS (41). This suggests that an increase in TMB leads to the distribution of antigen-presenting cells around the proximal tumor cells, thereby ameliorating the prognosis of patients undergoing immunotherapy.

2.3 MSI

MSI refers to the variations in the length of tandem repeats caused by defects in the DNA MMR system (42). Generally, tumors in which more than 30–40% of markers are mutated are termed as MSI-high (43). In a clinical study involving various types of solid malignancy, including gastroesophageal cancers, patients afflicted with MMR-deficient cancers and receiving pembrolizumab treatment exhibited favorable outcomes, with 53% achieving objective radiographic responses and 21% experiencing complete responses (44), indicating that ICIs are highly effective in the treatment of MSI-high patients regardless of the origin of the tumor tissue (44). Hence the FDA authorized the application of pembrolizumab in combating MSI-H solid tumors, marking the first time that the FDA has established a biomarker for treatment without restriction based on tumor type (45, 46).

Similar to the mechanism of TMB, MSI-H tumor cells generate more neoantigens, which in turn augments T cells' proliferation and activation, making such tumors more susceptible to anti-tumor immune responses (44, 45). A study of adenocarcinoma of esophagogastric junction (AEG) demonstrated that MSI-H tumors were associated with elevated level of $CD8^+$ T cells

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TABLE 2 Prognostic value of elevated PD-L1 expression in immunotherapy for EC.

Clinical trial	Phase	Pathological type	Status/ stage	ICIs Target	Treatment model	Detection method	Cut-off point	Prognostic value	Ref.
KEYNOTE-590 (NCT03189719)	3	EC	M/UA	PD-1	First-line	IHC 22C3	CPS: 10	Improved OS; Improved PFS	(11)
CheckMate 648 (NCT03143153)	3	ESCC	M/UA	PD-1	First-line	IHC 28-8	TPS: 1%	Improved OS; Improved PFS	(10)
ESCORT-1st (NCT03691090)	3	ESCC	A/M	PD-1	First-line	6E8 antibody	TPS: 1%	NSS	(12)
ORIENT-15 (NCT03748134)	3	ESCC	LA/M	PD-1	First-line	IHC 22C3	CPS: 1; 5; 10 TPS: 1%; 5%; 10%	NSS (prolonged duration of confirmed response, p = NA)	(13)
JUPITER-06 (NCT03829969)	3	ESCC	LA/M	PD-1	First-line	IHC JS311	CPS: 1; 10	NSS	(14)
RATIONALE-306 (NCT03783442)	3	ESCC	LA/M	PD-1	First-line	VENTANA SP263	TAP: 10%	NSS	(15)
EC-CRT-001 (NCT04005170)	2	ESCC	LA	PD-1	First-line	IHC 22C3	CPS: 10	NSS (improved CRR but p = 0.52)	(23)
KEYNOTE-181 (NCT02564263)	3	EC	LA/M	PD-1	Second-line	IHC 22C3	CPS: 10	Superior to chemotherapy	(9)
ESCORT (NCT03099382)	3	ESCC	A/M	PD-1	Second-line	6E8 antibody	TPS: 1%; 5%; 10%	NSS (improved OS but p = 0.13)	(16)
RATIONALE-302 (NCT03430843)	3	ESCC	LA/M	PD-1	Second-line	VENTANA SP263	TAP: 10%	NSS (improved OS but p = 0.21)	(17)
NCT02971956	2	EAC	A	PD-1	At least second-line	IHC 22C3	CPS: 1; 10	NSS (improved OS but p = 0.28; Improved PFS but p = 0.22)	(28)
NCT02730546	1b/2	AEG	cT_{1-3} N_{any} M_0	PD-1	NAT	IHC 22C3	CPS: 1; 10	Improved pCR	(29)
ChiCTR-1900026240	2	ESCC	Stage III/IVa	PD-1	NAT	IHC 22C3	CPS: 1 TPS: 50%	NSS	(30)
NCT04177797	2	ESCC	LA; Stage III/IVa	PD-1	NAT	IHC 22C3	CPS: 1 TPS: 1%	NSS	(31)
PERFECT (NCT03087864)	2	EAC	$<cT_{4b}N_0$ or cN_+M_0	PD-L1	NAT	IHC 22C3	CPS: 1; 10; 25	NSS	(32)
NCT02520453	2	ESCC	Stage II/III	PD-L1	NAT	VENTANA SP263	TPS: 1%	NSS (improved OS but p = 0.18; Improved DFS but p = 0.54)	(24)

A, advanced; AEG, adenocarcinoma of esophagogastric junction; CRR, complete response rate; DFS, disease-free survival; LA, locally advanced; M, metastatic; NA, not available; NAT, neoadjuvant therapy; NSS, not statistically significant; pCR, pathological complete response; TAP, tumor area positivity, UA, unresectable advanced.

infiltration in both the intratumoral region and invasive margin, as well as higher PD-L1 expression level in AEG cells, compared with microsatellite stable (MSS) tumors (47). By contrast, although MSI-low tumors also showed increased level of $CD8^+$ T cells in intratumoral area, no differences were observed in the invasive margin or in PD-L1 expression compared to MSS tumors (47). Similar results have also been observed in MSI-H colorectal cancer, which produces more neopeptides that are more readily recognized by the immune system than MSI-low tumor, and therefore eliciting an increased infiltration level of Th1 cells and $CD8^+$ T cells (48).

2.4 Abnormal DNA methylation

DNA methylation is a common epigenetic modification, characterized in mammals by the methylation of the C5 position

of cytosine to form 5-methylcytosine (49). Aberrant DNA methylation is a significant contributor to tumorigenesis and progression, and can serve as a prognostic biomarker for ESCC (50). For example, higher methylation levels in the promoters of miR129-2 and miR124-3 are associated with inferior response to neoadjuvant CRT of EC patients (51). However, studies discussing the relationship between DNA methylation and prognosis in the context of ICIs treatment are relatively limited. One study stratified 94 ESCC patients into two subgroups, S1 (n = 40) and S2 (n = 54), based on their DNA methylation and gene expression data (52). Genes differentially expressed between the two subgroups were identified as primarily involved in immune system regulation, immune cell activation, and cytokine function, according to KEGG/GO analysis. Taking their DNA methylation or gene expression data as the training set, the authors established a linear SVM model comprising 15 genes. Analysis of validation set data from 36 ESCC patients treated with PD-1/PD-L1 blockades plus chemotherapy was assembled. The final results demonstrated that the 15-gene expression signature exhibited a sensitivity of 68.8%, a specificity of 75%, and an efficacy of 72.2% in predicting responses to immunotherapy (52).

As mentioned previously, differences in DNA methylation and gene expression influenced the activation and immunological function of a wide range of immune cells in the S1 and S2 groups. The researchers investigated the condition of tumor microenvironment (TME) in each of the samples, observing that the patients in S2 group had higher levels of Tregs, resting memory $CD4^+$ T cells, Ths, macrophages and activated mast cells, as well as higher levels of immune checkpoint molecules, including CTLA-4 and lymphocyte activation gene-3 (LAG-3) (52). These findings indicate that aberrant DNA methylation can result in T cell exhaustion and suppression of anti-tumor immunity. Given the significant role of DNA methylation in immune responses against cancers, it is anticipated to be a valuable biomarker for EC immunotherapy (53).

2.5 Amplification of chromosome 11q13

The amplification of genomic region in chromosome 11q13 is among the most common aberrations observed in various malignant tumors, including ESCC (54). Chromosome 11q13 amplification contributes to promoting lymphangiogenesis, lymphatic metastasis, and suppression of immune response within the TME, thus hindering anti-cancer therapies (54, 55). In a clinical study involving advanced ESCC patients undergoing toripalimab therapy, those without 11q13 amplification (n=26) demonstrated significantly higher ORR (p = 0.024) and longer PFS (p = 0.025) than those with 11q13 amplification (n=24) (56), suggesting that chromosome 11q13 amplification may serve as an unfavorable biomarker for ESCC treated with PD-1 inhibitors (56), and similar result were observed in unresectable hepatocellular carcinoma (HCC) (57).

Chromosome 11q13 amplification includes the amplification of miR-548k and Cyclin D1 (CCND1), among others (54, 55, 58). MiR-548k promotes lymphangiogenesis by stimulating VEGFC secretion and activating ADAMTS1/VEGFC/VEGFR3 signaling pathway (54). Additionally, MiR-548k facilitates nodal metastasis by regulating the LF10/EGFR axis (54). CCND1 amplification leads to a state of immune exhaustion in the TME, which manifests as decreased densities of $CD8^+$ T cells, dendritic cells (DCs), and B cells and an elevated levels of Ths, Tregs, and myeloid-derived suppressor cells (MDSCs) (55). CCND1 exerts its function by activating CDK4/6 (57), and it is reported that the combined use of CDK4/6 inhibitors and anti-PD-1 antibodies significantly improves the survival outcomes in a mouse model of colon cancer (59). Moreover, chromosome 11q13 amplification is accompanied by an increased density of $Foxp3^+$ Tregs, which may contribute to hyperprogressive disease, a condition characterized by primary resistance to immunotherapy (57).

2.6 Amplification of MCL-1

Myeloid cell leukemia 1 (MCL-1) was isolated from a human myeloid leukemia cell line and belongs to the Bcl-2 protein family (60). MCL-1 amplification and overexpression is associated with the proliferation, drug resistance and inferior prognosis in various tumors including ESCC (61, 62), and inhibition of CPEB4-mediated MCL-1 translation can reverse the resistance to cisplatin in ESCC (63). Both MCL-1 and Bcl-xL belong to the bcl-2 family (60). Combination therapy targeting MCL-1 and Bcl-xL effectively eliminates melanoma cells from patients who experience relapse after PD-1 or CTLA-4 remedy, and curtails the self-renewal capability of melanoma cells (64).

In EC-CRT-001 trial involving ESCC patients treated with toripalimab plus CRT, univariable analyses of 16 genes of interest were conducted and only MCL-1 level was significantly associated with shorter OS (p = 0.03) and PFS (p = 0.024) (23). In *post-hoc* analysis, it emerged that patients with MCL-1 amplification demonstrated elevated levels of $PD\text{-}L1^+$ $CD8^+$ T cells and $PD\text{-}L1^+$ macrophages infiltration (23). In addition, a pan-cancer study found that the NANOG/HDAC1/MCL-1 axis mediates tumor resistance to PD-1 inhibitors, which could be reversed by silencing MCL-1 (65, 66). Collectively, these studies indicate a significant role for MCL-1 in mediating the immune-refractory state, thus suggesting the potential value of MCL-1 as both a biomarker and a therapeutic target in EC immunotherapy.

2.7 Long non-coding RNA (lncRNA)

long non-coding RNAs (lncRNAs), which is associated with tumor progression and immune evasion (67, 68), can be transcribed from both coding regions and regulatory regions of the tumor itself (69), or be delivered into cancer cells by TAMs via exosomes (70). Enhancers, as critical components of regulatory regions, are activated through demethylation and are subsequently transcribed into enhancer RNAs (eRNAs), a type of lncRNAs that are involved in the upregulation of corresponding target genes (71).

Gao et al. constructed an EDRGS score model based on 12 target genes in ESCC and found that patients with higher EDRGS exhibited superior responses to anti-PD-1 regimen ($p = 0.038$) (71). Bulk RNA-seq and scRNA-seq analyses revealed that EDRGS-high group exhibited elevated infiltration of $CD8^+$ T cells and NK cells, as well as upregulated levels of PD-1, LAG-3, TIM3, and TIGIT. In other words, EDRGS-high group denoted an immune-hot but immune-suppressive phenotype, accounting for improved response to ICIs (71). Additionally, it was found that eRNA AC005515.1 is co-expressed with several immune checkpoint genes, including CTLA4, Foxp3, and IDO1, and is positively correlated with the infiltration of $CD8^+$ T cells and M1 macrophages (72). Interestingly, patients with higher expression of AC005515.1 had increased TIDE scores and worse survival outcomes (72). This bioinformatics-based inference appears to be inconsistent with Gao's finding, highlighting the importance of further experiments and clinical studies to verify these results.

Another lncRNA, LINC02096, has been identified as a biomarker for ESCC immunotherapy by regulating immune evasion (73). Patients with elevated level of LINC02096 demonstrated inferior disease control rate (DCR) and ORR when undergoing anti-PD-1 monotherapy. In mouse model, knockdown of LINC02096 in TAMs upregulated the level of cytotoxic $CD8^+$ T cells, which rescued tumor progression and enhanced the treatment efficacy of anti-PD-1 antibody (73). Further research confirmed that high levels of LINC02096 are accumulated in ESCC cells with the involvement of exosomes secreted by TAMs and TNF-α. In tumor cells, LINC02096 inhibits the ubiquitination of histone methyltransferase MLL1, which enhances the levels of H3K4me3 in the promoter regions of PD-L1 and IDO-1, thereby undermining anti-tumor immunity (73).

3 Tumor-immune-microenvironment-associated biomarkers

The whole tumor-immune microenvironment (TIME) can be viewed as a biomarker for immunotherapy. TIME-associated biomarkers encompass various components, such as tumor-infiltrating lymphocytes (TILs), tumor-associated macrophages (TAMs), and cytokines (74).

3.1 TILs

3.1.1 Conventional immune biomarkers

A meta-analysis has confirmed that TILs overall can serve as a prognostic biomarker for OS in patients with EC (75). For surgically resected EC, patients with TIL-positive status tend to achieve favorable OS and DFS (21). However, treatments in the analyzed studies were not solely confined to ICIs therapy. Additionally, there is evidence that different TILs subsets may have distinct prognostic values (75). Therefore, the predictive role of TILs in EC immunotherapy warrants further elucidation.

In a clinical trial involving ESCC patients undergoing tislelizumab plus chemotherapy followed by esophagectomy, levels of $CD8^+$ T cells, Ths, Tregs, and mature DCs were significantly increased in pathological complete response (pCR) group, while the density of B cells and neutrophils significantly decreased (76). Another clinical study of locally advanced ESCC also detected a statistically significant correlation between $CD8^+$ T cells content, rather than other TIL subsets, and clinical response, with improved CRR ($p = 0.004$) and prolonged PFS ($p = 0.005$) (23). Another trial researching refractory EC demonstrated that patients with more abundant PD-1^+ $CD4^+$ T cells exhibited poorer radiological response ($p = 0.035$), and that the expression of PD-1 and T-cell immunoglobulin and mucin domain-3 (TIM-3) on $CD4^+$ T cells suggested early progression (28). We consider that the infiltration of a certain quantity of $CD8^+$ T cells is necessary for a physiological anti-tumor immune response; however, given the complex functions of diverse immune cells, how some immune cell types exert negative effects on EC immunotherapy requires further investigation.

3.1.2 Novel immune biomarkers

Novel immunotherapeutic targets have emerged in recent years, including LAG-3 and TIM-3, among others (5). However, due to the limited availability of clinical results related to these molecules, it is currently premature to reach conclusions regarding these factors. Therefore, we have included them as potential biomarkers and summarized relevant findings in the Discussion section of this review. TCF-1 expressed on $CD8^+$ T cells may serve as a novel immune biomarker. A study investigating pembrolizumab combined with CRT in ESCC observed that patients in the pCR group exhibited a higher level of TCF-1^+ cells than those in the non-pCR group ($p = 0.01$) (77). In melanoma, TCF-1^+ $CD8^+$ T cells are considered a positive biomarker, which will proliferate, self-renew, or differentiate into TCF-1^- $CD8^+$ T cells after treatment with ICIs (78). TCF-1 plays essential roles in maintaining the stem-like function of $CD8^+$ T cells and in intratumoral immune responses (79). Even when the influx of new T cells is blocked, melanoma mice with elevated TCF-1^+ TILs can still control tumor growth. Conversely, when TCF-1^+ TILs are suppressed, tumor control ability is lost (79).

3.2 TAMs

TAMs are immune cells with crucial roles in the TIME, whose density, distribution, and subtypes are correlated with the prognosis of EC (80–82). In a clinical trial investigating toripalimab plus chemotherapy in patients with locally advanced ESCC, responders showed a lower density of M2-TAMs (31). Another study involving camrelizumab plus CRT demonstrated that higher density of PD-$L1^-$ macrophages in the baseline tumor compartment was associated with improved PFS ($p = 0.032$) and in the on-treatment compartment indicated superior OS ($p = 0.018$) and PFS ($p = 0.028$) (41, 83). Through spatial multi-immunofluorescence, it was found that PD-$L1^-$ macrophages are

situated closer to tumor cells than PD-L1$^+$ macrophages, which may account for better clinical outcomes, to some extent (41). This study also found that the spatial distribution of TAMs was correlated with TMB, while previous reports have confirmed positive correlation between TAMs and PD-L1 levels in EC (84), and shown that TAMs could also secrete a variety of chemokines to regulate the TIME (85, 86), suggesting that the prognostic significance of TAMs may need to be judged in conjunction with other biomarkers (84). Notably, this study did not differentiate between M1 and M2 TAMs, nor did it investigate any association between the expression of CD68 or CD163 on macrophages and immunotherapy efficacy, which are areas warranting further research.

3.3 Cytokines

3.3.1 Interferon-γ (IFN-γ)

The PERFECT study tested for a six-gene IFN-γ signature in EAC patients receiving neoadjuvant immunotherapy based on previous experience and found that responders had higher levels of the baseline IFN-γ signature ($p = 0.043$) (32). Researchers conducted gene expression profile analysis of melanoma patients treated with pembrolizumab, and identified an IFN-γ-related signature as a prognostic marker by comparing responders and non-responders, further confirming its association with PFS and ORR in head and neck squamous cell carcinoma (HNSCC) and gastric cancer (GC) (87). These findings may be attributable to the involvement of IFN-γ in antigen presentation, chemokines secretion, and cytotoxicity, all of which are essential for the efficacy of immunotherapy (87). The upregulation of PD-L1 in the TME is established as partly originating from the effects of IFN-γ released by CD8$^+$ T cells, indicating that IFN-γ can affect other biomarkers to indirectly influence immunotherapy (88).

3.3.2 Chemokines

A study involving toripalimab plus chemotherapy in ESCC found that responders exhibited decreased CCL19 and elevated CXCL5 levels at baseline (31). CCL19 can activate the MEK1-ERK1/2 and PI3K-AKT pathways in M1-TAMs via CCR7, mediating their chemotaxis (89). However, although this finding regarding CXCL5 is consistent with the conclusion from a study of melanoma treated with nivolumab (90), the impact of CXCL5 on the TIME seems contradictory to its role as a positive biomarker, considering a study from skin cancers demonstrated that CXCL5 secreted by TAMs recruits MDSCs to the TME via the CXCR2-CXCL5 axis, exerting immunosuppressive effects (85). In addition, the infiltration of TAMs is reported to be modulated by the CCL2-CCR2 axis to affect PD-1 signaling pathway, leading to immune evasion (91), and CCL18 released by TAMs can promote tumor proliferation through activating the JAK2/STAT3 pathway (86), all of which contribute to the unsatisfactory prognosis of patients with ESCC.

4 Host-associated biomarkers

Host-associated biomarkers for cancer treatment include nutritional status (anemia, cachexia, etc.), peripheral blood immune substances, microbiomes, psychological disorders (including anxiety, depression, etc.), and endogenous hormones, among other factors. Promising results have been reported related to nutritional status, peripheral blood immune substances, antibiotic (ATB) use and its effects on microbiomes, and endogenous glucocorticoid in patients with EC.

4.1 Nutritional status

It is common for patients with upper gastrointestinal cancer to experience poor nutritional status, mainly due to reduced food intake and enhanced nutritional consumption by tumors (92). A recent retrospective study confirmed that the prognostic nutritional index (PNI) can serve as a biomarker for predicting the OS ($p = 0.047$) and PFS ($p = 0.020$) of EC patients undergoing anti-PD-1 inhibitors (93).

PNI, calculated from serum albumin content and total lymphocyte count, reflects the nutritional status and immunity of tumor patients (94). Albumin level reflects nutritional status, and a decrease in this factor indicates that the patient is in a state of malnutrition, which is a risk factor influencing prognosis (95). Lymphocytes exert anti-tumor immunity through cytokine-mediated cytotoxicity and insufficient lymphocytes results in lack of immune surveillance against tumors (96). Additionally, there are positive correlations between PNI and TILs status, CD8$^+$ cells density and Foxp3$^+$ cells density in EC patients, suggesting that lower PNI is associated with an unfavorable TIME, leading to poor response to immunotherapy (97).

4.2 Peripheral blood immune substances

Tumor-associated immunity can be categorized into immune responses within the TME and systemic immunity throughout the body, where the latter can be assessed by indicators such as neutrophil to lymphocyte ratio (NLR) and absolute neutrophil counts (ANC),among others (98). A real-world study of ESCC observed that an increase in NLR after ICIs treatment predicted decreased OS ($p = 0.004$) and inferior PFS ($p = 0.019$) (98). Similarly, the ORIENT-2 study showed that ESCC patients treated with sintilimab exhibited prolonged OS ($p < 0.001$) and PFS ($p = 0.006$) if the NLR was less than three at the sixth week (99). These findings may be attributable to the fact that neutrophils curtail the anti-tumor immunity mediated by NK cells and T cells, and secrete various cytokines, including IL-1 and IL-6, contributing to tumor proliferation (100–102).

Moreover, in neoadjuvant therapy combining toripalimab with chemotherapy for ESCC patients, although no relationship was

observed between NLR and patients' response, higher ANC and absolute natural killer cell counts were detected in responders (31). This phenomenon appears contradictory to the findings from patients with lung cancer, possibly due to the involvement of multiple chemotherapeutic agents in this study, which may have interfered with immunotherapy biomarker selection (103). Additionally, in ESCC patients treated with nivolumab, a rise in TIM-3$^+$ CD4$^+$ and TIM-3$^+$ CD8$^+$ T cells among peripheral blood mononuclear cells was observed in responders (104). Another study found that circulating CXCL10, interleukin 2 receptor α, and IL-6 were associated with pembrolizumab efficacy in treating EC, with favorable, unfavorable, and unfavorable relationships, respectively (28). These findings suggest that the prognostic significance of various immune substances in peripheral blood should not be neglected while TIME receives sufficient attention.

4.3 Antibiotic therapy and microbiomes

Microbiomes, including gut microbiota among others, can exert both protective and detrimental influences on cancer progression and therapeutic response (105). A meta-analysis incorporating retrospective studies of EC revealed that antibiotic (ATB) use from 60 days before to 30 days after ICIs treatment induced worse OS (P = 0.03) (106). This finding is consistent with the conclusion of a previous pan-cancer research (107), suggesting that ATB usage may lead to dysbiosis in patients' microbiomes, affecting their immune function and response to ICIs (98, 106, 108, 109).

The relationships between gut microbiota and host immune function, tumor occurrence and progression, anti-tumor immunity, and patients' prognosis are highly complex (105). Nevertheless, in general, the gut microbiota enhances antigen presentation mediated by DC cells, recruits and activates effector T cells to the TME, and reduces the density of Tregs and MDSCs (98), which provides a theoretical rationale for the beneficial role of normal gut microbiota in anti-tumor immunity. It is noteworthy that ATB use may not only affect patients' prognosis by altering the composition and diversity of intestinal flora. Patients receiving ATB may have inherently compromised immune function and a state of infection or susceptibility to infection, which could also contribute to poor outcomes in immunotherapy. On the other hand, dysbiosis of the gut microbiota may not only be induced by ATB usage. A study investigating camrelizumab plus chemotherapy in ESCC revealed that patients experienced a decrease in gut microbiota diversity following treatment (110). Therefore, the relationship among ATB administration, alterations in both gut microbiota and intratumoral microbiomes, and response to immunotherapy warrants further investigation.

4.4 Endogenous glucocorticoid

Excessive glucocorticoid can suppress the proliferation and differentiation of naive T cells, inhibit the CD28-CD80/CD86 co-stimulatory pathway, and disrupt immune surveillance function by affecting tumor-infiltrating immune cells (111, 112). A retrospective study encompassing advanced or metastatic pan-cancer, including EC, found that patients with high baseline endogenous glucocorticoid levels tended to exhibit lower ORR ($p < 0.001$) and had poorer durable clinical benefits (DCB) ($p = 0.001$), as well as shorter OS ($p < 0.001$) and PFS ($p < 0.001$) (113). Further research indicated that elevated glucocorticoid level is associated with lower infiltration levels of lymphocytes, CD4$^+$ T cells, and CD8$^+$ T cells, as well as increased NLR (113).

In other malignancies, glucocorticoid-related signaling pathways are generally detrimental factors for immunotherapy (114, 115). In pancreatic ductal adenocarcinoma, researchers have confirmed that the glucocorticoid receptor (GR) can upregulate the expression of PD-L1 and downregulate the expression of MHC-1 in tumor cells, thereby impairing cytotoxic T cells' infiltration and anti-tumor immune function (114). When GR is knocked down or inhibited, resistance to ICIs in mice will be reversed (114). In melanoma, HSD11B1 is an enzyme that can convert inert glucocorticoid into active glucocorticoid, and mice with high expression of HSD11B1 exhibit attenuated infiltration of CD4$^+$ T cells and CD8$^+$ T cells and are insensitive to PD-1 inhibitors (115). Blocking HSD11B1 leads to the decreased expression of CD206 and arginase-1 and the heightened expression of IL-12 in TAMs, as well as promoting secretion of IFN-γ by CD8$^+$ T cells, thereby enhancing the efficacy of PD-1 blockades (115).

4.5 Circulating tumor DNA (ctDNA)

Circulating tumor DNA (ctDNA) refers to cell-free DNA present in body fluids such as blood, synovial fluid, and cerebrospinal fluid (116). As a non-invasive biomarker, ctDNA has been widely applied to treatment monitoring in recent years (116). While the ctDNA status before sintilimab treatment (i.e., the baseline ctDNA level) is not statistically significant with major pathological response (MPR) ($p = 0.39$), patients with undetectable ctDNA are more likely to achieve pCR ($p = 0.008$) (117). It is noteworthy that more attention is paid to the dynamic changes of ctDNA content compared to its baseline level (118). It was observed that patients with undetectable ctDNA or ctDNA clearance post-ICIs therapy tend to obtain prolonged recurrence-free survival (RFS) and OS (119). Similarly, the EC-CRT-001 trial found that patients with detectable ctDNA during or after treatment, rather than at baseline, exhibited shorter OS and PFS, as well as inferior clinical complete response (cCR) (120). ctDNA clearance represents neoantigen-specific T cell responses. In a clinical trial investigating neoadjuvant nivolumab alone or plus relatlimab treatment, two patients who achieved complete pathological response had ctDNA clearance following ICIs induction. Both of them exhibited expansions of neoantigen-specific T cell clones, which were not observed in patients with persistently detectable ctDNA (119). Currently, further studies are underway to explore the prognostic value of ctDNA in EC immunotherapy (121, 122).

TABLE 3 Summary of prognostic biomarkers for Immunotherapy in EC.

Biomarker	Source of evidence	ICIs target	Prognostic value*	Mechanism
Tumor-cell-associated biomarkers				
PD-L1	Clinical trial	PD-1	Improved OS; Improved PFS; Improved pCR; Superior to chemotherapy	PD-1 - PD-L1 axis is the therapeutic target of PD-1 inhibitors. Higher PD-L1 expression stands for the greater potential for ICIs efficacy
TMB & MSI	Clinical trial; Clinical samples; Bioinformatics analysis	PD-1	Improved ORR; Improved CRR; Improved CBR	Cancer cells with increased mutations produce more neoantigens, which stimulates anti-tumor immune response and mediates the infiltration of $CD8^+$ T cells and the distance between APCs
Abnormal DNA methylation	Clinical samples; Bioinformatics analysis	PD-1; PD-L1	Unfavorable OS	S2 subgroup has an upregulated infiltration of Tregs, Ths, TAMs, activated mast cells and resting memory $CD4^+$ T cells, representing a state of immune exhaustion and suppression
Chromosome 11q13 amplification	Clinical trial; Clinical samples; Cell lines; Mouse models; Bioinformatics analysis	PD-1; PD-L1; CTLA-4	Unfavorable PFS; Unfavorable ORR	Chromosome 11q13 amplification consists of the amplification of miR-548k and CCND1: 1) MiR-548k promotes lymphangiogenesis by ADAMTS1/VEGFC/VEGFR3 axis and facilitates nodal metastasis by LF10/EGFR pathway; 2) CCND1 induces the exhaustion of anti-tumor immune cells and elevates the density of Ths, Tregs, MDSCs
MCL-1 amplification	Clinical trial; Mouse models; Bioinformatics analysis	PD-1	Unfavorable OS; Unfavorable PFS	1) MCL-1 amplification is associated with augmented level of $PD\text{-}L1^+$ $CD8^+$ T cells' and $PD\text{-}L1^+$ macrophages' infiltration; 2) NANOG/HDAC1/MCL-1 axis plays a pivotal role in displaying the resistant state against CTLs in TME
LncRNAs	Clinical samples; Cell lines; Mouse models; Bioinformatics analysis	PD-1	Unfavorable DCR; Unfavorable ORR	1) Influence the expression levels of target genes; 2) Elevate the expression levels of immune checkpoint genes, including PD-L1, IDO-1, LAG-3, TIM-3, etc. 3) Positively or negatively correlated with NK cells, $CD8^+$ T cells, and M1 macrophages
TIME-associated biomarkers				
TILs	Clinical trial	PD-1	CRR, PFS, pCR; Paradoxical	TILs plays both positive and negative roles in executing anti-tumor immunity, depending on their types, density, proportion, and gene expression profiles
$TCF\text{-}1^+$ T cells	Clinical trial; Clinical samples; Cell lines; Mouse models	PD-1; CTLA-4	Improved pCR	1) $TCF\text{-}1^+$ $CD8^+$ T cells will proliferate, self-renew and differentiate into $TCF\text{-}1^-$ $CD8^+$ T cells after treated with ICIs; 2) TCF-1 is essential for the stem-like function of $CD8^+$ T cells and intratumoral immune response
TAMs	Clinical trial; Clinical samples; Cell lines; Bioinformatics analysis	PD-1	$PD\text{-}L1^-$ TAMs: Improved OS; Improved PFS Reduced density of M2-TAMs: Improved MPR	1) The distance between $PD\text{-}L1^-$ TAMs and tumor cells is closer; 2) TAMs secrete chemokines such as CXCL5 and CCL18 to induce immune suppression and facilitate tumor proliferation; 3) TAMs are associated with the level of TMB and PD-L1 expression
Cytokines	Clinical trial; Clinical samples; Cell lines; Bioinformatics analysis	PD-1; PD-L1	Pathologic or clinical response; Paradoxical	1) IFN-γ-related genes are associated with antigen presentation, cytotoxicity, and chemokines secretion; 2) TAMs release CXCL5 to recruit MDSCs to TME via CXCR2-CXCL5 axis and release CCL18 to promote tumor proliferation via JAK2/STAT3 pathway; 3) CCL19 activates MEK1-ERK1/2 and PI3K-AKT pathway in M1-TAMs by CCR7 to mediate their chemotaxis; 4) CCL2-CCR2 axis mediates TAMs infiltration to affect PD-1 signaling in cancer cells, leading to their immune evasion

(Continued)

TABLE 3 Continued

Biomarker	Source of evidence	ICIs target	Prognostic value*	Mechanism
Host-associated biomarkers				
PNI	Clinical data;	PD-1	Improved OS; Improved PFS	1) Low albumin concentration reflects malnutrition status, which is regarded as a negative prognostic factor; 2) Lymphocytes play a predominant role in immune surveillance against tumor cells
NLR	Clinical trial; Clinical data	PD-1	Unfavorable OS; Unfavorable PFS	1) Neutrophils undermine anti-tumor immunity mediated by NK cells and T cells; 2) Cytokines (e.g., IL-1, IL-6) released by neutrophils promote tumor progression
ATB use	Clinical data; Meta-analysis	PD-1; PD-L1; CTLA-4	Unfavorable OS	Both ATB and ICIs treatment abate the amount and diversity of microbiomes. Microbiomes are conducive to antigen presentation and effector T cells' recruitment and activation
Endogenous glucocorticoid	Clinical data; Clinical samples; Cell lines; Mouse models	PD-1; PD-L1	Unfavorable OS; Unfavorable PFS; Unfavorable ORR	1) Elevated glucocorticoid is associated with lower infiltration level of $CD4^+$ T cells and $CD8^+$ T cells, as well as higher NLR; 2) More active glucocorticoid suppresses IFN-γ secretion from $CD8^+$ T cells and curbs inflammatory state of TAMs; 3) GR downregulates MHC-1 level of cancer cells and inhibits the infiltration of cytotoxic T cells
ctDNA	Clinical trial	PD-1; LAG-3	Unfavorable OS; Unfavorable PFS; Unfavorable cCR	ctDNA clearance represents neoantigen-specific T cell responses and expansions

APCs, antigen-presenting cells; CBR, clinical benefit rate; cCR, clinical complete response; CRR, complete response rate; CTLs, cytotoxic lymphocytes; DCR, disease control rate; GR, glucocorticoid receptor; LAG-3, lymphocyte activation gene-3; MDSCs, myeloid-derived suppressor cells; MPR, major pathologic response; pCR, pathologic complete response.
*Prognostic value refers to the predictive value of biomarkers when their expression, level or density are elevated unless otherwise specified.

5 Discussion

Despite variations in the most common pathological types across different geographical regions, EC poses significant threats and burdens to people worldwide (3). The emergence of ICIs has provided novel options for EC treatment, bringing promise for prolonged survival and improved quality of life for patients. ICIs are currently applied in first-line, second-line, adjuvant and neoadjuvant treatment patterns in EC (4, 123). However, not all EC patients can benefit equally from immunotherapy (5), highlighting the significance of identifying reliable biomarkers to discern subgroups who may respond better to immunotherapy. In this review we summarize tumor-cell-associated biomarkers, TIME-associated biomarkers, and host-associated biomarkers discovered in EC immunotherapy, as well as their possible underlying mechanisms (Table 3), with the goal of providing a theoretical basis for selection of suitable patients and administration of tailored treatments in the future.

In addition to summarizing well-established biomarkers such as PD-L1, TMB, MSI-H, and TILs, some relatively novel markers are also discussed in this review, including DNA methylation, amplification of chromosome 11q13 and specific genes, and cytokines' levels. We found that different studies have yielded conflicting conclusions regarding the prognostic significance of certain biomarker. Regarding PD-L1, many clinical studies failed to establish a statistically significant association between its expression and the efficacy of ICIs (Table 1). Similarly, it is also

TABLE 4 Prognostic value of potential biomarkers with elevated levels.

Biomarker	Source of evidence	Prognostic value	Possible mechanism	Ref.
Tumor-cell-associated biomarkers				
SOCS3	Bioinformatics analysis	Unfavorable OS	1) SOCS3 promotes the infiltration of CAFs, M2-TAMs, and Tregs; 2) SOCS3 methylation is negatively related to the dysfunction of T cells	(125)
METTL3	Bioinformatics analysis	Unfavorable OS; Unfavorable PFS; Unfavorable DFS	METTL3 correlates with immune genes and the infiltration of B cells, effector memory $CD8^+$ T cells, macrophages, NK cells and neutrophils	(126–128)

(Continued)

TABLE 4 Continued

Biomarker	Source of evidence	Prognostic value	Possible mechanism	Ref.
Tumor-cell-associated biomarkers				
B7-H3	Clinical samples	Unfavorable OS	B7-H3 positively correlates with the infiltration of Foxp3$^+$ Tregs and CD68$^+$ macrophages and negatively correlates with the density of CD3$^+$ T cells and CD8$^+$ T cells in TME	(129, 130)
TIME-associated biomarkers				
LAG-3	Clinical trial; Clinical samples	Improved OS; Improved PFS; Unfavorable RFS; Paradoxical	1) LAG-3 level is positively correlated with the density of CD3$^+$ TILs, CD4$^+$ TILs and CD8$^+$ TILs, and the ratio of CD4$^+$/CD8$^+$ TILs, indicating an inflammatory TIME; 2) LAG-3 is co-expressed with other immune checkpoints such as CTLA-4	(131–134)
TIM-3	Clinical samples; Bioinformatics analysis	Unfavorable OS	1) TIM-3 inhibits the function of CTLs and effector Th1 cells; 2) TIM-3 plays a role in the generation and differentiation of MDSCs; 3) TIM-3 suggests an augmented activity and apoptosis of Foxp3$^+$ Tregs	(135, 136)
TIGIT	Meta-analysis; Clinical samples; Cell lines; Bioinformatics analysis	Unfavorable OS; Unfavorable PFS	1) By biding to CD155, TIGIT suppresses the secretion of IL-12 and IFN-γ and stimulates the release of IL-10 in DC cells via ERK pathway; 2) TIGIT competes with the co-stimulatory receptor CD226 for binding to CD155, thereby inducing CD8$^+$ T cell exhaustion; 3) TIGIT$^+$ Tregs inhibit the function of Th1 and Th17 cells by IL-10 and fgl2	(136–139)
VISTA	Clinical samples	Favorable OS	1) VISTA pathway attenuates the level of cytokines including IL-2, IL-17, IFN-γ, CCL5; 2) VISTA is co-expressed with CD4 and CD68 in TILs	(140, 141)
MDSCs	Clinical samples; Cell lines; Mouse models	Unfavorable OS	1) MDSCs impair the function and proliferation of T cells and induce their apoptosis by arginase1, iNOS, IDO, HO-1, and NOX2; 2) MDSCs stimulate the proliferation and infiltration of Tregs by secreting IL-10 and IFN-γ; 3) CD14$^+$ HLA-DR$^{-/low}$ MDSCs exhibit higher expression of PD-L1 to suppress T cell proliferation	(142, 143)
Host-associated biomarkers				
Obesity	Meta-analysis; Clinical data; Mouse models	Improved OS; Improved PFS; Paradoxical	Adipose tissue releases leptin, TNF-α, IL-6, leading to dysfunction and exhaustion of immune cells, including elevated PD-1 expression on T cells, undermined NK cells and imbalance of the ratio of M1/M2 macrophages	(144, 145)
CCS	Clinical samples; Clinical data; Cell lines; Mouse models	Unfavorable OS; Unfavorable PFS; Unfavorable DCR	CCS is induced by a wide range of cytokines secreted by tumor or immune cells, including TNF-α, IL-1, IL-6, IL-8, TGF-β, which are considered negative factors for anti-tumor immunity. They lead to exhausted T cells, impaired NK cells and DCs, accumulated MDSCs, and increased Tregs and glucocorticoid level	(146, 147)
Distress	Meta-analysis; Clinical trial; Clinical samples	Unfavorable PFS; Unfavorable ORR Unfavorable DCR	1) Distress represents an activated HPA axis, leading to an elevated level of glucocorticoid, which is an adverse prognostic biomarker; 2) Depressive patients exhibit an enhanced COX-2-PGE2 axis, inducing the infiltration of MDSCs, Tregs and M2-TAMs; 3) Depressive patients show more MDSCs recruited by neuropeptide Y, less Tregs caused by scanty IL-2, and reduced CD8$^+$ T cells	(148–151)

B7-H3, B7 homologue 3; CAFs, cancer-associated fibroblasts; CCS, cancer cachexia syndrome; COX-2, cyclooxygenase-2; CTLs, cytotoxic T lymphocytes; DCR, disease control rate; DFS, disease-free survival; HPA, hypothalamus–pituitary–adrenal; MDSCs, myeloid-derived suppressor cells; METTL3, methyltransferase-like 3; PGE2, prostaglandin E2; RFS, recurrence-free survival; SOSC3, the suppressor of cytokine signaling 3; TGF-β, transforming growth factor-β; TIGIT, T cell immunoglobulin and ITIM domain; TNF-α, tumor necrosis factor-α; VISTA, V-domain Ig suppressor of T cell activation.

reported that TMB is insufficient to predict the outcomes of immunotherapy (31). These discrepancies might be attributed to the differences in detection methods, cut-off points selection, sample sizes, and treatment patterns. Recently a team has developed copy number alteration (CNA)-corrected TMB to predict the efficacy of PD-1 inhibitors plus chemotherapy (124), suggesting that biomarker standardization and adjustment warrants further exploration. The timing of detecting biomarkers' content and the attention to their dynamic changes are also crucial. When baseline levels of a biomarker are unrelated to efficacy, positive conclusions may be drawn from its post-treatment level, or from its dynamic alteration during therapy.

We have also summarized potential biomarkers for EC immunotherapy in this section based on the quality of evidence (Table 4). They fit into one of the following two features: 1) Although confirmed as associated with therapeutic efficacy in EC,

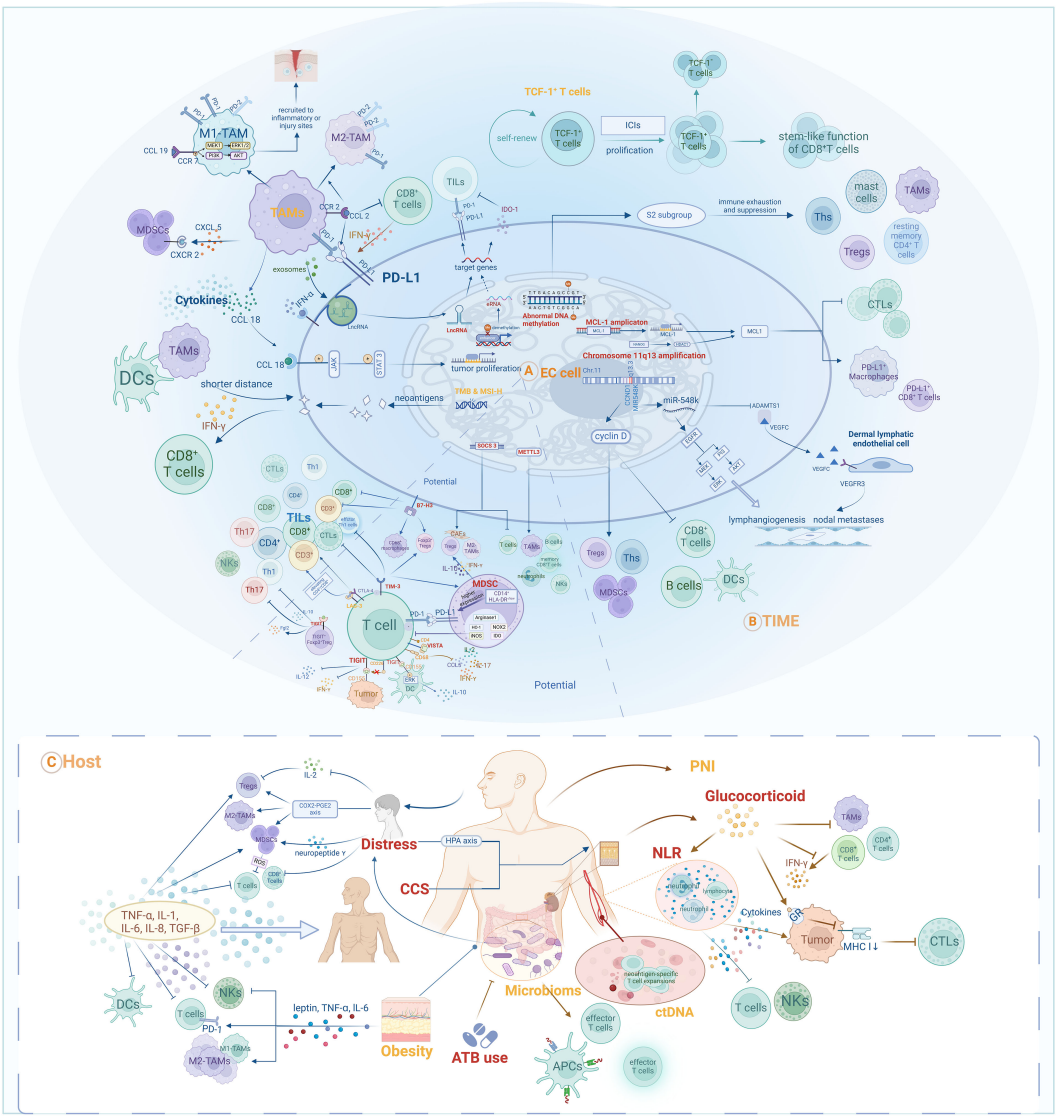

FIGURE 2
Prognostic and potential biomarkers for immunotherapy in EC and their interactive mechanisms: **(A)** Tumor-cell-associated biomarkers; **(B)** TIME-associated biomarkers; **(C)** Host-associated biomarkers.

evidence sources encompass all treatment methods, and are not confined to immunotherapy. Additionally, these markers affect prognosis through mechanisms involving the TIME or anti-tumor immunity; 2) Markers reported to be associated with the prognosis of immunotherapy in other types of cancers, primarily lung cancer and gastrointestinal cancers, while relevant research in EC is limited. We chose gastrointestinal tumors as a reference, since the esophagus and other gastrointestinal organs share similar histological structures and embryological origins. Lung cancer was also selected, both because of the anatomical proximity of the lungs and esophagus and considering the fact that studies of lung cancer are relatively abundant and advanced. There is a current trend in oncology research of validating results derived from lung cancer studies in other types of cancers.

Biomarkers summarized in the main sections of this review and potential biomarkers included in the Discussion section are illustrated in Figure 2. We found that various of these biomarkers can mutually interact to influence anti-tumor responses during immunotherapy. For instance, multiple biomarkers ultimately impact the responses of EC patients by mediating the quantity, activity, or phenotype of TILs. TAMs both influence the TIME by secreting chemokines to recruit MDSCs, and can also be modulated by chemokines, thereby assisting EC cells in evading anti-tumor immune responses (85, 86, 89, 91). These findings indicate that further research into the mechanisms underlying the activities of various biomarkers and to delineate their interactions will be of vital importance to provide comprehensive understanding of prognostic biomarkers in the context of EC immunotherapy.

Author contributions

XT: Writing – original draft, Writing – review & editing. MJ: Writing – original draft, Writing – review & editing. LW: Investigation, Methodology, Writing – review & editing. DZ: Investigation, Resources, Writing – review & editing. YY: Formal analysis, Supervision, Validation, Writing – review & editing. QS: Conceptualization, Formal analysis, Investigation, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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First-in-human phase 1 dose-escalation results with livmoniplimab, an antibody targeting the GARP:TGF-ß1 complex, as monotherapy and in combination with the anti–PD-1 antibody budigalimab in patients with advanced solid tumors

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Background: Transforming growth factor (TGF)-ß1 is a pleiotropic cytokine that can promote tumor growth and suppress antitumor immune responses. Latent TGF-ß1 associates with glycoprotein-A repetition predominant (GARP) on the surface of regulatory T cells prior to its activation and release. Livmoniplimab is a monoclonal antibody (mAb) that binds the GARP:TGF-ß1 complex to inhibit activation and release of TGF-ß1. It is in clinical development in combination with budigalimab, an anti-programmed cell death protein 1 Fc-modified mAb. The first-in-human, phase 1, dose-escalation results are presented herein (ClinicalTrials.gov: NCT03821935).

Methods: The dose-escalation phase enrolled adult patients with advanced solid tumors. Patients received escalating doses of livmoniplimab ranging from 3mg to 1500mg, once every 2 weeks (Q2W), as monotherapy or in combination with a 500mg fixed dose of budigalimab Q4W. The primary objective of the dose escalation was to determine the recommended phase 2 dose. Secondary

objectives were to assess safety and pharmacokinetics (PK), and exploratory objectives included evaluating preliminary efficacy.

Results: Fifty-seven patients enrolled in the dose escalation: 23 in monotherapy cohorts and 34 in combination therapy cohorts. Dose-limiting toxicities were limited, no maximum tolerated dose was reached, and the maximum administered dose of 1500mg was selected for dose expansion. The most common adverse events reported in monotherapy-treated patients were fatigue, anemia, and nausea, and those in combination therapy-treated patients were pruritus, fatigue, nausea, and anemia. Livmoniplimab exhibited dose-proportional PK, and peripheral blood biomarker data demonstrated saturation of the GARP:TGF-ß1 complex on platelets at livmoniplimab doses within the linear PK range. No objective tumor responses were observed in the monotherapy dose escalation. However, the objective response rate was 15% in the combination dose escalation, with a median response duration of 8.4 months.

Conclusion: Livmoniplimab was well-tolerated as monotherapy and in combination with budigalimab in the dose-escalation phase. Encouraging preliminary efficacy was demonstrated in the combination dose escalation in heavily pretreated patients, supporting further development of this novel drug combination in patients with advanced solid tumors.

KEYWORDS

advanced solid tumors, TGF-ß1, GARP, immunotherapy, anti-PD-1 antibody, combination drug therapy, investigational therapies, tumor microenvironment (TME)

1 Introduction

Transforming growth factor (TGF)-ß1 is a potent immunomodulatory cytokine that plays a key role in various cellular processes including cell proliferation, epithelial-to-mesenchymal transition and migration, and angiogenesis (1, 2). In oncogenesis, TGF-ß1 signaling pathways are hijacked by cancer cells to promote cancer progression (3, 4). In the tumor microenvironment (TME), TGF-ß1 promotes tumor growth by multiple mechanisms including: suppressing effector T cells, natural killer cells, and dendritic cells; inducing anti-inflammatory macrophage M2 polarization; and promoting tumor fibrosis via induction of cancer-associated fibroblasts, collagen proteins, and other extracellular matrix proteins (5). TGF-ß1 overexpression and signaling in cancer has been associated with poor prognosis and resistance to immune checkpoint inhibitors, including anti-programmed cell death protein 1 (PD-1)/PD-1 ligand 1 (PD-L1) therapies (6, 7).

TGF-ß1 is produced in a latent form, in which mature TGF-ß1 is complexed with a latency-associated peptide, thus preventing the mature TGF-ß1 from binding to its specific receptors and subsequent signaling (8). This latent TGF-ß1 complex associates with various latent TGF-ß binding proteins at the cell surface. One such protein is glycoprotein-A repetition predominant (GARP), expressed on the surface of immune cells, primarily $CD4^+$ regulatory T cells (Tregs) and platelets (9, 10), as well as some cancer cells (11–14). GARP binding to latent TGF-ß1 results in localization and concentration of the TGF-ß1 on the surface of immune cells (15), where TGF-ß1 activation and release is regulated by various integrins (15, 16).

Multiple therapeutic strategies have been developed to target TGF-ß expression and signaling, either by broadly targeting all TGF-ß isoforms, specifically targeting TGF-ß1 or TGF-ß2, or targeting the TGF-ß receptor. These include (a) anti-integrin agents that inhibit TGF-ß activation, (b) antibodies or antibody-based biotherapeutics against TGF-ß or its receptors that interfere with ligand-receptor interactions and downstream signaling, (c) small-molecule kinase inhibitors that interfere with TGF-ß receptor kinase activity and signaling, and d) antisense oligonucleotides (17–19). Despite promising preclinical antitumor activity in all cases, these TGF-ß–targeting agents have had mixed success in the clinic: some have failed due to toxicity or insufficient antitumor activity, some demonstrated encouraging preliminary clinical data that have yet to be confirmed in a registrational study, and others remain in early developmental stages. As a result, there is currently no TGF-ß–targeting agent approved in oncology (20). A potential limitation of these therapeutic approaches targeting TGF-ß pathways is that their

inhibition of TGF-ß signaling is not specific to the TME. Since TGF-ß1 is a pleiotropic cytokine that is expressed by most cells, systemically blocking TGF-ß1 activity may result in undesirable side effects, and inhibition locally in the TME may be beneficial.

Inhibiting TGF-ß1 activation and release from GARP on the surface of $CD4^+$ Tregs is a novel approach to target TGF-ß in a more site-restricted manner. Tregs are immune-suppressing cells that have been associated with poor outcomes in several tumor types (7) and resistance to checkpoint inhibitors (21–23). TGF-ß production by Tregs has been identified as a mechanism of immune suppression within the TME, and GARP may play a key role in facilitating localized TGF-ß release (19). GARP expression and TGF-ß1 release are increased in various solid tumors, including breast cancer (13), lung cancer (13), gastric cancer (12), colon cancer (13), and hepatocellular carcinoma (24).

Antibodies that bind to the GARP:TGF-ß1 complex and inhibit release of active TGF-ß1 were first developed by the laboratory of Prof Sophie Lucas in partnership with Argenx; these antibodies were shown to inhibit Treg immunosuppression in a xenogeneic graft-versus-host disease mouse model (25). Subsequently, the Lucas group demonstrated that antibodies against the mouse GARP:TGF-ß1 complex could overcome resistance to anti–PD-1 agents in a colon carcinoma mouse model and induce T-cell–mediated immunity that protected mice from tumor rechallenge (9).

Livmoniplimab is a first-in-class human immunoglobulin G4/k monoclonal antibody (mAb) that binds to the human GARP:TGF-ß1 complex and inhibits the release of mature TGF-ß1 [Figure 1 (26)]. It is being developed in combination with budigalimab (also known as ABBV-181), an investigational anti–PD-1 Fc-modified immunoglobulin G1 mAb that has demonstrated safety and efficacy in patients with non-small cell lung cancer and head and neck squamous cell carcinoma (27). Despite the broad application of anti–PD-1 antibodies in solid tumor immunotherapy, a considerable proportion of disease fails to respond to these agents or acquires resistance, and multiple lines of evidence support dual inhibition of PD-1 and TGF-ß as a therapeutic strategy. Mariathasan and colleagues observed that a TGF-ß gene signature in fibroblasts was associated with lack of response to atezolizumab in immune-excluded metastatic urothelial carcinoma (6). When gene expression analyses were performed on multiple cohorts from The Cancer Genome Atlas database, an overlap between markers of T-cell infiltration (typically associated with response to PD-1 blockade) and TGF-ß–related gene signatures was revealed, indicating that TGF-ß may be a mechanism of immune escape in these patients (AbbVie internal data). This concept was corroborated by mouse model data in which increased tumor growth inhibition and reinvigorated $CD8^+$ T-cell effector responses were demonstrated in mice treated with a combination of antibodies targeting PD-1 and the GARP:TGF-ß1 complex compared with either antibody alone (9). On the basis of this evidence, a clinical trial was designed to evaluate inhibition of the GARP:TGF-ß1 complex and PD-1. Herein, we present the results from the dose-escalation phase of this first-in-human (FIH) phase 1

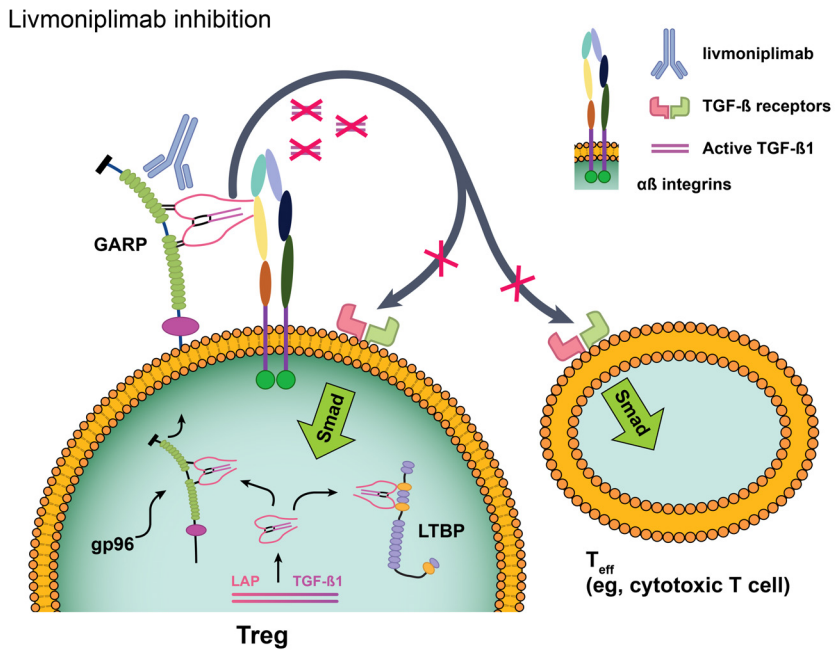

FIGURE 1
Livmoniplimab targets the GARP:TGF-ß complex and inhibits release of mature, active TGF-ß. Reproduced with edits under the terms of the Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/) from Metelli A, Salem M, Wallace CH, Wu BX, Li A, Li X, et al. Immunoregulatory functions and the therapeutic implications of GARP-TGF-ß in inflammation and cancer. *J Hematol Oncol* (2018) 11(1):24 (26). Changes were made to depict the livmoniplimab proposed mechanism of action. GARP, glycoprotein-A repetition predominant; LAP, latency-associated peptide; LTBP, latent TGF-ß binding protein; Smad, mothers against decapentaplegic family of transcription factors; Teff, effector T cell; TGF-ß, transforming growth factor ß; TME, tumor microenvironment; Treg, regulatory T cell.

study of livmoniplimab as monotherapy and in combination with budigalimab in patients with advanced solid tumors.

2 Methods

2.1 Study design

This is a phase 1, open-label, FIH, dose-escalation and dose-expansion study. Livmoniplimab was assessed via dose escalation as monotherapy and in combination with budigalimab in patients with locally advanced or metastatic solid tumors. Dose escalation was guided by a Bayesian optimal interval design based on the cumulative number of patients experiencing a dose-limiting toxicity (DLT) at each dose level. Dose expansion was designed to evaluate multiple cohorts of locally advanced or metastatic solid tumors.

For the dose-escalation phase, the primary objective was to determine the recommended phase 2 dose of livmoniplimab monotherapy and in combination with budigalimab. The secondary objective was to assess safety, tolerability, and pharmacokinetics (PK) of livmoniplimab as monotherapy and combined with budigalimab. Exploratory objectives included evaluating the preliminary efficacy of livmoniplimab as monotherapy and in combination with budigalimab and evaluating the pharmacodynamics (PD) and predictive biomarkers associated with PK, safety, and efficacy. The trial was registered with ClinicalTrials.gov (NCT03821935) and was approved by institutional review boards at each participating site prior to initiation. The study was performed in accordance with the International Conference on Harmonization Good Clinical Practice guidelines and the Declaration of Helsinki, with written informed consent obtained from all patients before study enrollment.

The first 2 livmoniplimab monotherapy cohorts, at dose levels 3mg and 10mg, enrolled a single patient. For livmoniplimab monotherapy dose cohorts at 30mg or higher, a minimum of 3 patients were enrolled. The combination dose-escalation phase began after ≥2 monotherapy dose levels were determined to be safe, with a minimum of 3 patients enrolled per cohort. Livmoniplimab was administered via intravenous infusion once every 2 weeks (Q2W), and in combination cohorts, budigalimab was administered at a 500mg fixed dose via intravenous infusion once every 4 weeks (Q4W). Patients in both arms received livmoniplimab, with or without budigalimab, until disease progression or intolerable toxicity.

2.2 Study population

The dose-escalation phase required that patients be ≥18 years of age with an advanced solid tumor considered refractory or intolerant to all existing therapies known to provide clinical benefit, unless patients were ineligible for or refused standard therapies. Patients were also required to have Eastern Cooperative Oncology Group performance status of 0 or 1 and adequate bone marrow, renal, hepatic, and coagulation function. Patients with unresolved adverse events (AEs) grade >1 from prior anticancer treatment (except alopecia), with clinically significant uncontrolled conditions or with uncontrolled metastases to the central nervous system, were excluded. Patients were also excluded if they had a history of any of the following: primary immunodeficiency, bone marrow or solid organ transplantation, clinical diagnosis of tuberculosis, active autoimmune disease, inflammatory bowel disease, interstitial lung disease or pneumonitis, myocarditis, Stevens-Johnson syndrome, toxic epidermal necrolysis, or drug reaction with eosinophilia and systemic symptoms.

2.3 Safety and efficacy assessments and statistics

Safety endpoints of treatment-emergent AEs (TEAEs; onset on or after the first dose and up to 90 days after the last dose), serious AEs, deaths, and changes in laboratory and vital sign parameters were assessed in all patients who received ≥1 dose of the study drug. DLTs were assessed for a period of 28 days following the first dose of livmoniplimab monotherapy or livmoniplimab and budigalimab combination therapy per the National Cancer Institute Common Terminology Criteria for AEs version 5.0. Patients who did not complete the full 28-day DLT observation period, for any reason other than a DLT, were considered non-DLT evaluable and were replaced at the same dose level. Patients were continuously monitored for known or expected immune-related toxicities.

Efficacy was evaluated per investigator assessments according to Response Evaluation Criteria in Solid Tumors (RECIST) v1.1 every 8 weeks for the first 12 months, then every 12 weeks until disease progression; all patients who received ≥1 dose of the study drug were considered. Patients were allowed to continue treatment beyond progression per RECIST v1.1 if they were absent of symptoms or signs of disease progression and had no decline in Eastern Cooperative Oncology Group performance status. Such patients were then evaluated using the modified RECIST v1.1 criteria for immune-based therapeutics. Objective response rate and its 2-sided 95% Clopper-Pearson (exact) confidence interval were calculated for each cohort on the basis of patients showing complete response or partial response (PR). Median duration of response and its 2-sided 95% confidence intervals were calculated for each cohort.

2.4 Platelet GARP:TGF-ß target engagement (TE) assay

Livmoniplimab saturation of the GARP:TGF-ß complex on peripheral blood platelets was assayed by immunostaining of isolated platelet-rich plasma (PRP). Whole blood samples were collected into K2*EDTA vacutainers and shipped to a central laboratory for analysis according to institutional review board-approved ethical guidelines. Blood collection tubes were centrifuged at 150×g for 15 minutes at 4°C without brake. The PRP supernatant layer was carefully collected and aliquoted prior to enumeration by an automated hematology analyzer. The PRP fractions were treated with dimethyl sulfoxide at a final concentration of 6% (v/v) and stored at – 80°C until immunostaining.

For immunostaining, PRP aliquots were thawed briefly at 37°C, washed with assay buffer (1% Human AB serum and 2 mM ethylenediaminetetraacetic acid in 500 mL of phosphate buffer saline), and stained with a platelet-specific mAb conjugated to a fluorescent fluorochrome, CD61-FITC from BioLegend (San Diego, CA). Two additional AbbVie proprietary reagents, 1E7-APC to detect GARP receptor levels and LHG10.6-PE to detect GARP: TGF-ß receptor levels, with accompanying isotype controls were included in the stain mixture to assess target engagement. Mean fluorescence intensities and quantitation beads for APC and PE (Bangs Laboratory) were used to determine GARP and GARP:TGF-ß levels, respectively, on purified platelets. Receptor levels were extrapolated from calibration curves generated from bead mean fluorescence intensity and mean equivalent soluble fluorochrome (MESF) density values. Longitudinal TE values were calculated using the equation: 100 * (1– [LHG10-PE_{MESF} Postdosing – Isotype-PE_{MESF} Postdosing]/[LHG10-PE_{MESF} Baseline – Isotype-PE_{MESF} Baseline]) and plotted using Prism (GraphPad 9). Assay validation and sample processing were conducted by MLM Medical Labs in Memphis, TN, in accordance with AbbVie guidance.

2.5 PK and antidrug antibody (ADA) assessments

Serial blood samples for measurements of livmoniplimab and budigalimab concentrations in serum were collected in cycles 1 and 3 prior to infusion, 15 minutes after the end of the respective infusion, and at 2 hours, 4 hours (only for livmoniplimab), 24 hours, 168 hours, and 336 hours, following the end of the respective infusion. PK samples were collected in all other cycles prior to infusion and 15 minutes after the end of the respective infusion. The lower limit of quantitation was 1.63 ng/mL and 50 ng/mL for livmoniplimab and budigalimab, respectively. Livmoniplimab and budigalimab serum concentrations were quantified using a validated bioanalytical assay and analyzed using noncompartmental analysis in Phoenix WinNonlin (version 8.3 Pharsight, Mountain View, CA). Peak serum concentrations, time to peak concentration, area under the curve to 336 hours, and terminal half-life were determined for livmoniplimab and budigalimab. Livmoniplimab and budigalimab blood samples for measurement of ADA were collected predose on day 1 of each cycle with an additional early ADA assessment on day 15 in cycle 1 only. All patients who received ≥1 dose of the study drug and had ≥1 valid postbaseline PK data were included in this analysis.

2.6 Exploratory blood PD biomarker assessments

Blood samples for exploratory biomarker assessment by flow cytometry were collected before infusion on day 1, day 8 and 15 of cycle 1, day 1(pre-dose) and 15 of cycle 2, day 1(pre-dose) of cycle 3. Memory T-cell frequencies and Ki67 proliferation were evaluated using validated flow cytometry assays (Covance Inc., USA) on freshly obtained anticoagulated blood as previously described (28).

3 Results

3.1 Translational PK/PD model to select FIH dose levels of livmoniplimab

A translational PK/PD model was used to predict the human PK of livmoniplimab and the corresponding target occupancy on platelets and tumor-infiltrating lymphocytes (TILs) on the basis of nonclinical data. Briefly, allometric scaling was used to predict the human PK parameters based on those estimated by fitting the data from a single-dose non-Good Laboratory Practice PK/PD study in cynomolgus monkeys to a 2-compartment model with target-mediated saturable clearance. The target engagement parameters estimated on the basis of the model fit were combined with measurements of target levels on platelets and TILs to calculate predicted target occupancy (%GARP-TGFß1 complexes bound by livmoniplimab) in human.

A maximum recommended starting dose for livmoniplimab of 3mg (0.05mg/kg for 60kg body weight) was selected on the basis of the model prediction of ≤80% maximum target occupancy on platelets in the peripheral blood and ≤15% on TILs (assuming the livmoniplimab concentration in the tumor is much less than that in the serum). In addition, the model predicted a duration of target occupancy of >10% on platelets for <5 days postdose at the maximum recommended starting dose. Dose escalations for the next 5 cohorts were based on ~3-fold increases. A maximum dose of 1500mg was selected on the basis of the model prediction of >99% target occupancy on both platelets and TILs. The final livmoniplimab dose levels evaluated were therefore 3mg, 10mg, 30mg, 100mg, 300mg, 1000mg, and 1500mg.

3.2 Patient demographics and baseline characteristics

Between March 2019 and February 2022, 23 patients were enrolled in the livmoniplimab monotherapy dose-escalation cohorts (3mg, N=1; 10mg, N=1; 30mg, N=3; 100mg, N=3; 300mg, N=3; 1000mg, N=4; 1500mg, N=8) and 34 patients enrolled in the livmoniplimab and budigalimab dose-escalation cohorts (livmoniplimab 10mg, N=4; 30mg, N=8; 100mg, N=3; 300mg, N=4; 1000mg, N=4; 1500mg, N=11; budigalimab 500mg fixed dose). Patient demographics and baseline disease characteristics are summarized in Table 1. Patients with a variety of solid tumors were enrolled in the dose-escalation phase; the most frequent tumor types in the monotherapy cohorts were non-small cell lung cancer (n=4), colorectal (n=3), and ovarian cancer (n=3), and in the combination therapy cohorts were colorectal (n=8), ovarian (n=7), and pancreatic cancer (n=4). Patients in the monotherapy cohorts had received a median of 4 (range 1, 10) prior lines of systemic therapies, and those in the combination therapy cohorts had received a median of 3 (range 0, 10) prior lines of systemic therapies. Eight (35%) and 10 (29%) patients receiving monotherapy and combination therapy, respectively, had received prior anti–PD-1 or anti–PD-L1 therapy.

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TABLE 1 Patient demographics and tumor baseline characteristics.

Livmoniplimab Monotherapy (Q2W)								
Livmoniplimab dosage	3mg (N=1)	10mg (N=1)	30mg (N=3)	100mg (N=3)	300mg (N=3)	1000mg (N=4)	1500mg (N=8)	Total (N=23)
Median age at baseline, years (range)	78.0 (78, 78)	54.0 (54, 54)	75.0 (47, 77)	71.0 (46, 73)	67.0 (46, 73)	66.5 (36, 74)	65.5 (49, 81)	67.0 (36, 81)
Sex, n (%)								
Male	0	0	0	3 (100)	1 (33)	3 (75)	1 (13)	8 (35)
Female	1 (100)	1 (100)	3 (100)	0	2 (67)	1 (25)	7 (87)	15 (65)
Race, n (%)								
White	1 (100)	1 (100)	1 (33)	3 (100)	1 (33)	1 (25)	3 (38)	11 (48)
Black or African American	0	0	0	0	1 (33)	1 (25)	0	2 (9)
Asian	0	0	2 (67)	0	1 (33)	2 (50)	5 (63)	10 (43)
ECOG performance status at baseline, n (%)								
0	1 (100)	1 (100)	2 (67)	1 (33)	1 (33)	1 (25)	4 (50)	11 (48)
1	0	0	1 (33)	2 (67)	2 (67)	3 (75)	4 (50)	12 (52)
Primary cancer type, n (%)								
TNBC	0	0	1 (33)	0	0	0	1 (13)	2 (9)
Pancreatic	0	0	0	0	1 (33)	0	0	1 (4)
Urothelial	0	0	0	0	0	0	0	0
HCC	0	0	0	0	0	0	0	0
NSCLC	0	0	1 (33)	1 (33)	0	0	2 (25)	4 (17)
Ovarian	1 (100)	1 (100)	0	0	1 (33)	0	0	3 (13)
Colorectal	0	0	0	0	0	0	3 (38)	3 (13)
Breast cancer (non-TNBC)	0	0	0	0	0	0	1 (13)	1 (4)
Head and neck (HNSCC)	0	0	0	0	0	0	0	0
Other solid tumors[a]	0	0	1 (33)	2 (67)	1 (33)	4 (100)	1 (13)	9 (39)
Median prior lines of systemic therapy, n (range)	6.0 (6, 6)	3.0 (3, 3)	5.0 (4, 9)	1.0 (1, 4)	3.0 (3, 10)	2.0 (1, 5)	5.0 (1, 8)	4.0 (1, 10)
Received prior anti–PD-(L)1 therapy,[b] n (%)								
Yes	0	1 (100)	1 (33)	1 (33)	0	2 (50)	3 (38)	8 (35)
No	1 (100)	0	2 (67)	2 (67)	3 (100)	2 (50)	5 (62)	15 (65)

Livmoniplimab (Q2W) + Budigalimab (500mg Q4W) Combination Therapy							
Livmoniplimab dosage	10mg (N=4)	30mg (N=8)	100mg (N=3)	300mg (N=4)	1000mg (N=4)	1500mg (N=11)	Total (N=34)
Median age at baseline, years (range)	50.0 (46, 62)	53.5 (39, 72)	52.0 (50, 63)	63.5 (62, 71)	67.5 (48, 77)	53.0 (20, 70)	57.5 (20, 77)
Sex, n (%)							
Male	2 (50)	6 (75)	1 (33)	1 (25)	0	1 (9)	11 (32)
Female	2 (50)	2 (25)	2 (67)	3 (75)	4 (100)	10 (91)	23 (68)
Race, n (%)							
White	3 (75)	7 (88)	2 (67)	3 (75)	3 (75)	6 (55)	24 (71)
Black or African American	1 (25)	1 (13)	0	0	0	1 (9)	3 (9)
Asian	0	0	1 (33)	1 (25)	1 (25)	4 (36)	7 (21)
ECOG performance status at baseline, n (%)							
0	4 (100)	2 (25)	3 (100)	3 (75)	0	8 (73)	20 (59)
1	0	6 (75)	0	1 (25)	4 (100)	3 (27)	14 (41)
Primary cancer type, n (%)							
TNBC	0	0	0	0	0	0	0
Pancreatic	1 (25)	1 (13)	0	1 (25)	0	1 (9)	4 (12)
Urothelial	0	0	0	0	0	1 (9)	1 (3)
HCC	0	0	0	0	0	0	0
NSCLC	0	0	0	0	1 (25)	0	1 (3)
Ovarian	1 (25)	0	1 (33)	0	0	5 (46)	7 (21)
Colorectal	2 (50)	2 (25)	2 (67)	2 (50)	0	0	8 (24)
Breast cancer (non-TNBC)	0	1 (13)	0	0	1 (25)	0	2 (6)

(Continued)

TABLE 1 Continued

Livmoniplimab (Q2W) + Budigalimab (500mg Q4W) Combination Therapy							
Livmoniplimab dosage	10mg (N=4)	30mg (N=8)	100mg (N=3)	300mg (N=4)	1000mg (N=4)	1500mg (N=11)	Total (N=34)
Head and neck (HNSCC)	0	0	0	0	0	0	0
Other solid tumors[a]	0	4 (50)	0	1 (25)	2 (50)	4 (36)	11 (32)
Median prior lines of systemic therapy, n (range)	3.0 (1, 7)	4.0 (2, 10)	6.0 (2, 10)	2.5 (2, 3)	3.5 (2, 6)	3.0 (0, 7)	3.0 (0, 10)
Received prior anti–PD-(L)1 therapy,[b] **n (%)**							
Yes	1 (25)	3 (38)	1 (33)	2 (50)	1 (25)	2 (18)	10 (29)
No	3 (75)	5 (62)	2 (67)	2 (50)	3 (75)	9 (82)	24 (71)

[a]Other solid tumor types include sarcoma, mesothelioma, endometrial cancer, uterine cancer, gastric/gastroesophageal junction adenocarcinoma, prostate cancer, papillary adenocarcinoma, gastrointestinal stromal tumor, hemangiopericytoma, renal cell carcinoma, adrenocortical carcinoma, orbital sebaceous gland cancer, and ampullary adenocarcinoma. [b]Patient received either prior anti–PD-1 or anti–PD-L1 therapy.

ECOG, Eastern Cooperative Oncology Group; HCC, hepatocellular carcinoma; HNSCC, head and neck squamous cell carcinoma; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein 1; PD-L1, PD-1 ligand 1; Q2W, once every 2 weeks; Q4W, once every 4 weeks; TNBC, triple-negative breast cancer.

3.3 Drug exposure

In the livmoniplimab monotherapy dose escalation, patients received a median of 2 cycles and the median duration of exposure was 43 days. Patients in the combination therapy dose escalation received a median of 2.5 cycles, and were exposed to livmoniplimab and budigalimab for a median duration of 54 days and 44 days, respectively. At the time of data cutoff on 30 March 2023, all 23 patients enrolled in the monotherapy dose-escalation cohorts discontinued treatment, most commonly due to disease progression (87%); 33 (97%) patients enrolled in the combination therapy dose escalation discontinued treatment, with disease progression being the most common reason (59%). Details of patient drug exposure are summarized in Supplementary Table S1.

3.4 PK and ADA analysis

As of August 2022, all 57 patients enrolled in the dose-escalation phase who received livmoniplimab monotherapy or combination therapy with budigalimab have preliminary PK data available. Mean livmoniplimab serum concentration-versus-time profiles from cycle 1 (after the first dose) from monotherapy and combination therapy escalation cohorts are presented in Figures 2A and B, respectively. The preliminary mean PK parameters for the monotherapy cohorts are presented in Supplementary Table S2A and for the combination cohorts in Supplementary Table S2B. Livmoniplimab exhibits dose-proportional PK across the dose range of 30 to 1500mg, where complete GARP:TGF-ß1 target saturation in circulation over the treatment period was observed (Figures 2C, D). Approximately 2-fold accumulation was observed on a Q2W administration schedule in cycle 3 compared with cycle 1. Livmoniplimab or budigalimab PK (data on file) was not impacted by their coadministration. No treatment-emergent ADAs were reported for either livmoniplimab (N=32) at doses >30mg Q2W or budigalimab (N=34).

3.5 GARP:TGF-ß platelet TE

Since activated Tregs that upregulate the GARP:TGF-ß1 complex are challenging to detect in circulation, a surrogate TE assay was developed and validated on purified peripheral blood platelets to determine the extent of livmoniplimab saturation of the GARP:TGF-ß1 complex after intravenous administration. In Figures 2C and D, longitudinal plots depict the degree of saturation as early as 2 hours postdosing with livmoniplimab in the indicated dosing cohorts receiving monotherapy and combination therapy, respectively. The single patient who received 3mg livmoniplimab monotherapy attained complete saturation at 2hr postdosing, which then minimally desaturated at cycle 1 day 8. In contrast, all higher monotherapy dosing cohorts sustained complete saturation in circulation after livmoniplimab administration across the 2-week dosing interval. In the combination therapy arm, all dosing cohorts attained complete saturation 2hr postdosing with livmoniplimab; only the lowest combination cohort receiving 10mg livmoniplimab recorded partial desaturation at cycle 1 day 15.

3.6 PD biomarkers

Blood PD biomarkers were longitudinally evaluated by flow cytometry and analyzed according to dose and clinical response status. An increase in proliferating $Ki67^+$ $CD8^+$ T cells post-treatment was noted in both monotherapy and combination therapy arms with some of the largest increases associated with clinical responders in the combination arms at both low and higher doses (Figure 3A). Further analysis of the clinical responders from the combination arms revealed an increase in activated central memory and central effector T cells post-treatment with a peak at C2D1 when compared with patients who had stable disease or patients who experienced progression upon treatment in either monotherapy or combination therapy arms (Figures 3B, C). Soluble markers, including cytokines and TGF-ß1, were measured in circulation, and modest changes were observed that were independent of the dose or response (data not shown).

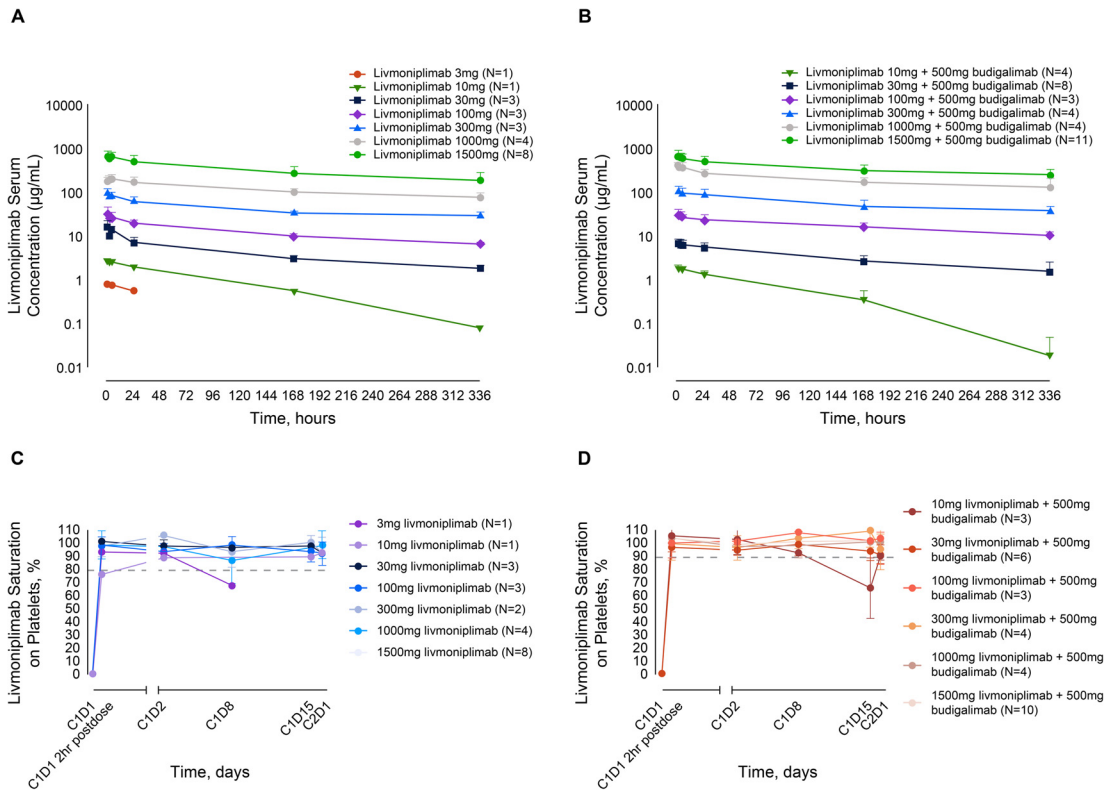

FIGURE 2
Livmoniplimab PK and TE profiles. **(A)**: Livmoniplimab Q2W PK profile for monotherapy dose escalation cohorts. **(B)**: Livmoniplimab Q2W PK profile for livmoniplimab and budigalimab combination therapy cohorts. **(C)**: GARP:TGF-β platelet TE for livmoniplimab Q2W monotherapy dose escalation cohorts. **(D)**: GARP:TGF-β platelet TE for livmoniplimab Q2W and budigalimab combination therapy cohorts. On the basis of the assay validation characterization (data not shown), complete TE was established at 80% saturation. C, cycle; D, day; GARP, glycoprotein-A repetition predominant; PK, pharmacokinetics; Q2W, once every 2 weeks; TE, target engagement; TGF-ß, transforming growth factor ß.

3.7 Safety

Safety results from dose escalation are summarized in Table 2. In total, 22 (96%) patients receiving livmoniplimab monotherapy experienced a TEAE; the most common TEAEs were fatigue (44%), anemia (35%), and nausea (30%). Livmoniplimab treatment-related AEs (TRAEs) were observed in 16 (70%) patients, with fatigue (22%) and anemia (13%) the most common. Grade 3 or 4 TEAEs occurred in 9 (39%) patients, with the most common being anemia (9%) and atrial fibrillation (9%). One monotherapy-treated patient reported a serious AE deemed related to the study drug – dermatitis, after receiving 1500mg livmoniplimab. Two (9%) patients had a TRAE resulting in treatment interruption, including thrombocytopenia and dermatitis each in 1 patient; no patients had a TRAE resulting in discontinuation in the monotherapy dose escalation.

In the combination therapy dose escalation, 34 (100%) patients reported TEAEs, with the most common being pruritus (47%), fatigue (41%), nausea (41%), and anemia (38%). Livmoniplimab TRAEs were reported in 25 (74%) patients, the most common being pruritus (35%), maculopapular rash (27%), and fatigue (24%). Budigalimab TRAEs were reported in 24 (71%) patients, with pruritus (35%), maculopapular rash (27%), and fatigue (24%) the most common. Grade 3 or 4 TEAEs were reported in 23 (68%) patients; anemia (12%), malignant neoplasm progression, and decreased neutrophil count (9% each) were the most common. Study drug-related serious AEs were experienced by 5 (15%) patients receiving combination therapy, with no single term reported in more than 1 patient. Nine (27%) patients had an AE related to either livmoniplimab or budigalimab resulting in treatment interruption, with the most common being maculopapular rash, in 4 patients. Five (15%) patients had a TRAE resulting in discontinuation in the combination therapy dose escalation, including maculopapular rash in 2 patients and pruritus, urticaria, and nephritis each in 1 patient.

No patients experienced a DLT in the livmoniplimab monotherapy dose escalation; 1 patient (3%) in the combination dose escalation experienced a DLT of increased alanine aminotransferase. There were no deaths related to either livmoniplimab or budigalimab. The maximum tolerated dose for livmoniplimab as monotherapy or in combination with budigalimab was not reached, and the maximum administered dose of 1500mg was selected for dose expansion.

3.8 Efficacy

Antitumor efficacy per investigator assessment is shown in Table 3. In the monotherapy cohorts, no objective responses were

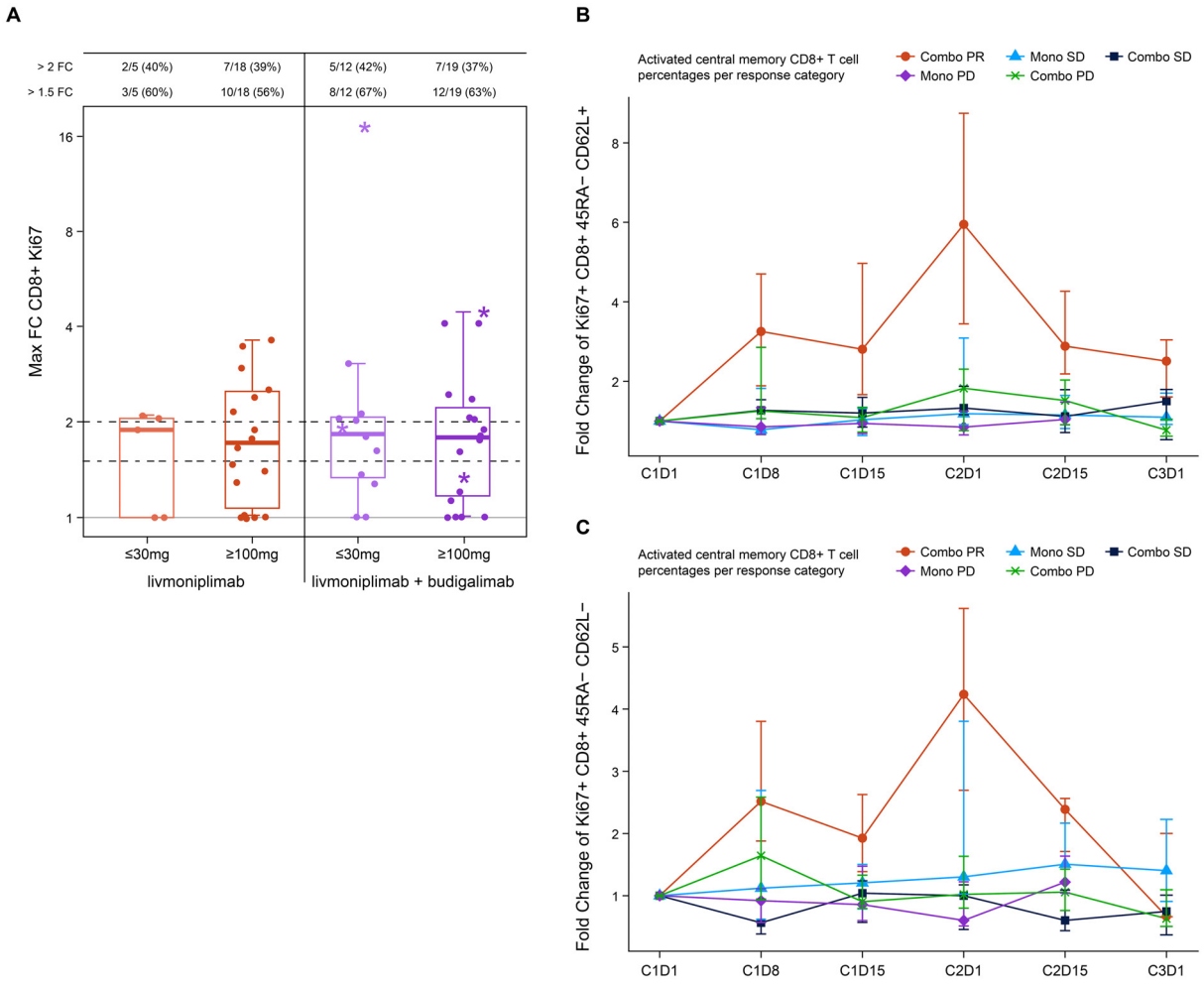

FIGURE 3
Pharmacodynamic changes induced by livmoniplimab measured by immunophenotyping. **(A)** Activated Ki67$^+$ CD8$^+$ T cells frequency fold change in monotherapy and combination therapy arms. Star denotes clinical responders. **(B)** Fold change of Ki67$^+$ CD8$^+$ 45RA$^-$ CD62L$^+$ activated central memory T-cell frequencies. **(C)** Fold change of Ki67$^+$ CD8$^+$ 45RA$^-$ CD62L$^-$ activated central memory T-cell frequencies. Combo, combination therapy; FC, fold change; Mono, monotherapy; PD, progressive disease; PR, partial response; SD, stable disease.

observed; 7 (30%) and 14 (61%) patients experienced stable disease and progressive disease, respectively. In the combination therapy cohorts, the confirmed objective response rate was 15%; 5 (15%), 9 (27%), and 18 (53%) patients had PR, stable disease, and progressive disease, respectively. Tumor response to study drug(s), measured as percentage change from baseline target lesions over time per assessment by the investigator, is depicted for each response-evaluable patient in dose escalation in Figure 4. The median duration of objective response for patients treated with combination therapy was 8.4 months.

Responses were observed in multiple solid tumor types across several livmoniplimab dose levels, ranging from 30mg to 1500mg, in combination with a 500mg fixed dose of budigalimab. One responder was a patient with PD-1–naive gastroesophageal junction adenocarcinoma enrolled in the 30mg livmoniplimab combination cohort. Two responders had colorectal cancer; one was microsatellite stable, PD-1 naive, and was enrolled in the 30mg livmoniplimab combination cohort, and another, who was microsatellite instability low (retrospective tumor tissue testing by whole exome sequencing at AbbVie), was enrolled in the 100mg livmoniplimab combination cohort and received, and responded to, prior PD-1 and cytotoxic T-lymphocyte antigen 4 combination checkpoint inhibitor therapy. The last 2 responders were patients with PD-1–naive ovarian cancer treated with 1500mg livmoniplimab combination therapy.

An additional 5 patients who were enrolled in combination therapy dose-escalation cohorts had durable stable disease for approximately 6 months or longer; one of these patients received 10mg livmoniplimab and 4 received 1500mg. These include 1 patient with PD-1–relapsed microsatellite-stable colorectal cancer who had experienced stable disease previously with a combination of anti–PD-1 and anti-cytotoxic T-lymphocyte antigen 4, 1 patient with PD-1–naive alveolar sarcoma, 1 patient with PD-1–relapsed urothelial cancer who had stable disease to prior anti–PD-1, and 2 patients with PD-1–naive ovarian cancer. One of the patients with ovarian cancer converted to an unconfirmed PR after almost a year on study before discontinuing due to an AE. Interestingly, these patients with ovarian cancer who had durable stable disease or PRs had granulosa cell histology, a tumor type for which the importance

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TABLE 2 Safety.

Livmoniplimab Monotherapy (Q2W)								
Livmoniplimab dosage	3mg (N=1)	10mg (N=1)	30mg (N=3)	100mg (N=3)	300mg (N=3)	1000mg (N=4)	1500mg (N=8)	Total (N=23)
Any TEAE, n (%)[a,b]	1 (100)	1 (100)	3 (100)	3 (100)	3 (100)	3 (75)	8 (100)	22 (96)
Fatigue	0	1 (100)	1 (33)	3 (100)	1 (33)	0	4 (50)	10 (44)
Anemia	0	0	2 (67)	0	1 (33)	1 (25)	4 (50)	8 (35)
Nausea	1 (100)	0	1 (33)	2 (67)	1 (33)	0	2 (25)	7 (30)
Tumor pain	1 (100)	0	0	1 (33)	0	2 (50)	2 (25)	6 (26)
Diarrhea	0	0	0	2 (67)	1 (33)	1 (25)	1 (13)	5 (22)
Increased AST	0	0	1 (33)	0	0	1 (25)	2 (25)	4 (17)
Decreased appetite	1 (100)	0	0	1 (33)	0	0	2 (25)	4 (17)
Vomiting	1 (100)	1 (100)	0	1 (33)	0	0	1 (13)	4 (17)
Increased GGT	0	0	1 (33)	0	0	1 (25)	2 (25)	4 (17)
Dizziness	0	1 (100)	1 (33)	2 (67)	0	0	0	4 (17)
Disease progression	0	0	1 (33)	0	0	0	2 (25)	3 (13)
Arthralgia	1 (100)	0	0	1 (33)	0	0	1 (13)	3 (13)
Peripheral edema	0	1 (100)	0	0	0	1 (25)	1 (13)	3 (13)
Dehydration	0	0	0	0	1 (33)	1 (25)	1 (13)	3 (13)
Increased blood alkaline phosphatase	0	0	0	0	0	1 (25)	2 (25)	3 (13)
Pain	0	0	0	1 (33)	1 (33)	1 (25)	0	3 (13)
TRAEs leading to either study drug interruption	0	0	0	0	1 (33)	0	1 (13)	2 (9)
TRAEs leading to either study drug discontinuation	0	0	0	0	0	0	0	0
Any grade 3 or 4 TEAE, n (%)[c]	0	0	1 (33)	0	1 (33)	2 (50)	5 (63)	9 (39)
Anemia	0	0	1 (33)	0	1 (33)	0	0	2 (9)
Atrial fibrillation	0	0	1 (33)	0	0	0	1 (13)	2 (9)
Any SAEs related to study drugs, n (%)	0	0	0	0	0	0	1 (13)	1 (4)
Dermatitis	0	0	0	0	0	0	1 (13)	1 (4)

Livmoniplimab (Q2W) + Budigalimab (500mg Q4W) Combination Therapy							
Livmoniplimab dosage	10mg (N=4)	30mg (N=8)	100mg (N=3)	300mg (N=4)	1000mg (N=4)	1500mg (N=11)	Total (N=34)
Any TEAE, n (%)[a,b]	4 (100)	8 (100)	3 (100)	4 (100)	4 (100)	11 (100)	34 (100)
Pruritis	3 (75)	2 (25)	0	2 (50)	2 (50)	7 (64)	16 (47)
Fatigue	0	5 (63)	2 (67)	3 (75)	1 (25)	3 (27)	14 (41)
Nausea	0	4 (50)	2 (67)	1 (25)	3 (75)	4 (36)	14 (41)
Anemia	1 (25)	3 (38)	0	1 (25)	3 (75)	5 (46)	13 (38)
Maculopapular rash	1 (25)	3 (38)	0	0	1 (25)	4 (36)	9 (27)
Diarrhea	0	1 (13)	1 (33)	3 (75)	0	3 (27)	8 (24)
Malignant neoplasm progression	0	3 (38)	0	2 (50)	1 (25)	1 (9)	7 (21)
Rash	0	1 (13)	1 (33)	2 (50)	0	3 (27)	7 (21)
Increased AST	0	2 (25)	1 (33)	0	0	4 (36)	7 (21)
Arthralgia	0	1 (13)	1 (33)	0	1 (25)	4 (36)	7 (21)
Dry skin	2 (50)	1 (13)	0	0	0	4 (36)	7 (21)
Increased ALT	0	2 (25)	1 (33)	0	0	3 (27)	6 (18)
Constipation	0	2 (25)	0	1 (25)	0	3 (27)	6 (18)
Decreased appetite	0	1 (13)	1 (33)	0	1 (25)	3 (27)	6 (18)
Headache	0	0	1 (33)	2 (50)	0	3 (27)	6 (18)
Dehydration	1 (25)	2 (25)	0	1 (25)	0	2 (18)	6 (18)
Vomiting	0	2 (25)	0	2 (50)	0	1 (9)	5 (15)
Pyrexia	0	3 (38)	0	1 (25)	0	1 (9)	5 (15)
Peripheral edema	0	2 (25)	0	0	1 (25)	2 (18)	5 (15)
Tumor pain	0	2 (25)	1 (33)	1 (25)	0	1 (9)	5 (15)
Cough	1 (25)	0	1 (33)	1 (25)	1 (25)	1 (9)	5 (15)
Hypomagnesemia	0	1 (13)	1 (33)	0	0	3 (27)	5 (15)
Increased blood creatinine	1 (25)	2 (25)	0	0	0	1 (9)	4 (12)
Myalgia	0	1 (13)	1 (33)	0	1 (25)	1 (9)	4 (12)
Ascites	0	1 (13)	0	1 (25)	1 (25)	1 (9)	4 (12)
Abdominal pain	0	1 (13)	0	0	0	0	1 (3)
Dyspnea	0	2 (25)	1 (33)	0	0	1 (9)	4 (12)
TRAEs leading to either study drug interruption	0	1 (13)	1 (33)	0	1 (25)	6 (55)	9 (27)
TRAEs leading to either study drug discontinuation	0	1 (13)	0	0	0	4 (37)	5 (15)

(Continued)

TABLE 2 Continued

Livmoniplimab (Q2W) + Budigalimab (500mg Q4W) Combination Therapy							
Livmoniplimab dosage	10mg (N=4)	30mg (N=8)	100mg (N=3)	300mg (N=4)	1000mg (N=4)	1500mg (N=11)	Total (N=34)
Any grade 3 or 4 TEAE, n (%)[c]	3 (75)	4 (50)	1 (33)	4 (100)	3 (75)	8 (73)	23 (68)
Anemia	0	0	0	0	2 (50)	2 (18)	4 (12)
Malignant neoplasm progression	0	2 (25)	0	1 (25)	0	0	3 (9)
Decreased neutrophil count	1 (25)	0	0	0	1 (25)	1 (9)	3 (9)
Increased ALT	0	1 (13)	0	0	0	1 (9)	2 (6)
Increased AST	0	1 (13)	0	0	0	1 (9)	2 (6)
Diarrhea	0	0	0	2 (50)	0	0	2 (6)
Tumor pain	0	1 (13)	0	0	0	1 (9)	2 (6)
Any SAEs related to study drugs, n (%)	0	1 (13)	0	0	0	4 (36)	5 (15)
Increased ALT	0	0	0	0	0	1 (9)	1 (3)
Diabetic ketoacidosis	0	0	0	0	0	1 (9)	1 (3)
Arthralgia	0	0	0	0	0	1 (9)	1 (3)
Autoimmune enteropathy	0	1 (13)	0	0	0	0	1 (3)
Decreased ejection fraction	0	0	0	0	0	1 (9)	1 (3)
Enterocolitis	0	0	0	0	0	1 (9)	1 (3)
Hypotension	0	0	0	0	0	1 (9)	1 (3)
Malaise	0	0	0	0	0	1 (9)	1 (3)

[a]Occurring in >10% of total patients. [b]TEAE defined as AEs with onset on or after the first dose and up to 90 days after the last dose date. [c]Occurring in >5% of total patients. Preferred terms were coded using MedDRA dictionary version 26.0. A patient who reports 1 or more events under the same preferred term is counted only once in that preferred term.
AE, adverse event; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase; MedDRA, Medical Dictionary for Regulatory Activities; Q2W, once every 2 weeks; Q4W, once every 4 weeks; SAE, serious adverse event; TEAE, treatment-emergent adverse event; TRAE, treatment-related adverse event.

of TGF-ß signaling in tumorigenesis has been previously demonstrated (29), thus warranting further investigation.

4 Discussion

Many novel therapeutics targeting different components of the TGF-ß signaling pathway have entered the clinic to date and remain in various stages of clinical development. Galunisertib and vactosertib are small-molecule TGF-ß receptor 1 kinase inhibitors that have been evaluated in several solid tumor types as monotherapy and in combination with anti–PD-1 or anti–PD-L1 antibodies, radiation therapy, or chemotherapy. Galunisertib development appears to have been discontinued following limited-efficacy data readouts (30–32). While some clinical responses have been observed with vactosertib, the contribution of components has not been published to date (33–36). LY3022859, a small-molecule inhibitor that targets the TGF-ß receptor 2, was discontinued following uncontrolled cytokine release (37).

Different mAbs targeting the TGF-ß pathway have been tested in the clinic as well. NIS793 is a TGF-ß inhibitory mAb being developed in combination with spartalizumab, an anti–PD-1 mAb, or chemotherapy. Clinical responses to NIS793 were observed in a phase 1 dose-escalation and dose-expansion study (38). NIS793 continues to be investigated in a phase 3 study in combination with chemotherapy in metastatic pancreatic ductal adenocarcinoma (39) and in a phase 2 study in colorectal cancer (40). Fresolimumab is another anti–TGF-ß mAb that demonstrated limited response during early clinical trials in patients with solid tumors, including renal cell carcinoma and melanoma (41). However, development of this mAb appears to have been discontinued in oncology. Another TGF-ß–targeting mAb, SAR439459, was discontinued after a FIH study due to lack of efficacy and a substantial risk of bleeding, particularly in patients with hepatocellular carcinoma (42). Utilizing a different approach instead of targeting mature TGF-ß, SRK-181 is a mAb specific for the latent form of TGF-ß1, to prevent its activation. It was well-tolerated and showed preliminary efficacy in a phase 1 trial in advanced solid tumors (43).

Therapeutic modalities beyond small molecules and antibodies have been employed as well. Bintrafusp alfa is a bifunctional fusion protein comprising a human anti–PD-L1 antibody fused to the soluble extracellular domain of TGF-ß receptor II and referred to as a "TGF-ß trap." This novel therapeutic generated much excitement when preliminary data demonstrated a high objective response rate in PD-L1–high non-small cell lung cancer and other solid tumors (44, 45). Unfortunately, these early results failed to replicate in later phase 2 and phase 3 studies (46–49). Some trials of bintrafusp alfa have yet to report results, including several National Cancer Institute-sponsored and single-institution studies, according to ClinicalTrials.gov. Cilengitide is an αvß3 and αvß5 integrin inhibitor evaluated in solid tumors, including glioblastoma and head and neck squamous cell carcinoma, that failed to improve overall survival in randomized phase 2 and 3 studies (50, 51). Antisense oligonucleotides have been developed to block TGF-ß1 or TGF-ß2, with the latter entering the clinic for solid tumors including glioma (17, 18).

Livmoniplimab is a first-in-class antibody that targets the GARP:TGF-ß1 complex to block the release of active TGF-ß1. It has a differentiated mechanism compared with other antibodies, small molecules, and protein- or nucleotide-based therapeutics targeting TGF-ß that have entered the clinic to date. Livmoniplimab specifically inhibits TGF-ß1 in a GARP-dependent context, which may increase the therapeutic index and/or the tumor-selectivity of this antibody compared with agents that target all TGF-ß isoforms or broadly target TGF-ß1. Whereas bintrafusp alfa requires co-localization of TGF-ß and PD-

TABLE 3 Confirmed response per RECIST v1.1 as assessed by investigator.

Livmoniplimab Monotherapy (Q2W)								
Livmoniplimab dosage	3mg (N=1)	10mg (N=1)	30mg (N=3)	100mg (N=3)	300mg (N=3)	1000mg (N=4)	1500mg (N=8)	Total (N=23)
Objective response rate (CR + PR)[a]								
N (%)	0	0	0	0	0	0	0	0
95% CI[b]	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)
Best overall response per RECIST v1.1[c]								
CR	0	0	0	0	0	0	0	0
PR	0	0	0	0	0	0	0	0
SD[d]	0	0	0	2 (67)	2 (67)	0	3 (38)	7 (30)
PD	1 (100)	1 (100)	3 (100)	1 (33)	1 (33)	3 (75)	4 (50)	14 (61)
Not evaluable	0	0	0	0	0	0	0	0
Not assessed[e]	0	0	0	0	0	1 (25)	1 (13)	2 (9)
Duration of objective response[f]								
Median (months)	NR	NR	NR	NR	NR	NR	NR	NR
95% CI[g]	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)	(-, -)
Livmoniplimab (Q2W) + Budigalimab (500mg Q4W) Combination Therapy								
Livmoniplimab dosage		10mg (N=4)	30mg (N=8)	100mg (N=3)	300mg (N=4)	1000mg (N=4)	1500mg (N=11)	Total (N=34)
Objective response rate (CR + PR)[a]								
N (%)		0	2 (25)	1 (33)	0	0	2 (18)	5 (15)
95% CI[b]		(-, -)	(3.2, 65.1)	(0.8, 90.6)	(-, -)	(-, -)	(2.3, 51.8)	(5.0, 31.1)
Best overall response per RECIST v1.1[c]								
CR		0	0	0	0	0	0	0
PR		0	2 (25)	1 (33)	0	0	2 (18)	5 (15)
SD[d]		3 (75)	2 (25)	0	0	0	4 (36)	9 (27)
PD		1 (25)	4 (50)	2 (67)	3 (75)	4 (100)	4 (36)	18 (53)
Not evaluable		0	0	0	0	0	1 (9)	1 (3)
Not assessed[e]		0	0	0	1 (25)	0	0	1 (3)
Duration of objective response[f]								
Median (months)		NR	7.5	5.5	NR	NR	NR	8.4
95% CI[g]		(-, -)	(3.65, -)	(-, -)	(-, -)	(-, -)	(-, -)	(3.65, -)

[a]Confirmed objective response based on 2 consecutive response assessments at least 28 days apart. [b]From exact binomial distribution. [c]Based on response assessment visits prior to subsequent anticancer therapy. [d]Based on response assessment visit at least 35 days after first dose of study drug. [e]Patients discontinued study with no postbaseline response assessment (or scan), or patients recently enrolled and did not reach the first postbaseline tumor assessment time point yet and captured under "Other reason for not assessed" category. [f]Duration of response is defined as the time from the date of first documented CR or PR to the documented date of PD or death, whichever occurs first. Patients who neither progressed nor died or received subsequent anticancer therapy are censored at the last evaluable disease assessment. Patients with subsequent anticancer therapy are censored at the date of last tumor assessment prior to the start of the new therapy. [g]Based on Kaplan-Meier estimates.

CI, confidence interval; CR, complete response; NR, not reached; PD, progressive disease; PR, partial response; Q2W, once every 2 weeks; Q4W, once every 4 weeks; RECIST, Response Evaluation Criteria in Solid Tumors version 1.1; SD, stable disease.

1 ligands and targets these proteins with a fixed 1:1 dose ratio, the combination of livmoniplimab and budigalimab allows for independent inhibition of the immunosuppressive TGF-ß1 and PD-1 pathways and dose optimization of each agent for sustained target engagement. In addition, targeting TGF-ß1 derived from GARP-expressing Tregs may focus livmoniplimab drug activity on tumors in which Tregs are elevated.

This FIH dose-escalation study of livmoniplimab as monotherapy and in combination with budigalimab enrolled patients with advanced solid tumors who had received a median of 3–4 prior lines of therapy. Peripheral blood biomarker data demonstrated that saturation of the GARP:TGF-ß1 complex on circulating platelets occurred at livmoniplimab doses within the linear PK range (30mg–1500mg), with no treatment-emergent ADA reported for either livmoniplimab (at 30mg to 1500mg range) or budigalimab. The modest changes in TGF-ß1 levels in circulation observed following treatment with livmoniplimab may be difficult to interpret due to the different sources and forms of TGF-ß and may require development of more specific assays. Overall, the clinical PK/PD data observed in the dose escalation aligned with the values predicted in preclinical modeling. Livmoniplimab demonstrated a tolerable safety profile as monotherapy and in combination with budigalimab. The most common TEAEs in monotherapy-treated patients were fatigue, anemia, and nausea, and those in combination therapy-treated patients were pruritus, fatigue, nausea, and anemia. DLTs were limited and no maximum tolerated dose was reached. The maximum administered dose of livmoniplimab was selected for the dose-expansion phase, in order to generate additional biomarker,

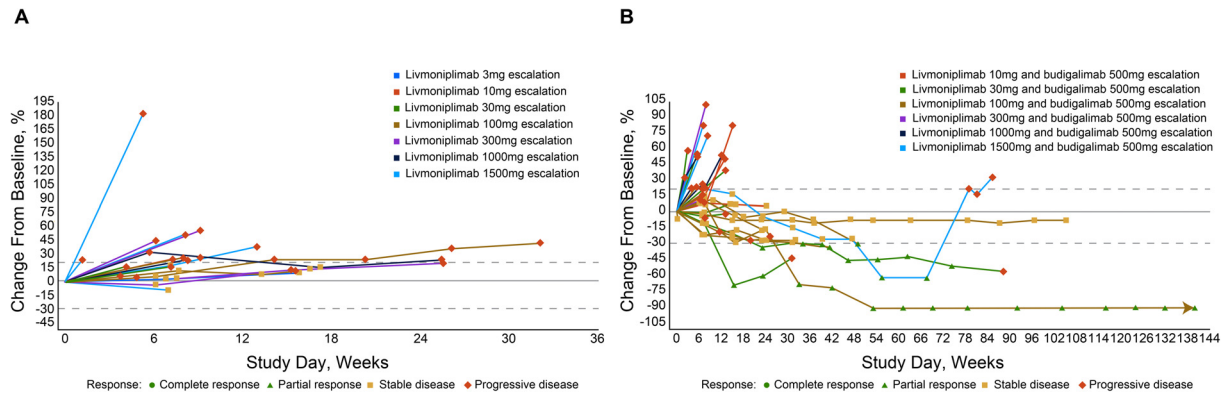

FIGURE 4
Percentage change in target lesion sum diameter measurements from baseline over time per investigator assessment in response-evaluable set (efficacy-evaluable patients defined as patients who have received at least 1 dose of study drug and have either had at least 1 postdose tumor assessment or discontinued treatment due to AE, progressive disease, or death); per RECIST v1.1 and iRECIST. **(A)**: Livmoniplimab monotherapy (Q2W) cohorts (N=22). **(B)**: Livmoniplimab (Q2W) and budigalimab combination therapy cohorts (N=34). → Denotes patients still on treatment. One patient did not have on-study tumor measurement data due to early death. AE, adverse event; iRECIST, modified RECIST v1.1 criteria for immune-based therapeutics; Q2W, once every 2 weeks; RECIST, Response Evaluation Criteria in Solid Tumors.

safety, and efficacy data in cohorts of prespecified solid tumors and select doses for future dose optimization studies.

Encouraging preliminary efficacy was observed in the combination dose escalation in heavily pretreated, advanced solid tumors. While no radiographic responses were observed with livmoniplimab monotherapy, this is consistent with preclinical data in mouse models with GARP:TGF-ß1 surrogate antibodies demonstrating little to no monotherapy activity (de Streel et al., 2020 (9) and AbbVie internal data). Of 34 patients enrolled in the combination dose escalation, 5 patients (15%) experienced confirmed objective responses per investigator RECIST v1.1 assessment, with a median duration of response of 8.4 months. Responses and durable stable disease were observed across multiple solid tumor types including gastroesophageal junction adenocarcinoma, colorectal adenocarcinoma, ovarian cancer, alveolar sarcoma, and urothelial cancer in both PD-1–naive patients and those with prior anti–PD-1 exposure. Livmoniplimab was assessed in solid tumor models preclinically and in patients with advanced solid tumors in this phase 1 study. However, TGF-ß1 dysregulation has been described for hematologic cancers and there are reports of elevated Tregs associated with poor prognosis in these cancers (52–54). Thus, it remains possible that livmoniplimab would exhibit clinical activity beyond solid tumors, particularly in hematologic cancers with elevated GARP-expressing Tregs.

Overall, these dose-escalation data demonstrate encouraging clinical activity and tolerable safety with livmoniplimab, a novel GARP:TGF-ß1 mAb, in combination with budigalimab, an anti–PD-1 Fc-modified mAb. Clinical activity was observed across a range of livmoniplimab doses, from 30mg to 1500mg, where linear PK and target saturation of platelets in circulation was observed. However, it is anticipated that livmoniplimab doses higher than 30mg would be required to achieve sufficient exposure in the TME for complete TE of GARP:TGF-ß1 complex on relevant cell types and to inhibit local, active TGF-ß1 release in a sustained manner across solid tumor indications. Further exploration of this novel drug combination is warranted, with the dose-expansion phase and dose-optimization studies currently enrolling patients with multiple solid tumor types.

Data availability statement

AbbVie is committed to responsible data sharing regarding the clinical trials we sponsor. This includes access to anonymized, individual, and trial-level data (analysis data sets), as well as other information (eg, protocols, clinical study reports, or analysis plans), as long as the trials are not part of an ongoing or planned regulatory submission. This includes requests for clinical trial data for unlicensed products and indications. These clinical trial data can be requested by any qualified researchers who engage in rigorous, independent, scientific research, and will be provided following review and approval of a research proposal, Statistical Analysis Plan, and execution of a Data Sharing Agreement. Data requests can be submitted at any time after approval in the US and Europe and after acceptance of this manuscript for publication. The data will be accessible for 12 months, with possible extensions considered. For more information on the process or to submit a request, visit the following link: https://vivli.org/ourmember/abbvie/.

Ethics statement

The studies involving humans were approved by Razak – University Health Network Research Ethics Board, under review file number 19-5351.0.1. The studies were conducted in accordance with the local legislation and institutional requirements. The

participants provided their written informed consent to participate in this study.

Author contributions

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Conflict of interest

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Publisher's note

Supplementary material

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Glossary

%CV	coefficient of variation
ADA	antidrug antibody
AE	adverse event
ALT	alanine aminotransferase
AR	accumulation ratio
AST	aspartate aminotransferase
C	cycle
CI	confidence interval
CR	complete response
D	day
DLT	dose-limiting toxicity
ECOG	Eastern Cooperative Oncology Group
FIH	first-in-human
GARP	glycoprotein-A repetition predominant
GGT	gamma-glutamyltransferase
HCC	hepatocellular carcinoma
HNSCC	head and neck squamous cell carcinoma
iRECIST	modified RECIST v1.1 criteria for immune-based therapeutics
LAP	latency-associated peptide
LTBP	latent TGF-β binding protein
mAb	monoclonal antibody
MedDRA	Medical Dictionary for Regulatory Activities
MESF	mean equivalent soluble fluorochrome
NR	not reached
NSCLC	non-small cell lung cancer
PD	pharmacodynamics
PD-1	programmed cell death protein 1
PD-L1	PD-1 ligand 1
PK	pharmacokinetics
PR	partial response
PRP	platelet-rich plasma
Q2W	once every 2 weeks
Q4W	once every 4 weeks
RECIST	Response Evaluation Criteria in Solid Tumors
SAE	serious adverse event
SD	standard deviation
Smad	mothers against decapentaplegic family of transcription factors
$t_{1/2}$	terminal phase elimination half-life
TE	target engagement
TEAE	treatment-emergent adverse event
T_{eff}	effector T cell
TGF	transforming growth factor
TIL	tumor-infiltrating lymphocyte
TME	tumor microenvironment
TNBC	triple-negative breast cancer
TRAE	treatment-related adverse event
Treg	regulatory T cell

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