ENGINEERED TARGETED CANCER IMMUNOTHERAPIES

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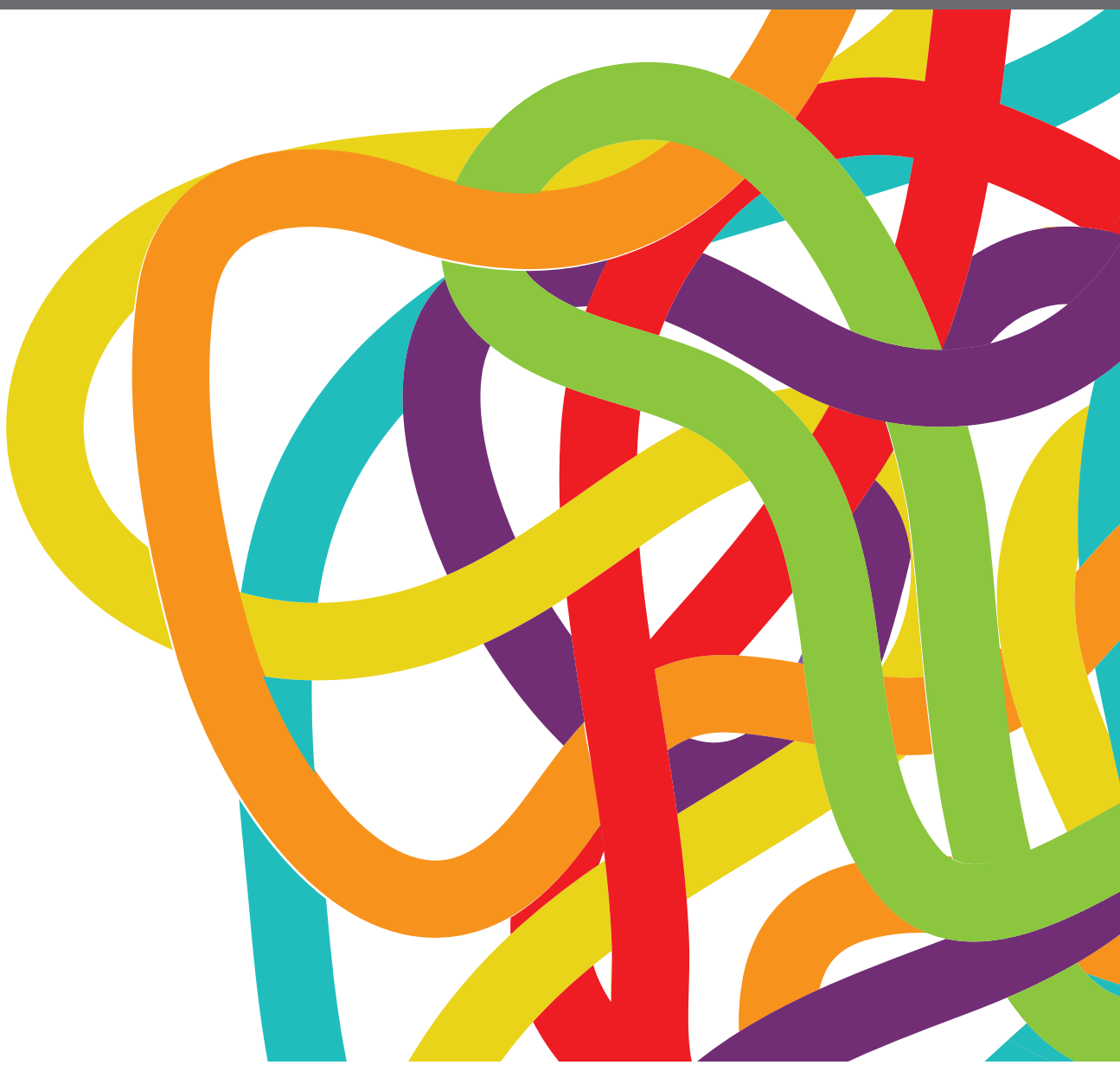

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ENGINEERED TARGETED CANCER IMMUNOTHERAPIES

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EDITORIAL
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Editorial: Engineered Targeted Cancer Immunotherapies

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Editorial on the Research Topic:

Engineered Targeted Cancer Immunotherapies

Conventional cancer therapies, including surgery, radiotherapy and chemotherapy showed good effects in the treatment of patients with early-stage cancers, but they often fail to cure many patients that develop metastasis in different organs.

To overcome this issue more selective therapies, such as immunotherapy, have been developed in the last few decades.

The aim of immunotherapy is to enhance the power of immune system to target cancer, leading to a selective killing of cancer cells and a concomitant preservation of normal tissues.

Unfortunately, cancer cells use several mechanisms to impair the efficacy of immunotherapy, such as expression of neo-antigens, over-expression of immunosuppressive molecules (IDO, PD-L1), accumulation of myeloid-derived suppressor cells (MDSCs) and regulatory T cells in the tumor microenvironment (TME).

To improve immunotherapy efficacy and to overcome the inhibitory activity of the TME on the immune system, engineered targeted cancer immunotherapies have been developed. These include bispecific monoclonal antibodies, immunotoxins, fusion proteins, chimeric antigen receptor (CAR)-T cells, gene therapy and monoclonal antibodies (mAbs) with antibody-dependent cell-mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) activity.

CAR-T cell technology is based on the isolation of patient's T lymphocytes, which are then engineered to express chimeric antigen receptors (CARs). The modified T lymphocytes can recognize and kill cancer cells in a manner that does not involve the major histocompatibility complex (MHC). After proliferation *in vitro*, CAR-T cells are reinfused into the patient (Lin et al.).

CAR-T cells achieved promising results as immunotherapy, especially against hematological malignancies, where they showed impressive response with high target specificity.

In this regard, in the review from Gambella et al. is reported that CAR-T cells targeting CD19 showed promising results in the treatment of diffuse large B-cell lymphoma (Gambella et al.).

In their original research, Wang et al. observed that Bryostatin, a member of a family of cyclic polyketides, which interacts with the diacylglycerol biding site of the C-1 regulatory domain of protein kinase C, activates CAR T-cell antigen-non-specific killing (CTAK), and CAR-T NK-like killing for Pre-B acute lymphocytic leukemia (ALL) through the modulation of both CD19 and CD22 expression on leukemia cells. This modulation allows for a greater degree of CAR-mediated leukemia cell killing. (Wang et al.).

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However, in patients with solid tumors, CAR-T cell therapy did not achieve yet a good objective response and this phenomenon is due to the ability of TME of solid tumors to inactivate CAR-T cells.

All existing CAR-T cells available on the market are autologous (made with same patient-derived T lymphocytes) to avoid severe alloimmune rejection due to a mismatch of MHC between the donor and the recipient.

As explained in the review from Lin et al., to improve the efficacy of CAR-T cells, costimulatory molecules, such as CD28 or 4-1BB, were incorporated into CAR structure to promote CAR-T cells survival and functionality *in vivo* (second and third generation CAR). In addition, CAR-T cells have been further engineered to secrete cytokines (fourth generation CAR) which allow to CAR-T to be more viable and to activates other immune cells (Lin et al.).

In their review, Zhang et al. showed the importance to use gene-edited interleukin CAR-T cells therapy as a novel strategy for the treatment of malignancies. The most used cytokines used to construct fourth generation CAR are interleukins including IL-7, IL-12, IL-15, IL-18, IL-21 and IL-23. These CAR-T cells include co-expression of single interleukin, two interleukins, interleukin combined with other cytokines, interleukin receptors, interleukin subunits, and fusion inverted cytokine receptors (ICR). There are several Phase I and Phase I/II clinical trials evaluating the safety and efficacy of gene-edited interleukin-CAR-T (fourth generation CAR), involving hematological tumors and solid tumors (Zhang et al.). Another efficient gene editing process to improve efficacy of CAR-T cells is the CRISPR/Cas9 strategy for the editing of human primary NK and T Cells as reported by Elmas et al. For example, CRISPR/Cas9 has been used to knock down TGF-β receptor II (TGFBR2) to reduce CAR-T cells exhaustion and to enhance CAR-T cells anti-tumor activity. In addition, CRISPR/cas9 knock down of granulocyte-macrophage colony-stimulating factor (GM-CSF) was useful to decrease cytokine release syndrome (CRS) and neuroinflammation linked to CAR-T cell therapy (Elmas et al.).

Despite these improvements, there are still some safety concerns on the use of autologous CAR-T cells, including CRS and neurotoxicity caused by CAR-T cells overactivation. In addition, autologous CAR-T cells have high cost and intensive manufacturing process, which slow down their quick availability for the patient.

Lin et al. in their review explained that one strategy to further improve CAR-T cells safety and efficacy is to employ universal CAR-T (UCAR-T) cell therapy, which consist of allogeneic CAR-T cells that are taken from healthy donors. UCAR-T cells share the same engineering process and mechanisms of action of autologous CAR-T, but are cheaper than autologous CAR-T, have a much less intensive manufacturing process, can be immediately available to cancer patients and showed promising results in treating T-cells malignancies. To reduce the Graft-Versus-Host Disease (GvHD) and rejection, UCAR-T cells underwent to additional gene editing processes, such as knock out of the TCR, genetic ablation of MHC-I and/or MHC-II and editing of CD7 to prevent the fratricide in CD7 UCAR-T cells (Lin et al.).

A more recent and promising approach is the employment of chimeric antigen receptor-engineered NK (CAR-NK) cells. In their review, Baysal et al. reported that CAR-NK can be obtained either through lenti-/retroviral transduction of primary adult natural killer (NK) cells or through the engineered immortalized NK-92 cells. CAR-NK cells have several advantages over CAR-T cells, including more robustness, reduction of frequency of cytokine release syndrome, suppression of GvHD induced by CAR-T cells (Baysal et al.). In this regard, in recent years, several clinical trials have investigated the use of CAR-NK cells as therapeutic approach against hematological malignancies and indicated the possibility of adopting CAR-NK therapy for patients with high-risk B cell lymphoma and leukemia. CAR-NK cells can also be equipped with on-board cytokines, such as IL-15, to enhance both persistence and cytotoxicity against tumor cells (Gambella et al.).

Beyond T cells and NK cells, also macrophages can be engineered to improve cancer immunotherapy.

In the review from Ding et al. are reported different methods to create engineered macrophages for cancer therapy *via* nanotechnology and genetic manipulation. Since macrophages have a great ability to infiltrate tumors, a promising strategy to deliver anti-cancer drugs in the TME is to load macrophages with nanoparticles (NPs). NPs can deliver a variety of anticancer agents, such as chemotherapeutic drugs, targeted drugs, messenger RNA, small interfering RNA, and the CRISPR/Cas9 genetic editing system, and many studies have demonstrated that NP-loaded macrophages (NPL-Ms) can deliver the anti-cancer drug in a more efficient manner to tumor cells, leading to a strong antitumor effect. In addition, the review from Ding et al. showed also that macrophages engineered to express CARs can efficiently migrate to tumor sites and to kill tumor cells through phagocytosis. After reaching TME, these engineered macrophages can significantly subvert TME immunosuppressive activity and, in turn, enhance T cell-mediated anticancer immune responses (Ding et al.).

Another strategy to improve immunotherapy is to engineer mAbs targeting tumor antigens.

Important targets of anti-cancer mAbs are pathways mediated by the epidermal growth factor receptor (EGFR), CD20, vascular endothelial growth factor (VEGF), and the programmed cell death protein-1 (PD-1)/programmed cell death protein-1 ligand (PD-L1).

Although the immunotherapy with mAbs has increased survival of cancer patients, the lack of tumor antigens, uncontrolled activation of oncogenes, increased activity of regulatory T cells and MDSCs in the TME can lead to the resistance to immune checkpoints inhibitors (ICIs)-based therapy and to its subsequent failure.

To overcome this issue, mAbs were engineered to have different mechanisms of action. In this regard, mAbs able to mediate ADCC may contribute to improve the clinical response of cancer patients treated with ICIs.

Examples of clinically approved mAbs that can mediate ADCC include trastuzumab, rituximab, cetuximab, avelumab.

Baysal et al. reported that one interesting strategy to potentiate the ADCC activity mediated by mAbs is the employment of adoptive NK cells to restore NK cell functionality of cancer patients that is often impaired by immunosuppressive activity of TME. Authors suggested that a

promising approach, in evaluation in different clinical trials, is the combination between cetuximab, which targets the epidermal growth factor receptor (EGFR) expressed in breast, lung, colorectal, head and neck cancers, and adoptive transfer of autologous or allogenic expanded NK cells (Baysal et al.).

The employment of allogenic expanded NK cells has the advantage of being a good alternative to autologous NK cells due to the limited number of patient-derived NK cells. Other benefits of allogenic NK cells include the possibility to obtain NK cells from healthy donors and the ability to produce high quantity of engineered NK cell lines with a greater antitumor activity.

Baysal et al. also reported that the anti-tumor activity of allogenic NK cells in combination with cetuximab can be enhanced by stimulation of NK cells with cytokines such as IL-2, IL-12, IL-15, IL-21. Stimulation of NK cells with these cytokines leads to enhancement of the antitumor effects of NK cells against various tumor types and significantly increases cytokine and chemokine secretions which, in turn, stimulate the infiltration of CD8+ T cells into the tumor. Several clinical trials showed promising clinical responses and a tolerable safety profile using cetuximab in combination with NK stimulated with these cytokines in different cancer types (Baysal et al.).

In their original research article, Klewinghaus et al. suggested that another efficient strategy to kill $EGFR^+$ cells could be the employment of cattle-derived ultralong CDR-H3 common light chain bispecific antibodies targeting EGFR on tumor cells as well as natural cytotoxicity receptor NKp30 on NK cells. These engineered bispecific antibodies elicited potent NK cell killing of EGFR-overexpressing tumor cells as well as robust release of proinflammatory cytokine interferon-γ (IFN- γ) *in vitro*. Since IFN-γ can inhibit suppressive immune cell subsets and redirect NK, NKT and T cell trafficking into tumors, the stimulation of NK cells to release IFN-γ by these types of bispecific antibodies might be a promising strategy to improve antibody-based immunotherapy in clinic (Klewinghaus et al.).

Chasov et al. reported promising new humoral and cell-based immunotherapies for targeting p53 mutant cancers.

Authors showed that the peptide neoantigens from a proteolytically processed mutant p53 protein are presented by APCs to B and T cells to activate the immune response. To this end, an interesting approach is based on bispecific TCRm antibodies that bind to both TCR and the peptide on MHC (pMHC) presenting the mutant p53 antigen. The scope of this approach is to enhance the presentation to T cells of mutant p53 peptides to stimulate T cells to destroy cancer cells bearing mutant p53 without affecting the normal cells with wild type p53 (Chasov et al.).

Kooti et al. reported studies showing that oncolytic viruses (OVs) can represent a valid alternative to CARs and engineered mAbs to kill cancer cells.

Oncolytic viruses (OVs) include a group of viruses that selectively recognize and kill malignant cells, without affecting the surrounding health cells. OVs can kill cancer cells through several mechanisms, including direct cytotoxicity, induction of immune-mediated cytotoxicity and disruption of tumor vasculature.

In addition, OVs can favor recruitment of immune cells, such as cytotoxic T lymphocytes, dendritic cells, NK cells and phagocytic cells in the TME to induce immune cell death of cancer cells. To improve their efficacy OVs are often engineered to express immune-stimulatory (IL-2, IL-4, IL-12 and GM-CSF) and pro-apoptotic (tumor necrosis factor alpha, p53 and TRAIL) genes (Kooti et al.).

The treatment of hepatocellular carcinoma (HCC), one of the most common malignancies globally, and multiple myeloma (MM), is benefiting from some of engineered cancer immunotherapies mentioned above. In the review from Miao et al. is reported how ICIs (anti PD-1/PD-L1 and cytotoxic T-lymphocyte antigen-4 (CTLA-4) mAbs, alone or in combination), tumor vaccines, engineered NK cells, CAR-T cells are widely used in clinic and in clinical trials for the treatment of HCC (Miao et al.).

Similarly, the review from Guo et al. showed that, for thetreatment of MM, the most promising engineered cancer immunotherapies evaluated in clinical trials are antibody-drug conjugates (ADCs), second-generation CAR-T cells and CAR-NK cells (Guo et al.).

The big challenge now is to evaluate the combination of engineered targeted cancer immunotherapies with conventional treatment methods to evaluate if this strategy can produce synergistic effects and a better efficacy for the treatment of blood and solid tumors.

AUTHOR CONTRIBUTIONS

All authors listed have made a direct and intellectual contribution to the work and approved the submitted version.

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Conflict of Interest: MF is an employee of Precision Biologics, Inc., a biotech company producing monoclonal antibodies for the treatment of solid tumors (Phase 1/2 clinical trials). MF holds a patent for one of these monoclonal antibodies.

The remaining author declares 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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REVIEW
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Gene-Edited Interleukin CAR-T Cells Therapy in the Treatment of Malignancies: Present and Future

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In recent years, chimeric antigen receptor T cells (CAR-T cells) have been faced with the problems of weak proliferation and poor persistence in the treatment of some malignancies. Researchers have been trying to perfect the function of CAR-T by genetically modifying its structure. In addition to the participation of T cell receptor (TCR) and costimulatory signals, immune cytokines also exert a decisive role in the activation and proliferation of T cells. Therefore, genetic engineering strategies were used to generate cytokines to enhance tumor killing function of CAR-T cells. When CAR-T cells are in contact with target tumor tissue, the proliferation ability and persistence of T cells can be improved by structurally or inductively releasing immunoregulatory molecules to the tumor region. There are a large number of CAR-T cells studies on gene-edited cytokines, and the most common cytokines involved are interleukins (IL-7, IL-12, IL-15, IL-18, IL-21, IL-23). Methods for the construction of gene-edited interleukin CAR-T cells include co-expression of single interleukin, two interleukin, interleukin combined with other cytokines, interleukin receptors, interleukin subunits, and fusion inverted cytokine receptors (ICR). Preclinical and clinical trials have yielded positive results, and many more are under way. By reading a large number of literatures, we summarized the functional characteristics of some members of the interleukin family related to tumor immunotherapy, and described the research status of gene-edited interleukin CAR-T cells in the treatment of malignant tumors. The objective is to explore the optimized strategy of gene edited interleukin-CAR-T cell function.

Keywords: CAR-T cells, interleukin, gene-edited, immunotherapy, TME, malignant tumor

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INTRODUCTION

CAR-T cells technology has achieved gratifying results in the clinical treatment of hematologic malignancies (1, 2). However, it has hit a bottleneck in treating solid tumors (3–6). Studies have shown that the inhibitory tumor microenvironment (TME) of solid tumors can inactivate CAR-T cells (7). The full activation and amplification of normal T cells require not only T cell receptor signals and costimulatory signals, but also the synergistic action of immune cytokines. Current theories suggest that the immunosuppressive TME of solid tumors is mainly characterized by the suppression of immune cell function, So it weakens CAR-T cells tumor immunity (8, 9).

To overcome this challenge, multiple strategies have been applied to optimize CAR-T cells technology. Immune cytokines are the basis of T cells' immune function, and they have been demonstrated that they can significantly improve the antitumor activity of CAR T cells (10). Therefore, the researchers created a fourth generation of CAR-T cells by gene modifying the structure of CAR-T cells using immune cytokines (11, 12).

Interleukin is a type of cytokine produced by multiple immune cells and used by these immune cells. Some members of the interleukin family exert multifarious roles in the anti-tumor process as growth factors of T cells. At present, many gene-edited interleukin CAR-T cells have achieved positive efficacy in the treatment of malignant tumors in preclinical studies, and related clinical studies are ongoing. With the structural optimization of gene-edited interleukin CAR-T cells, its efficacy in overcoming the immunosuppressive TME is also increasing. Here, we shown the correlation between the above families of interleukin and tumor immunotherapy, and summarize the research progress of their application for CAR-T cells technology. Finally, the optimization of gene-edited interleukin-CAR T cells in anti-tumor therapy was discussed.

MEMBERS OF THE INTERLEUKIN FAMILY AND TUMOR IMMUNITY

Last decade, with the development of tumor immunotherapy, the function of interleukin in tumor immunotherapy has attracted more and more attention from researchers. A large number of tumor immunotherapy techniques began to use interleukin to improve the immune response of tumor. **Table 1** shows part of the interleukin family and their functions related to tumor immunotherapy.

Correlation Between IL-1 Family Members and Tumor Immunity

The IL-1 family mainly includes IL-1, IL-18, IL-33, and IL-36. They initiate a powerful inflammatory and immune response by binding to specific receptors in the IL-1 receptor family. These immunomodulatory molecules are generated by immune cells and regulate the function of these immune cells. Therefore, they are closely related to tumor immunity.

IL-1 is a pro-inflammatory cytokine, which includes two subtypes of IL-1α and IL-1β, and regulates adaptive immune response mainly through binding with its receptor (IL-1R) in the body. IL-1α acts as a local alarm in the event of cell damage, while IL-1β release can also occur in the circulation and is strictly controlled. IL-1β is primarily derived from myeloid cells and is upregulated and associated with disease progression in many different types of cancer, such as colon and lung malignancies. Cancer cells also drive tumor-associated inflammatory macrophages to produce IL-1β, which inhibits tumor immune response through IL-1β-mediated accumulation of myeloid derived suppressor cells (MDSCs). Therefore, current clinical studies have focused on the role of antagonistic IL-1 β activity in anti-tumor (13). These results indicate that IL-1β acts on adaptive immunity and may indirectly modulate T cell immune response to tumor.

TABLE 1 | Summary of cytokines related to tumor immunotherapy in the interleukin family.

Interleukins	Tumor immune-related functions	Receptors	The associated immune cells	Associated activation pathway
IL-1 family				
IL-1	Proinflammatory, regulating adaptive immune response	IL-1R	DCs, T cells	NF-κB (13)
IL-18	T cell are activated by enhancing endogenous TCR	IL-18Rα/IL-18Rβ	CD8 $^{+}$T cells, NK cells	NF-κB (14)
IL-33	Bidirectional regulation of tumor immune response	ST2	Th cells, NK cells, Treg cells	NF-κB,MAP (15)
IL-36	Promote DCs maturation and indirectly promote T cell proliferation	IL-36R	DCs,T cells	NF-κB,MAP (16)
IL-2 family				
IL-2	Regulate the proliferation and apoptosis of activated T cells	IL-2Rα/IL-2Rβ	T cells, NK cells, monocyte macrophages, B cells	STAT5 (17–19)
IL-4	Regulates the function of Th1 and Th2 cells	IL-4R	Th cells,	STAT6 (20)
IL-7	Promote T cell proliferation and maintain cell homeostasis	IL-7Rα	Naive and memory T cells	STAT5 (21, 22)
IL-9	Promote the proliferation and activation of T cells	IL-9R	CD8+ T cells, NK T cells	STAT1, STAT3, STAT5 (23)
IL-15	Promote T cell proliferation and maintain cell homeostasis	IL-15Rα/IL-2Rβ	CD8 +T cells,NK cells	STAT5 (24)
IL-21	Modulate effector function of CD8+ T cells and polarization of CD4+ T Th cells	IL-21R	CD8+ T cells, CD4+ T cells, NK T cells	STAT3 (25, 26)
IL-6/12 family				
IL-6	Regulates immune response and inflammation	IL-6R	T cells	STAT3 (27)
IL-12	Enhance the IFN-γ secretion function of Th17 cells and cytotoxic effect of NK cells and T cells, stimulate T cell differentiation	IL-12Rβ1/IL-12Rβ2	NK cells, NK T cells, CD8+T cells	STAT4 (28)
IL-23	Promotes memory T cell proliferation	IL-23R	T cells	STAT3 (29)
IL-27	Affects antigen presentation and regulates the differentiation and activation of Th cells	gp130/WSX-1	Treg cells	STAT1, STAT3 (30)
IL-35	Promotes immunosuppression by inhibiting the differentiation of Th1 and Th17 cells	IL-12Rβ2/ gp130/WSX-1	Treg cells	STAT1, STAT3, STAT5 (30)

IL-18 is also an important pro-inflammatory and immunomodulatory cytokine (31), which activates T cell proliferation and IFN-γ secretion by enhancing endogenous TCR. It can also promote more effective tumor killing by enhancing the expression of Fas ligands in immune cells (32). Besides, studies have demonstrated that IL-18 improves T cell function without causing severe dose-limiting toxicity (33, 34). Therefore, IL-18 is a promising candidate cytokine for gene-edited CAR-T cells.

As an inflammatory factor, IL-33 plays multiple roles in tumor immunity. In 2015, a study found that IL-33 was identified as a ligand for oncogenic inhibitory receptor 2 (ST2) (35). IL-33 plays an immunomodulatory role by interacting with ST2. IL-33 can act on multitudinous immune cells, such as Th1, Th2, NK and regulatory T cells (Tregs) (15). Therefore, IL-33 has a bidirectional regulatory function of different cancer immune cells. Three subtypes of IL-36, known as IL-36α, IL-36β, and IL-36γ, have different functions. IL-36 has been shown to promote upregulation of CD80 and CD86, markers of DCs activation, and promote DCs maturation (36). The immunoregulatory function of IL-36α is to directly promote the proliferation of CD4+T cells (37). IL-36β promotes T cell proliferation by promoting the production of IL-12 and IL-18 by DCs (38). The function of IL-36γ is to induce CD4+T cells to secrete IFN-γ, IL-4 and IL-17 (39).Therefore, IL-36 also exerts a bidirectional regulatory role in the process of tumor immunity, and has both activation and inhibition effects.

Correlation Between IL-2 Family Members and Tumor Immunity

The IL-2 family is part of the receptor γc family, which belongs to type I cytokines, and they contain many interleukins. Its members mainly include IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21,and all of them play immunomodulatory functions through the JAK-STAT pathway (40, 41). And these cytokines exert vital functions in the regulation of immune cells.

IL-2 is a T cells growth factor that enhances the cytolytic activity of NK cells (17). It promotes Tregs differentiation, which regulates the adaptive immune response (18). At present, IL-2 is the main cytokine used to culture T cells for immunotherapy. Nevertheless, T cells cultured by IL-2 showed phenotypic heterogeneity and were mainly composed of effector memory cells that had full functional effects but were sensitive to death. IL-4 is mainly involved in the function regulation of Th2 cells, so it is known as Th2 cytokine. It can promote tumor progression by down-regulating Th1 signaling and directly inactivating CD8+T cells (42). Shuku-ei Ito et al. investigated the effect of neutralizing IL-4 on tumor immunity (20), the results suggested that an IL-4 antibody can enhance anti-tumor immunity. Therefore, IL-4 can be used as a target for tumor immunotherapy due to its role in the tumor microenvironment.

IL-7 is the most important tumor immune-related cytokine in the γc family, and its function is mainly to regulate naive T cells and memory T cells homeostasis (21, 22). Studies have confirmed that IL-7-induced signal transduction defect is the main reason for affecting T cell development in severe combined immunodeficiency disorder (SCID) patients (43) and in patients with SCID caused by JAK3 mutation (44, 45). IL-7 is an indispensable cytokine for T cell growth, therefore, IL-7 has also become a popular cytokine in gene-edited CAR-T cells research. IL-9 is also an important tumor immune-related cytokine, mainly produced by Th9 cells (46, 47). IL-9 derived from Th9 cells can improve the tumor killing function of CD8+ T cells and NK T cells by promoting secretion of IFN-γ (48, 49). Therefore, Th9 cells have been shown to have an antitumor effect in most solid tumors (50). However, it has been shown to be tumorigenic in most hematologic tumors (51).

As an immunoregulatory cytokine, IL-15 is an important homeostasis cytokine of CD8+T cells and NK cells. The main function of IL-15 is to promote the growth of memory CD8+T cells (52, 53). Therefore, L-15 has been used in several studies to optimize the structure of CAR-T cells. However, IL-15 must form the IL-15/IL-15Rα complex in order to exert its tumor immune function. IL-15/IL-15Rα complex has poor stability and can bind to IL-15Rβγ to decrease tumor immune efficacy (54). Therefore, the stability of IL-15/IL-15Rα complex is essential for IL-15 to perform tumor immune function. The researchers used several strategies to improve the stability of IL-15 function. One strategy is to extend the persistence of the IL-15/IL-15Rα complex by fusion with the IgG Fc domain, resulting in more persistent induction of CD8+T cells and NK cells (55). Another strategy is to enhance the capability of IL-15 through a fusion protein that is conjugated to human IL-15 through the ligosome in the terminal cytokine binding domain of human IL-15RαNH$_2$ and has similar biological activity to that described above (54).

IL-21 is a multifunctional cytokine, exerts a vital role in regulating the function of CD8+ T cells (25). IL-21 can improve the activity of CD8+ T cells, making it potentially valuable in cancer immunotherapy (56). Besides, a recent study on pancreatic cancer found that IL-21 also has an anti-tumor effect by enhancing NK cell function (57). IL-21 has also been used in studies of CAR-T for its ability to positively regulate tumor-associated immune cells.

Correlation Between IL-6/12 Family Members and Tumor Immunity

The family members include typical members IL-6, IL-12, IL-23, IL-27, and IL-35. Cytokines in the IL-12 family influence the outcome of cancer, infection, and inflammatory disease. Most of the members are produced by DCs, macrophages, endothelial cells, T lymphocytes, and tumor cells (58),which conduct downstream signal transduction through JAK protein and STAT. They regulate tumor immunity in both direct and indirect ways.

IL-6 is a pleiotropic cytokine,affects T cell activation, amplification, survival, and polarization (59). Studies have shown that during the inflammatory process, IL-6 signaling has been found to promote the expression of T cell attractor chemokines (60). IL-6 can also regulate the surface expression of Fas receptor through up-regulating anti-apoptotic factors by STAT3, thereby inhibiting T cell apoptosis (61, 62). IL-6 has been also demonstrated to participate in the accumulation of

MDSCs in tumors (29). In addition, IL-6 exerts vital roles in the acute immune response. When stimulated by local inflammation, IL-6 can promotes the production of acute phase proteins by acting on the liver (63). IL-6 is an important factor affecting liver cells, hematopoietic progenitor cells, cardiovascular, endocrine and nervous system homeostasis (64). Therefore, a large number of CAR T clinical trials have shown that high serum IL-6 levels are associated with cytokine release syndrome (CRS), and IL-6 is a monitoring indicator in the clinical diagnosis and treatment of CRS (65).

As an inflammatory cytokine, IL-12 is mainly generated by DCs cells and macrophages. Studies have demonstrated that IL-12 can improve the activation of Th1 and Th17 cells (66) and enhance the cytolysis ability of CD8+T cells (67). Therefore, IL-12 is expected to be successful in adoptive immunotherapy of tumors due to its positive regulation of tumor immune properties. IL-23 is constituted of IL-23αp19 and IL-12βp40 (29), and facilitates the proliferation of memory T cells, especially Th17 cells expressing the its receptor (IL-23R) (68–70). IL-23 activates the tumor immune response to inhibit tumor progress, which has given rise to the application of IL-23 in the treatment of tumors by gene-edited CAR-T.

IL-27 is an effective immunomodulatory cytokine, which mainly has anti-inflammatory and inhibitory properties in immunomodulatory regulation, especially in inhibiting Th2 and Th17 differentiation. However, recent studies comparing these results have also demonstrated that IL-27 promotes the growth and survival of Tregs (30). Myeloid and epithelial cells treated with IL-27 also showed enhanced antigen presentation by upregulating MHCI and MHCII as well as costimulatory molecules (71). Therefore, IL-27 is also a major regulator of TME. IL-35 is an effective regulatory cytokine, mainly secreted by Tregs. IL-35 can convert T cell into the regulatory cell population that produces IL-35, which is called the induction of Tregs-IL-35 (69, 72). IL-35 inhibited function of Th1 and Th17 cells by promoting the expansion of Tregs (72, 73). Therefore, IL-35 is an immunosuppressive cytokine and exerts important roles in promoting tumor progression.

CORRELATION STUDY OF GENE-EDITED INTERLEUKIN CAR-T CELLS IN THE TREATMENT OF MALIGNANT TUMORS

The researchers genetically engineered these cytokines to modulate CAR-T activity to better kill tumor cells. At present, a great number of preclinical studies have confirmed that gene-edited co-expression of cytokines such as IL 7, IL 12, IL 15, IL 18, IL21, and IL 23 can enhance the antitumor activity of CAR-T (**Table 2**). Simultaneously, clinical trials of gene-edited interleukin-CAR-T for malignancies are under way at several medical centers around the world (**Table 3**), involving hematological tumors and solid tumors, to evaluate its effective dose and safety.

IL-7

IL-7 has been widely used in tumor immunotherapy to enhance the anti-tumor immune response of T cells (91, 92). Studies have shown that IL7 not only promotes CD8+ T cell proliferation and reduces T cell apoptosis and depletion by enhancing Bcl-2 expression, but also increases the phenotype of poorly differentiated CAR-T cells, thus improving the persistence and viability of CAR-T cells (75, 93). There were also clinical trials (NCT00586391, NCT00709033) that amplified CAR-T cells with IL-7 and IL15 *in vitro*, and then confirmed these findings by phenotypic analysis of CAR-T cells (94).Cong He et al. (75) constructed gene-edited IL-7 CAR-T cells targeting NKG2D, and found that co-expressing IL-7 enhanced the proliferation and persistence of NKG2D-CAR-T cells *in vitro* and *in vivo*. In order to further optimize the construction of CAR-T cells, researchers have used IL-7 in combination with other cytokines to modify

TABLE 2 | Summary of preclinical studies on the use of CAR-T cells co-expressing cytokines in the treatment of malignant tumors.

Tumor	Targeted antigen	Gene-edited cytokines	Reference
Lung cancer, pancreatic ductal adenocarcinoma	hCD20, Mesothelin	IL-7 and CCL19	Keishi Adachi et al. (74)
prostatic cancer	NKG2D	IL-7	Cong He et al. (75)
hepatic carcinoma	GPC3	IL-7 and PH20	Xingcheng Xiong et al. (76)
breast carcinoma	AXL	C7R	Zhenhui Zhao et al. (77)
Colorectal cancer, pancreatic cancer, stomach cancer	CEA	IL-12	Xiaowei Chi et al. (78)
lymphoma	CD19	IL-12	Gray Kueberuwa et al. (79)
hepatic carcinoma	glypican-3 (GPC3)	IL-12	Ying Liu et al. (80)
ovarian cancer	Muc-16	IL-12	Oladapo O.Yeku et al. (81)
leukemia	CD19	IL-15	Lenka V. Hurton et al. (82)
Cerebral endothelioma	VEGFR-2	IL-15	Evripidis Lanitis et al. (83)
melanoma	CD19	IL-18	Biliang Hu et al. (84)
hepatic carcinoma	GPC3	IL-21	Yi Wang et al. (85)
chronic lymphocytic leukemia	CD19	IL-21	Štach M et al. (86)
hepatic carcinoma	GPC3	IL-15 and IL-21	Batra S. A et al. (87)
neuroblastoma	GD2	IL-23	Xingcong Ma et al. (88)
prostatic cancer	PSMA	IL-23	Dawei Wang et al. (89)
hepatic carcinoma	GPC3	4/21 ICR	Yi Wang et al. (85)
pancreatic cancer	PSCA	4/7 ICR	Somala Mohammed et al. (90)

TABLE 3 | Clinical trial summary of gene-edited interleukin CAR-T cells.

Targeted antigen	Tumor	Gene-edited cytokines	Patients (n)	Clinical stage	Identifying code (ClinicalTrials.gov)	Sponsor	Status
EGFR	metastatic colorectal cancer	IL-12	20	I	NCT03542799	Shenzhen Second People's Hospital, China	Not yet recruiting
CD19	Diffuse large B cell lymphoma	IL7 and CCL19	24	I	NCT04381741	The Second Affiliated Hospital of Zhejiang University, China	Recruiting
Nectin4/FAP	Nectin4 positive late malignant solid tumor	IL7 and CCL19, or IL12	30	I	NCT03932565	The Sixth Affiliated Hospital of Wenzhou Medical University, China	Recruiting
CD19	lymphoma	IL-7 and IL -15	20	I/II	NCT02652910	Xinqiao Hospital, Chongqing City, China	Unknown status
GD2	neuroblastoma	IL -15	18	I	NCT03721068	Rineberg Comprehensive Cancer Center, USA	Recruiting
CD19/CD20	lymphoma	IL-7 and IL-15	32	I/II	NCT04186520	Medical College of Wisconsin, USA	Recruiting
GD2	Neuroblastoma, osteosarcoma	C7R	94	I	NCT03635632	Baylor College of Medicine, USA	Recruiting
GD2	neuroglioma	C7R	34	I	NCT04099797	Baylor College of Medicine, USA	Recruiting
GPC3	Multiple solid tumors (liver cancer, sarcoma, etc.)	IL -15	24	I	NCT04377932	Baylor College of Medicine, USA	Not yet recruiting
GPC3	Multiple solid tumors (liver cancer, sarcoma, etc.)	IL -15 and IL-21	24	I	NCT04715191	Baylor College of Medicine, USA	Not yet recruiting
CD138, integrin β7, CS1, CD38 and BCMA	multiple myeloma	IL7 and CCL19	30	I	NCT03778346	The Sixth Affiliated Hospital of Wenzhou Medical University, China	Recruiting
CD19	lymphoma	IL -18	30	I	NCT04684563	University of Pennsylvania, USA	Not yet recruiting
CD5	T-cell Acute Lymphoblastic Leukemia T-cell Non-Hodgkin Lymphoma	IL15/IL15 sushi	20	I	NCT04594135	Peking University Shenzhen Hospital Shenzhen, Guangdong, China	Recruiting
MUC16	Multiple solid tumors	IL-12	18	I	NCT02498912	Kettering Cancer Center, USA	Active, not recruiting

All clinical trials were download at www.clinicaltrials.gov (access date: March 04, 2021).

CAR-T cells, and achieved promising results in preclinical study. For instance, Keishi Adachi et al. (74) constructed CAR-T cells that co-expressing IL-7 and CCL19, and found that multiple cytokines significantly improved tumor infiltration and survival of CAR-T cells. More robust antitumor activity and durability than conventional CAR-T has been realized in studies targeting solid malignant tumors. These related clinical trials are ongoing, such as targeting CD19 CAR-T trial for lymphoma (NCT04381741); targeting NECTIN4/FAP CAR-T for advanced malignant solid tumors (NCT03932565). Similarly,Xingcheng Xiong et al. (76) constructed CAR-T cells co-expressing IL-7 and hyaluronidase(PH20) in the preclinical study of targeting GPC3 CAR-T cells for liver cancer, and the results showed that the co-expression of IL-7 and PH20 may obviously improve the efficacy of CAR-T cells for solid tumors. Other clinical studies of co-expressing IL-7 and IL-15 CAR-T cells for lymphoma are also ongoing (NCT02652910, NCT04186520), aiming to test the hypothesis that co-expressing IL-7 and IL-15 CAR-T cells persist for longer after infusion in patients with lymphoma. And whether the persistence of CAR-T cells improves the anti-lymphoma efficacy.

Furthermore, IL-7 receptor (C7R) was also used for the construction of gene-edited CAR-T. A recent study confirmed the significant antitumor activity of co-expressing C7R CAR-T cells against neuroblastoma and glioblastoma (95). Two clinical trials (NCT03635632, NCT04099797) of CAR-T co-expressing C7R targeting GD2 in the treatment of neuroblastoma, osteosarcoma, and glioma are currently under way, the purpose of the studies was to find the maximum safe dose of GD2-C7R CAR-T cells and assess how long they can be detected in the blood and their effect on tumors.

IL-12

Because IL-12 can effectively mobilize the immune system, it has become one of the cytokines that mediate anti-tumor activity (96–98). A series of preclinical studies have demonstrated that IL-12 has antitumor activity by degrading tumors or prolonging survival in tumor-bearing animals (99). Giulia Agliardi et al. (100) conducted a preclinical study on the treatment of glioblastoma multiforme (GBM) by combining CAR-T cells with local injection of IL-12. The results showed that CAR-T therapy combined with local injection of IL-12 resulted in a more durable antitumor response than CAR-T therapy alone. The study also demonstrated that IL-12 not only enhanced the cytotoxicity of CAR-T cells, but also remodeled TME, promoted the infiltration of pro-inflammatory CD4+ T cells, and reduced the number of Tregs. However, systemic use of IL-12 can cause serious and unexpected side effects, which greatly limits its clinical use (101, 102). In the face of this challenge, the researchers have been trying to construct gene-edited IL-12

CAR-T cells in an effort to enhance anti-tumor activity while mitigating its side effects (103, 104). Ying Liu et al. (80) designed targeting GPC3 CAR-T cells and IL12-GPC3-CAR-T cells. This study demonstrated that IL12-GPC3-CAR-T cells were more capable of lysis of GPC3+ tumor cells and secreted more cytokines than GPC3-CAR-T cells. IL-12-GPC3- CAR-T cells showed a stronger antitumor effect in tumor-bearing mice due to increased infiltration and persistence of T cells by IL-12. Similarly, Gray Kueberuwa et al. (79) used CAR-T cells expressing murine IL-12 (IL12-CD19-CAR-T cells) to show eradication of B-cell lymphoma with a long-term survival rate. They also demonstrated that IL12-CD19-CAR-T cells not only kill CD19+ tumor cells directly, but also recruit host immune cells for an anticancer immune response. This finding may enable gene-edited IL-12 CAR-T cells to be used in the treatment of malignancy without the need for lymphatic clearance, so that these cells can be better used for anti-tumor immunity.

Fengtao You et al. (105) constructed CAR T cells targeting MUC1 co-expressing IL-12 (MUC1-IL-12-CAR T cells) and targeted CAR T cells modified with MUC1 (MUC1-CAR T cells) for use in seminal vesicle carcinoma in Phase I clinical trials (NCT02587689). MUC1-IL-12-CAR-T cells using MUC1 normal SCFV sequence SM3; MUC1-CAR T cells use the mutated SM3 scFv sequence. Two CAR T cells were injected locally into two separate metastatic lesions of the same seminal vesicle carcinoma patient. The results showed that MUC1-CAR T cells effectively induced tumor necrosis, while MUC1-IL-12 CAR T cells treated lesions showed no tumor necrosis. Of course, the purpose of this clinical study was to demonstrate the importance of SCFV in CAR T cell therapy. But it also demonstrated the safety of gene-edited IL-12 CAR T cells for clinical use. Two clinical trials (NCT03542799 and NCT02498912) are currently evaluating the safety and feasibility of co-expressing IL-12 CAR-T cells in patients with solid tumors, as well as evaluating the maximum tolerated dose.

IL-15

The tumor immune function of IL-15 is mainly to maintain CD8+ memory T cell homeostasis and inhibit activation-induced cell death (106). Therefore, gene-edited IL-15 CAR-T cells have been demonstrated to be superior in the treatment of malignant tumors. Evripidis Lanitis et al. (83) used retroviral vectors to encode co-expressed mouse interleuk-15 CAR-T cells (IL-15-CAR-T) targeting tumor blood vessels. Results showed that co-expression of IL-15 not only enhanced the tumor infiltration and control of tumor growth, but also enhanced the effect of IL-15 on tumor growth. Furthermore, TME was optimized (activation of NK cells and reduction of M2 macrophages). Further studies showed that the expression of Bcl-2 in CAR-T cells expressing IL-15 was up-regulated, while the expression of PD-1 was down-regulated. Analogously, Lenka V. Hurton et al. (82)designed co-expressing IL-15 CAR-T cells using gene-edited technology, which demonstrated a strong killing effect against CD19+ leukemia in preclinical experiments. The study analyzed the phenotype of proliferating T cells and found that the most persistent T cell phenotype was consistent with that of T memory stem cells. The results demonstrated that IL15 signaling could maintain T memory stem cells persistence. Which lays a theoretical foundation for the further application of IL-15 in optimizing CAR-T cells construction.

Gene-edited IL-15 has also shown enhanced antitumor activity of CAR T cells in clinical trials. Jia Feng et al. (107) modified CD5-targeted CAR-T cells by means of genetic engineering to secrete IL-15/IL-15 Sushi(IL-15 protein linked to the IL-15Rα sushi domain of the IL-15 receptor) Complex. In a phase I clinical trial (NCT04594135), these CAR-T cells were tested for safety and efficacy in a patient with refractory lymphoblastic lymphoma with central nervous system infiltration. In the trial, symptoms of central nervous system compression were significantly reduced after 3 weeks of treatment with IL-15-CD5-CAR-T cells, and soft tissue mass shadow was significantly reduced after 8 weeks of treatment. These results suggest that gene-engineered IL-15 CAR-T cells are an effective treatment for T cell malignancies, especially in patients with central nervous system involvement. At present, clinical trials (NCT03721068, NCT04377932) are under way to treat multiple solid tumors (liver cancer, sarcoma, fibroblastoma). The goal of these studies is to determine the maximum safe dose of CAR-T cells and how long they last in the body. To understand the side effects and evaluate its efficacy in solid tumors.

IL-18

Previous studies have shown that the structural expression of IL-18 by CAR-T cells significantly enhances the antitumor activity of CAR-T cells (84). Biliang Hu et al. (84) constructed CD19-IL-18 CAR-T cells using transgenic technology to conduct *in vivo* anti-tumor studies. CD19-IL-18 CAR-T cells significantly enhanced the proliferation of CAR-T cells. And effectively enhance the anti-tumor effect of melanoma mice. The study confirmed that the proliferation of IL-18-secreting CAR- T cells in the transplanted model was significantly enhanced, which was dependent on the IL-18R signaling pathways. This finding provides a strategy for the use of CAR-T cells in solid tumors. Since, Yong Huang et al. (14) also found that exogenous IL-18 could improve the anti-tumor function of HER2-specific CAR-T cells *in vitro* and *in vivo*, not only in immunodeficient mice, but also in immunotolerant mice. In addition, Markus Chmielewski et al. (108) found that the anti-tumor process of CAR-T cells co-expressing IL-18 was accompanied by the overall change of tumor immune microenvironment. Specifically, the number of M1 macrophages and NK cells increased, while the number of Tregs, inhibitory DC and M2 macrophages decreased, indicating that IL-18 has the function of recruiting peripheral immune cells to participate in anti-tumor combat. University of Pennsylvania team is currently conducting a clinical trial (NCT04684563) of co-expressing IL-18 CAR-T cells targeting CD-19 in the treatment of lymphoma. The primary objective of this study is to evaluate the maximum safe dose.

IL-21

IL-21 can enhance tumor immune response mediated by T cells. Li Du et al. (109) found that the addition of IL-21 in the

preparation of CAR-T cells could improve the T cell transfection efficiency by reducing the expression of IFN-γ in activated T cells. They also shown that exogenous IL-21 improved the cytotoxicity of CAR-T cells by enhancing the enrichment and amplification of poorly differentiated CAR-T cells. This finding lays a foundation for the application of IL-21 to optimize the structure of CAR-T cells. ŠTach, M et al. (86) constructed gene-edited IL-21 CAR-T cells targeting CD19, and studied the effect of IL-21 on its function. The results showed that IL-21 enhanced the expansion of CAR-T cells, and prevented the differentiation of CAR-T cells into late memory phenotype. Besides, gene-edited IL-21 promoted tumor infiltrating of CD19 CAR-T cells, leading to tumor growth retarded. Yi Wang et al. (85) constructed 4/21 ICR-CAR-T cells and reversed the efficacy of IL-4 against CAR-T cells in the environment of hepatocellular carcinoma(HCC) through the IL-21 pathway. The 4/21 ICR has been shown to activate the STAT3 pathway, thereby promoting Th17-like polarization of CAR-T cells *in vitro* and enhancing the toxicity of targeted HCC cells. IL-21 is the one that ultimately plays a direct role in promoting the anti-tumor function of CAR-T cells. A clinical trial of co-expressing IL-15 and IL-21 targeting GPC3 in multiple solid tumors (NCT04715191) is ongoing at Baylor College of Medicine. The objective of this study was to determine the maximum safe dose of CAR-T cells and to determine their survival time and side effects *in vivo*. At the same time, the efficacy was evaluated.

IL-23

Gene-edited IL-23 CAR-T cells have been relatively infrequently studied, but have yielded significant results. Dawei Wang et al. (89) designed co-expressing IL-23 targeting prostate specific membrane antigen(PSMA) CAR-T cells and studied their antitumor functions. This study confirmed that *in vitro* proliferation and cytokine secretion of co-expressing IL-23 CAR-T cells were significantly higher than that of conventional CAR-T cells. Co-expressing IL-23 CAR-T cells also showed higher tumor clearance and faster weight recovery *in vivo*. Furthermore, it has been demonstrated that T cells upregulate IL-23α p19 subunit but not p40 subunit under TCR stimulation. Therefore, some researchers constructed CAR-T cells co-expressing the p40 subunit, and found that T cells obtained selective proliferative activity through the IL-23 signaling pathway. Compared with conventional CAR-T cells, P40-CAR-T cells showed superior antitumor activity (88). The therapeutic efficacy of p40-CAR-T cells in xenotransplantation of tumor-bearing mice was superior to that of conventional CAR-T cells.

STRUCTURE DEVELOPMENT AND OPTIMIZATION OF GENE-EDITED INTERLEUKIN CAR-T CELLS

At present, the construction of gene-edited interleukin-CAR-T cell structure is diversified in the process of gradual optimization. The main construction methods for studying gene-edited interleukin-associated CAR-T include: co-expression of a single interleukin, two interleukin, interleukin combined with other cytokines, interleukin receptor, co-expression of interleukin subunit, and fusion ICR. The specific construction method is shown in **Figure 1**.

To enhance the tumor killing ability of CAR-T, researchers constructed CAR-T by gene-edited an interleukin that positively

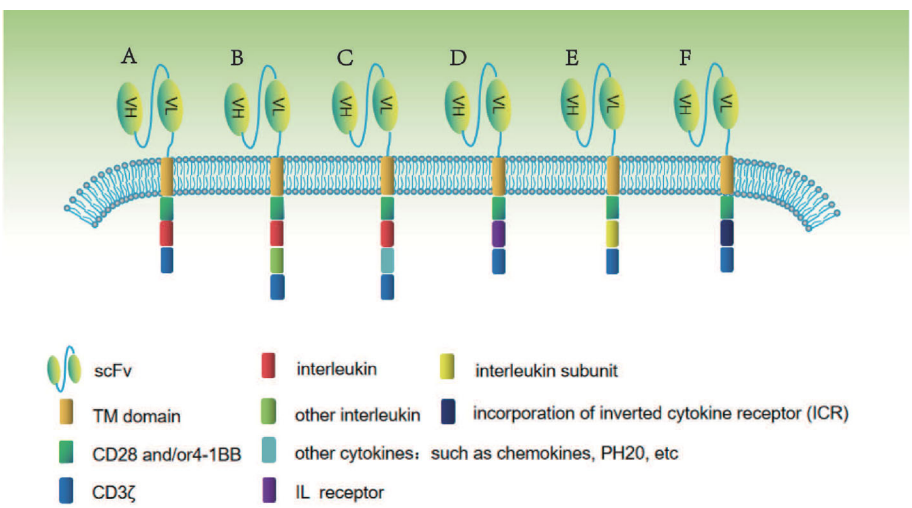

FIGURE 1 | In this figure, different methods of constructing gene-edited interleukin-CAR T cells are shown. **(A)** Co-expression of a single interleukin. **(B)** Co-expression of two interleukins. **(C)** Co-expression of interleukin combined with other cytokines. **(D)** Co-expression of interleukin receptor. **(E)** Co-expression of interleukin subunit. **(F)** Co-expression of fusion interleukin ICR.

regulates T cell function, initially primarily for hematological tumors, and later for solid tumors. There are many relevant preclinical and clinical studies, as shown in **Tables 2** and **3**. For example, in 2018, Gray Kueberuwa et al. (79) constructed targeting CD19 IL-12-CAR-T cells in a preclinical study on the treatment of lymphoma, and the CAR-T cells expressing IL-12 in the trial not only killed CD19+ tumor cells directly, but also recruited other immune cells of the host for anti-tumor immune response. In 2020, Cong He et al. (75) constructed a CAR-T targeting NKG2D co-expressing IL-7, and in the treatment of prostate cancer, it was found that IL-7 production enhanced the expansion of CAR-T cells and inhibited their apoptosis. Later, researchers attempted to construct bileukin and interleukin combined with other cytokine CAR-T to enhance its tumor killing function. Andreas A. Hombach et al. (110) constructed co-expressing IL-7 and IL12 CAR-T cells, and the constructional production of IL-7 and IL-12 has been shown to enhance the expansion and persistence of CAR-T cells in preclinical studies of colorectal cancer. In 2018, Keishi Adachi team (74) constructed co-expressing IL-7 and CCL19 CAR-T cells, and demonstrated excellent tumor-killing activity in multiple solid tumors. Interestingly, researchers constructed both the co-expressing of IL-7 (IL-17-CAR) and the co-expressing of CCL19 (CCL19-CAR) T cells, and found *in vivo* that these two types of CAR-T cells were comparable to conventional CAR-T cells in killing tumors. This study demonstrates the limited ability of gene-edited individual interleukin CAR-T cells to enhance anti-tumor function. Furthermore, this suggests the importance of cytokine collaboration in enhancing CAR-T function. In 2020, Xingcheng Xiong and his team (76) constructed co-expressing IL-7 and PH20 CAR-T cells. Because the co-expressing PH20 can effectively degrade extracellular matrix, and enhance the tumor infiltration function of CAR-T cells. The study has demonstrated that co-expressing IL-7 and PH20 CAR-T cells can significantly improve their antitumor activity in multiple solid tumors. Therefore, the construction of multiple interleukin and interleukin combined with other cytokines gene-edited CAR-T cells is an important direction to conquer solid tumors in the future.

Side reaction should be considered while CAR-T cells improve immune function, after all, interleukin hypersaturation activation as cytokines is harmful to the body. Researchers constructed CAR-T cells that co-expressing interleukin receptors and applied the limited interleukin ligand in the tumor microenvironment to brake their functional release. Zhenhui Zhao et al. (77) constructed co-expressing IL-7 receptor(C7R) CAR-T cells, which shown good tumor killing effect *in vitro* in the preclinical experiment of treating triple-negative breast cancer. However, *in vivo*, C7R-CAR-T cells have not demonstrated any advantage over conventional CAR-T cells. Which may be influenced by the density of IL-7 ligand in tumor tissues. Xingcong Ma et al. (88) Constructed co-expressing IL-23 subunit (p40) CAR-T cells (p40-CAR-T) that in order to avoid the body damage caused by overactivation of cytokines. The results showed that p40-CAR-T cells had stronger antitumor activity compared to conventional CAR-T cells, and more importantly, showed fewer side effects compared to CAR-T cells co-expressing other interleukin *in vivo* trials. This study tells us that on the way to improve CAR-T function, we should not blindly increase the secretion of cytokines, but should achieve accurate co-expression and reduce meaningless harmful expression.

In the face of tumor inhibition microenvironment, most of the current studies are aimed at improving tumor killing functions by increasing the secretion of cytokines that positively regulate CAR-T function. However, this structural design ignores the value of immunosuppressive cytokines in the tumor immune microenvironment. Ann M Leen et al. (111) constructed CAR-T cells co-expressing the fusion ICR, and IL-4/IL-7 ICR (4/7 ICR) contained the IL-4 receptor ectodomain and the IL7 receptor endodomain. The study demonstrated that 4/7 ICR can be used to protect CAR-T cells from IL-4 inhibition. The 4/7 ICR accepts immunosuppressive IL-4 but converts its downstream signals into immune-stimulating IL-7 receptors. In contact with IL-4, CAR-T cells can maintain Th1 phenotype a strong antitumor activity *in vivo*. Then, Somala Mohammed et al. (90) generated CAR-T cells targeting prostate stem cell antigen (PSCA) 4/7 ICR-CAR-T cells, which demonstrated that 4/7 ICR-CAR-T cells grew normally in an IL-4-rich microenvironment, thereby enhancing their antitumor activity. Subsequently, Yi Wang et al. (85) reported a novel IL-4/IL-21 ICR (4/21 ICR) that improved the tumor killing efficacy of CAR-T cells through a mechanism different from that of the 4/7 ICR. This study demonstrated that 4/21 ICR activates the STAT3 pathway in response to IL-4 stimulation, promoting Th17-like polarization and tumor-targeted cytotoxicity of CAR-T cells *in vitro*. In addition, 4/21 ICR-CAR-T cells also showed strong antitumor activity against IL-4 positive tumors *in vivo*. Therefore, gene-edited ICR CAR-T cells are a promising clinical practice for the treatment of solid tumors.

POTENTIAL TOXICITY OF GENE-EDITED INTERLEUKIN CAR-T CELLS

As mentioned above, gene-edited interleukin-CAR-T cell technology is optimized not only to enhance the function of CAR-T cells, but also to consider the cytotoxic effects of interleukin-over release. A phase 1 clinical trial of CD5-IL15/IL15 sushi CAR-T cells in refractory lymphoblastic lymphoma (NCT04594135) has been published (107). The patient was found to be well tolerated by infused CAR-T cells, causing only grade I CRS toxicity. Levels of ferritin and high-sensitivity C-reactive protein were briefly elevated. By detecting the cytokine level of patients in the first month, it was found that the expression of cytokines remained relatively stable. IL-15 levels also did not rise significantly after the infusion. CD5-IL15/IL15 sushi CAR-T cells secreted IL15/IL15 sushi complex in the body, which may lead to excessive IL-15 levels throughout the body. However, this was not observed in patients. This study demonstrates that gene-edited IL-15 CAR-T in the treatment of refractory lymphoblastic lymphoma causes mild CRS and is fully tolerated by the body. Besides, in the phase I clinical trial

(NCT02587689) of MUC1-IL-12-CAR T cells constructed by Fengtao You for the treatment of seminal vesicle carcinoma, patients only started to experience mild headache, fever, muscle pain, nasal congestion and abdominal distention discomfort. From 6 to 12 days after the intratumoral injection, all discomfort disappeared and the body temperature returned to normal. Transient CRS was detected after intratumor injection, with a 10-fold increase in IL-6 and an approximately 60% increase in TNF-α (105). This study also confirmed that the side effects produced by MUC1-IL-12-CAR-T cells can be tolerated by the body. More clinical trials are needed to test the potential cytotoxicity of gene-edited interleukin-CAR-T cells before they can be widely used in the clinic.

DISCUSSION

Adoptive immunotherapy based on CAR-T cells has proved to be a promising strategy for the treatment of hematological malignant tumor. However, this clinical success has not been fully realized in solid tumors largely because of the hostile TME of solid tumors. Tumor immunosuppressive microenvironments limit the proliferation and persistence of CAR-T cells, and often impair the anti-tumor efficacy of CAR-T cells. Immunoregulatory cytokines, which are critical components of T cell activation, proliferation (10). Interleukin plays different roles in tumor immunity. They regulate the activation, proliferation and apoptosis of T cells, but also recruit peripheral immune cells to participate in tumor immunity. In the absence of these factors, even if the selected target is very good, CAR-T cells will not produce a complete and lasting killing effect on the tumor. Therefore, the above cytokines are used as cytokines for gene modification of CAR structures, and preclinical studies have also demonstrated that modified CAR-T cells can further enhance the efficacy of CAR-T cells by secreting cytokines.

In addition, the present study demonstrated that partial interleukin not only improves the function of CAR-T cells, but also engages the host peripheral immune cells to participate in the anti-tumor battle (79, 83, 100). This finding is critical because the current clinical use of CAR-T cell technology requires that host lymphatic clearance protocols provide adequate space for CAR-T. The current preclinical trial demonstrates that gene-edited interleukin-CAR-T cells can eliminate this step (79). This leads to the possibility that, on the one hand, the clinical treatment of the patient alleviates the pain of chemotherapy, and on the other hand, the anticancer activity of these immune cells can be utilized by genetically modifying IL secreted by CAR-T.

The CAR-T immunotherapy of genetically modified cytokines also faces the problem of dose limiting toxicity. When cytokines are produced in large quantities and corresponding receptors are reduced in the tumor microenvironment, peripheral tolerance is increased. Studies have demonstrated that genetically modified T cells lead to overexpression of the IL-7 receptor, thereby enhancing the antitumor activity of genetically modified IL-7 CAR-T and reducing the dose limiting toxicity (112). This may be one of the reasons why there are many studies on CAR-T co-expression of IL-7 at present. In response to this challenge, researchers developed CAR-T cells that genetically edited the interleukin-cell receptor and interleukin-subunit, which can effectively limit the over release of cytokines and prevent the development of CRS. However, the treatment of malignancies with gene-edited single interleukin CAR-T cells may also present problems of immune tolerance or cytokine inactivation. Therefore, the researchers began to gene-edited multiple cytokines to construct CAR-T cells, enabling the cytokines to enhance the synergistic action of CAR-T cells to kill tumor cells.

Gene-edited ICR CAR-T cells were developed to further enhance their antitumor activity while overcoming tumor immunosuppressor factors. The 4/7 ICR and 4/21 ICR CAR-T cell technologies rely on inhibitory regulatory cytokines to activate positive regulatory cytokines to perform immune regulatory functions. It can effectively reverse the inhibitory cytokine signal to the positive regulatory signal. Thus, CAR-T can better adapt to the tumor immunosuppressive microenvironment. However, the activation of ICR is limited by the expression of inhibitory factors in tumor tissues, and it is difficult to activate ICR once tumor tissues do not express targeted inhibitory cytokines. If combined with gene-edited interleukin and ICR to construct CAR-T cells, it may achieve the purpose of reversing the inhibitory signal and enhancing the positive signal. This may be an effective strategy for gene-edited interleukin-CAR T cells to conquer solid tumors. At present, there are a few studies in this area, and more preclinical studies are needed to verify its efficacy.

Currently, studies related to gene-edited interleukin CAR-T have achieved some results, but there is still a long way to go before it can be fully used in clinical trials. First, cytokines such as interleukin not only act on CAR-T cells, but also act on other immune cells, such as recruiting peripheral immune cells to participate in tumor immunity. However, it is difficult to achieve in immunocompromised mice, as part of the current pre-clinical trials are *in vivo* studies using immunocompromised mice. Secondly, the clinical treatment of gene-edited interleukin CAR-T has the possibility of CRS, because overstimulation of interleukin release, when tumor tissue receptor density cannot be satisfied, will inevitably increase the load of peripheral circulation. All the above need to be verified by further clinical studies. At present, most of the relevant clinical trials are in recruitment, and some of them have not been started yet. It is hoped that the relevant clinical research will achieve gratifying results.

CONCLUSIONS

In summary, as immune regulatory factors, interleukin family members exert important functions in the activation and functional regulation of immune cells. In published preclinical and clinical studies, gene-edited interleukin CAR-T has been shown to enhance tumor killing in the treatment of malignancies with tolerable side effects. With the development of gene-edited technology and the development of researches on the interleukin family, gene-edited interleukin CAR-T technology in the treatment of malignant tumors will be able to achieve encouraging results.

AUTHOR CONTRIBUTIONS

All authors conceptualized and wrote the manuscript. LY conceived and modified the structure of this review. ZZ and ML additionally performed literature and data analysis. All authors contributed to the article and approved the submitted version.

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REVIEW
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Promising New Tools for Targeting p53 Mutant Cancers: Humoral and Cell-Based Immunotherapies

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Transcription factor and oncosuppressor protein p53 is considered as one of the most promising molecular targets that remains a high-hanging fruit in cancer therapy. *TP53* gene encoding the p53 protein is known to be the most frequently mutated gene in human cancers. The loss of transcriptional functions caused by mutations in p53 protein leads to deactivation of intrinsic tumor suppressive responses associated with wild-type (WT) p53 and acquisition of new pro-oncogenic properties such as enhanced cell proliferation, metastasis and chemoresistance. Hotspot mutations of p53 are often immunogenic and elicit intratumoral T cell responses to mutant p53 neoantigens, thus suggesting this protein as an attractive candidate for targeted anti-cancer immunotherapies. In this review we discuss the possible use of p53 antigens as molecular targets in immunotherapy, including the application of T cell receptor mimic (TCRm) monoclonal antibodies (mAbs) as a novel powerful approach.

Keywords: p53, mutation, neoantigen, T cell, T cell receptor, T cell receptor mimic antibody, immunotherapy, combined therapy

Abbreviations: ACT, adoptive cell therapy; ADC, antibody drug conjugate; ADCC, antibody-dependent cell-mediated cytotoxicity; APC, antigen-presenting cell; BiKE, bispecific killer cell engager antibody; BiTE, bispecific T cell engager; BsAb, bispecific antibody; CAR, chimeric antigen receptor; CRISPR, clustered regularly interspaced short palindromic repeats; DART, dual affinity retargeting antibody; DBD, DNA binding domain; DC, dendritic cell; ECM, extracellular matrix; ERAP1, endoplasmic reticulum aminopeptidase 1; HC, heavy chain; HLA, human leukocyte antigen Ig, immunoglobulin; IL-2, interleukin 2; mAb, monoclonal antibody MDM2, murine double minute 2; MAC, membrane attack antibody; MHC, major histocompatibility complex; NK, natural killer; NKG2D, natural killer group 2 member D; PBL, peripheral blood lymphocyte; PBMC, peripheral blood mononuclear cell; REP, rapid expansion phase; scFv, single-chain variable fragment; SV40, simian virus 40; TAA, tumor-associated antigen; TCR, T cell receptor; TCRm, T cell receptor mimic; TIL, tumor-infiltrating lymphocyte; TLR, toll-like receptor; TME, tumor microenvironment; TMG, tandem minigene; TriKE, trispecific killer cell engager antibody; TSA, tumor-specific antigen.

INTRODUCTION

The tumor suppressor p53 is a protein that performs its cellular functions through transcriptional regulation of genes involved in DNA repair, senescence and apoptosis. The p53 protein is widely known as the "guardian of the genome" that prevents the propagation of cells harboring genetic aberrations, e.g. mutations. *TP53* gene encoding p53 protein is arguably the most frequently altered gene in human cancer (1). The loss of wild-type (WT) p53 functions is the primary outcome of *TP53* mutations that deprives cells of intrinsic tumor suppressive responses, such as senescence and apoptosis. The intracellular p53 level is tightly regulated by its negative regulator murine double minute 2 (MDM2) ubiquitin ligase, primarily through ubiquitination followed by proteasomal degradation. In most human cancers p53 is deactivated either due to loss-of-function mutations or because of the overexpression of MDM2.

The p53 protein is known to trigger immune-related cellular mechanisms and evidence from studying the humoral immune responses in cancer patients testifies that both WT and mutant p53 neoepitopes are recognized by the immune system (2). Recent data revealed that p53 hotspot mutations are immunogenic and elicit intratumoral T cell responses to a range of neoantigens, thus suggesting this protein as an attractive target for anticancer immunotherapies (3).

Antibody-based therapy targets tumor-specific and tumor-associated antigens (TAAs) expressed on the cell surface. However, the majority of such TAAs are localized within the cell which makes them not amenable for such therapies. Intracellular proteins are proteolytically processed by the proteasome to yield 8 to 11 amino acid-long fragments in the cytosol. These peptides are bound in the groove of major histocompatibility complex (MHC) class I molecules, also called human leukocyte antigen (HLA), and presented on the cell surface as peptide/HLA complexes, which enables their recognition by T cell receptors (TCRs) of the T cells. However, the use of soluble TCR domains as therapeutic agents has been hindered by their inherent low affinity and instability as recombinant molecules (4, 5). To this end, T cell receptor mimic (TCRm) antibodies (Abs) recognizing epitopes similar to peptide/HLA complexes have been developed (6–8).

In this review, we discuss the role of p53 (both WT and mutant) in modulation of the immune response during tumor development and its recruitment as a target antigen in immunotherapy, including the novel promising approaches based on TCRm Abs.

RESPONSE OF p53 TO IMMUNE SIGNALING

The discovery of p53 in 1979 in association with simian virus 40 (SV40) large T antigen uncovered the crucial role of the protein in viral etiology and immunology of cancer. The joint efforts of the scientific community revealed p53 as the multifaceted molecular actor and resulted in an avalanche of published articles with over 12 000 entries in Pubmed (9).

The p53 protein is an essential component of the innate immune response mediating clearance of damaged cells and defense against external influence (10). The mechanisms of p53 activity involve regulation of the immune landscape by modulating inflammation, senescence and immunity in the surrounding tumor microenvironment (TME), including tumor stroma, extracellular matrix (ECM) and associated immune cells infiltrate (11).

Some immune-associated cellular mechanisms triggered by p53 become dysfunctional when the protein is mutated, and can result in enhanced neoangiogenesis and ECM remodeling, disruption of innate tumor immunity, genotoxic stress response of the toll-like receptor (TLR) pathway, formation of pro-tumor macrophage signature and altered cell-mediated immunity in cancer (12).

Dysfunction of p53 is also associated with the development of autoimmune diseases and often involves overexpression of the *Foxp3* gene in Treg cells (regulatory subpopulation of T cells). TCR signaling was reported to induce upregulation of p53 and downstream transcription activation of *Foxp3* which contributed to p53-mediated Treg cell induction in mice (13).

Cooperation of signals regulating with expression of p53 and induction of natural killer group 2 member D (NKG2D) ligand in tumor cells was associated with their predisposition for immune evasion (14). Additionally, p53 regulates the expression of NKG2D ligands ULBP1 and ULBP2, either positively as a transcriptional target or negatively through the upregulation of miR-34a (11). An important immune checkpoint molecule attenuating the immune response programmed cell death ligand 1 (PD-L1 or CD274) was also found to be regulated by p53. Specifically, p53 modulates the tumor immune response by regulating the expression of miR34, which directly binds to the 3′ untranslated region of the PD-L1 encoding gene (15).

The p53 was also shown to regulate toll-like receptor (TLR) innate immunity genes altering the immune system in response to the DNA stress in cancer cells (16). The human TLR family consists of ten members that regulate adaptor proteins, kinases and effector transcription factors that ultimately induce expression of pro-inflammatory mediators such as cytokines, chemokines and interferons. Targeting of TLR3 and TLR9 by p53 activates their expression and initiates apoptosis (17).

Additionally, p53 regulates endogenous antigen presentation through transcriptional control of aminopeptidase ERAP1 and peptide transporter TAP1. Antigen presentation by MHC class I and class II proteins plays a pivotal role in the adaptive branch of the immune system. Both MHC classes share the task of presenting neoantigen peptides on the cell surface for recognition by T cells. Prior to presentation, peptides are processed from cell's own endogenous proteins or from exogenous proteins uptaken into the endo-lysosomal system (**Figure 1**). MHCI-associated peptides are generated by proteasomal proteolysis and their translocation into the endoplasmic reticulum requires both TAP1 and TAP2. The p53-driven activation of TAP1 in response to DNA damage increases the pMHCI levels on tumor cells (18). Whereas ERAP1

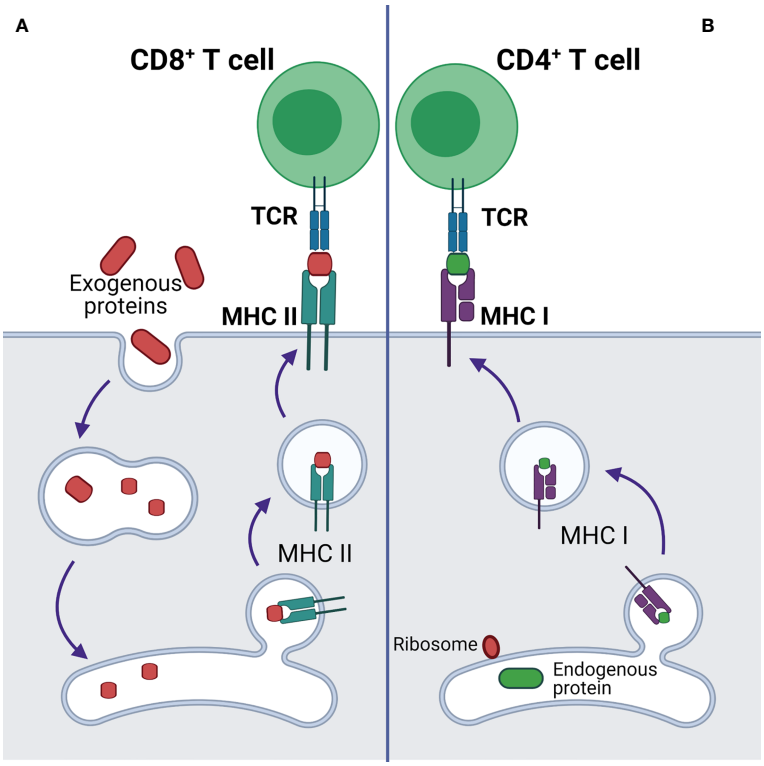

FIGURE 1 | Antigen presentation by MHCI and MHCII complexes. **(A)** Presentation of exogenous antigen to CD4+ T cell by MHCII after lysosomal protein processing. **(B)** Presentation of endogenous antigen (endogenous mutant protein or exogenous protein, e.g. viral protein) to CD8+ T cell by MHCI.

detaches oligopeptides from the proteasome to ensure their correct length (usually 8-10 amino acids) for MHCI loading (**Figure 2**) (19).

ADOPTIVE T CELL-BASED IMMUNOTHERAPY

Human cancer is often accompanied by genetic mutations, especially in *TP53*, with each patient carrying their own set of mutations resulting in neoantigen-specific T cell responses. This knowledge can be utilized to develop personalized therapies depending on the tumor genetic profile (20). One of the main treatment modalities within cancer immunotherapy is the adoptive cell therapy (ACT) approach based on autologous or allogeneic tumor-specific cytotoxic T cells. Within the paradigm of this therapeutic approach the cell product is infused into cancer patients with the goal of locating, recognizing and destroying tumor cells (21). Tumor-infiltrating lymphocytes (TILs) represent the oldest branch of ACT, the so-called "blind" approach that includes cultivation, expansion and subsequent transfusion of TILs without their prior selection. Initially TILs are isolated from homogenized tumor tissues or sentinel lymph nodes, then cultured with IL-2 in the presence of tumor lysate as an antigen source and gamma irradiated peripheral blood mononuclear cells (PBMCs) as feeder cells (22). Finally, following the rapid expansion phase (REP) TILs suspension could be infused back into the patient as an autologous cell therapy (23). Adoptive immunotherapy also involves the use of tumor vaccines made from autologous or allogeneic antigen-presenting cells (e.g. dendritic cells) containing private neoepitopes of tumor-associated antigens (24). One of the most prominent and promising examples of ACT is the chimeric antigen receptor (CAR) T cell immunotherapy for the treatment of hematologic B cell malignancies (25, 26).

Neoplastic tumor growth resulting from accumulation of genomic alterations is controlled by the immune system. The mutations often result in translation of abnormal proteins that may be further processed into new immunogenic T cell epitopes (i.e. neoantigens) and serve as potential targets for the T cell based therapies. Neoantigens are short peptides presented on the surface of tumor cells by the pMHC complex. Patient's own peripheral T cells or TILs may be used as a cell source for the antigen-specific expansion or could be transduced with the artificial TCR specific to the neoantigen of choice. HLA encoding genes are highly variable between individuals and were suggested to a primary role in determining the cancer susceptibility (27). Recent data suggested that the HLA affinity to neoantigen peptides may differ significantly depending on the mutation status unrelated to genotype variation and couldn't be

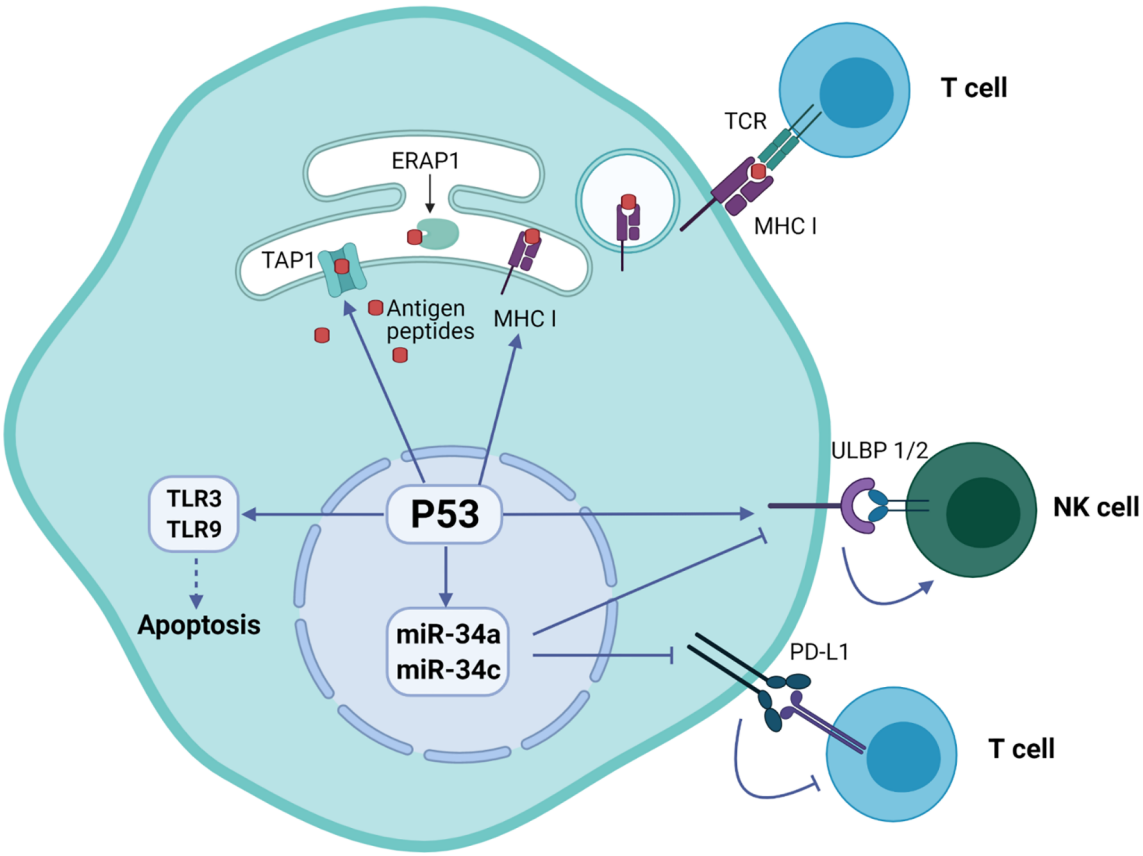

FIGURE 2 | Regulation of immune system functions by p53 protein in tumor cells. The p53 protein is involved in the presentation of endogenous peptides through regulation of TAP1 and ERAP1. In addition, p53 regulates the expression of NKG2D ligands ULBP1 and ULBP2, as well as inhibition of expression PD-L1 ligand through miR-34 microRNA precursor family.

directly correlated with the immunogenic properties of those neoantigens (28). The issue of neoantigen prediction, identification, and characterization based on genome sequencing data remains unresolved and requires significant efforts at technical and bioinformatic levels.

MUTANT p53 AS AN ANTIGEN IN CANCER IMMUNOTHERAPY

The *TP53* gene, encoding the p53 tumor suppressor protein, is the most commonly mutated gene in human cancer. Involvement of mutant p53 in malignant inflammation associated with immune dysfunction and the ability of adaptive immune system to respond to mutations in p53 makes this protein an appropriate target for cancer immunotherapy (29). *TP53* missense mutations in pancreatic ductal adenocarcinoma (PDAC) cells were found to increase the extent of fibrosis and reduce the infiltration of cytotoxic CD8+ T cells (30). The inhibition of mutant p53 functions may potentially sensitize PDAC tumors to anticancer treatments, including immunotherapy, therefore reduced infiltration of CD8+ T cells may augment the ability of PDAC tumors to evade the immune system.

Recent data suggest that mutant p53 peptides serve as suitable neoantigens for both CD4+ and CD8+ TCRs (3). The authors employed a high-throughput approach to generate a tandem minigene (TMG) library containing *TP53* mutations that was used to electroporate immature dendritic cells for subsequent co-culturing with TILs. This allowed identification of TILs populations reactive to the mutations frequently occurring at certain p53 hotspots (31). Peripheral blood lymphocytes (PBLs) were isolated from lung cancer patients with mutant p53 (R175H, Y220C, R248W) tumors by sorting antigen-experienced CD4+ and CD8+ T cells. The T cells were then stimulated with mutant p53 peptides *in vitro* to validate the recognition and specificity of the immune response. As a result, T cells with mutant p53-specific TCRs were confirmed to recognize naturally processed p53 neoepitopes *in vitro*. The same research group demonstrated specific T cell responses to *TP53* "hotspot" mutation neoantigens (Y220C, G245S) in patients with metastatic ovarian cancer (32).

Two molecular features often distinguish tumors with mutant p53: overexpression of this otherwise tightly regulated protein

and neo-epitope mutations (33, 34). Processed mutant p53 proteins get exposed on the surface of malignant cells as pMHC for immunosurveillance by T cells.

According to the recent data the hepatocellular carcinoma patients carrying *TP53* neoantigens were associated with better prognosis, higher CD8+ lymphocyte infiltration and enhanced immune cytolytic activity (35). Therefore *TP53* neoantigens may affect survival prognosis by regulating anti-tumor immunity and may be considered as promising targets for hepatocellular carcinoma immunotherapy.

The relationship between the tumor mutation burden (TMB), including *TP53* mutations, and clinical relevance was analyzed using the expression data of 546 head and neck squamous cell carcinoma (HNSCC) patients from the Cancer Genome Atlas database (36). The immune-related genes prognostic model was created indicating that high TMB was associated with worse prognosis in HNSCC patients. In addition, macrophages, CD8+ and CD4+ T cells appeared to be the most commonly infiltrated subtypes of immune cells in HNSCC.

The mutant p53-derived peptides have been employed as targets in various immunotherapy strategies some of which are currently in clinical trials (**Table 1**), including anti-cancer vaccines and soluble recombinant TCRs. For example, ALT-801, a biologic drug composed of interleukin-2 (IL-2) genetically fused to a soluble humanized TCR specific to a p53-derived antigen, is currently in phase II clinical trials in combination with gemcitabine (bladder cancer) and cisplatin (metastatic melanoma) (37, 38).

THERAPEUTIC MONOCLONAL ANTIBODIES

B and T cells are two classes of lymphocytes playing a key role in the adaptive immune response. Antibodies produced by B cells are usually specific to cell surface or soluble antigens and are unable to penetrate intracellular environment. TCRs recognize target neoantigens in the form of a peptide presented on MHCI or MHCII. The peptides presented on MHCI are normally proteolytic fragments of endogenously processed proteins originating from the cells displaying the pMHCI complex, whereas the peptides on pMHCII usually originate from extracellular proteins taken up and processed by the pMHC-displaying cell through a variety of mechanisms (**Figure 1**) (39).

The specificity and versatility of antibodies has positioned them as highly valuable tools for biological research and various medical applications, including diagnostics and therapy (40). Antibodies and TCRs have high affinities for their pMHC targets in nanomolar and micromolar ranges, respectively (41). Therapeutic monoclonal antibody-based therapy is more flexible and versatile than adoptive T cell-based immunotherapy, since antibodies do not need to be individually tailor-made for each patient and therefore are more accessible at a much lower cost. Antibody therapy also allows easier dosage control and adjusted treatment regimens depending on the patient's response. Multiple antibody-based drugs such as rituximab, bevacizumab, trastuzumab have proven exceptional utility for cancer therapy (42).

About 50% of all human cancers possess p53 mutations most of which are missense and localized in the DNA-binding domain (DBD) of the protein (1). Most of the mutant p53 proteins are unable to bind DNA and transactivate expression of downstream genes such as *MDM2* which in turn regulates the p53 levels through the autoregulatory loop, thereby resulting in increased levels of the mutant p53 protein in tumor cells (43). Elevated p53 levels can trigger an immune response and cause the production of antibodies (Abs) which appears to be an early event in some cancers (44).

Antibodies against p53 protein have been detected in approximately 17% cases of breast cancer in women (45). In total about 30% of individuals with various cancers were estimated to have detectable anti-p53 Abs (46). High levels of anti-p53 Abs have been detected in patients with premalignant and malignant lesions, and this parameter could be used as a

TABLE 1 | The list of clinical stage therapies targeting p53 mutant cancers.

Target (Diagnosis)	Therapy	National clinical trial number	Number of patients	Transduced cells/vector	Phase
p53-derived peptides in the context of HLA-A2 (Metastatic melanoma)	ALT-801 (IL-2 genetically fused to a humanized soluble TCR), Cisplatin	NCT01029873	25		II
p53-derived peptides in the context of HLA-A2 (Non-muscle invasive bladder cancer)	ALT-801, Gemcitabine	NCT01625260	52		II
(Metastatic Breast Cancer Malignant Melanoma)	DC vaccine	NCT00978913	31	DCs transfected with mRNA encoding Survivin, hTERT and p53	I
(Head and Neck Squamous Cell Carcinoma Lymphoma)	Recombinant human p53 adenovirus (Ad-p53) with anti-PD-1/anti-PD-L1	NCT03544723	40	Ad-p53	II
(Metastatic breast cancer with mutated p53)	Ad-p53-DC ᴨvaccine, 1-methyl-d-tryptophan	NCT01042535	44	Ad-p53 transduced DCs	II
(Lung Cancer)	Ad-p53-DC vaccine, Nivolumab, Ipilimumab	NCT03406715	14	Ad-p53 transduced DCs	II
(Kidney Cancer) (Melanoma)	Anti-p53 TCR PBLs, Ad-p53-DC vaccine, Aldesleukin	NCT00704938	3	Anti-p53 TCR- transduced PBLs Ad-p53 transduced DCs	II
(Melanoma with p53 overexpression)	Anti-p53 TCR	NCT00393029	12	Anti-p53 TCR- transduced PBLs	II
(Fallopian Tube Carcinoma) (Ovarian Carcinoma) (Peritoneal Carcinoma)	p53-MVA (modified vaccinia Ankara), Pembrolizumab	NCT03113487	28		II

biological marker for early cancer diagnostics (47). Additionally, detection of anti-p53 Abs in saliva has also been reported providing an easier and non-invasive prognostics approach (48).

The anti-p53 Abs usually recognize immunodominant epitopes at both termini of p53, although this is not where the missense mutations are normally located (49). Most of these Abs do not recognize the DBD region where missense mutations often occur and therefore are unable to specifically distinguish between WT and mutant forms of the protein.

BISPECIFIC ANTIBODIES

Bispecific antibodies (BsAbs) represent a class of monoclonal Abs capable of simultaneous binding two antigens. A subtype of BsAbs called bispecific T cell engagers (BiTEs) has been developed to simultaneously bind tumor-expressed antigens (e.g. BCMA, CD19) and CD3 on T cells (50). The BiTE-mediated interaction of tumor cell with cytotoxic T cell activates proliferation of the latter, thereby increasing the overall number of effector T cells and strengthening the lysis of malignant tumor cells. BiTEs were demonstrated to form such cytolytic synapse with CD8 T cells in a manner independent from MHCI expression on tumor cells (51).

The BiTE binding domains are represented by two single-chain variable fragment (scFv) regions of monoclonal antibodies joined by a flexible peptide linker. One scFv binding domain can be modified to target the surface antigen of interest, while the other domain is always specific to CD3 of TCR. Blinatumomab was the first BiTE approved by the US Food and Drug Administration to treat acute lymphoblastic leukemia (52).

Multiple varieties of the BiTE approach were also developed to diversify the landscape of targeted therapies. These include dual affinity retargeting antibodies (DARTs), as well as bi- and tri-specific killer cell engager antibodies (BiKEs and TriKEs) (51). DARTs use a diabody backbone with the addition of a C-terminal disulfide bridge for improved stabilization. When compared to their equivalent BiTEs CD19-specific DARTs yielded a stronger B cell lysis and T cell-activation (53). BiKEs utilize the innate immune system by harnessing natural killer (NK) cells *via* CD16. Similar to BiKEs, TriKEs consist of a bispecific antibody that recognizes CD16 on NK cells and CD33 on myeloid cancer cells, and in addition they also contain a modified human IL-15 crosslinker (54).

TCR MIMIC ANTIBODIES AS AN INNOVATIVE CLASS OF THERAPEUTICS

A novel class of antibodies binding pMHC often referred to as TCR mimic (TCRm) or TCR-like antibodies represent a highly promising therapeutic modality against cancers associated with mutant p53 (55). In contrast to therapeutic Abs that usually bind soluble or cell surface antigens, the TCRm Abs provide a complementary strategy by effectively targeting the pMHC complexes that present the processed target neoantigen peptides. In recent years multiple TCRm Abs have been developed to target various tumor antigen epitopes in the context of MHC (56, 57). In addition, TCRm Abs have also been explored as candidates for delivery of antibody drug conjugates (ADCs) since pMHC-TCRm Ab complexes can be effectively internalized (58).

TCR MIMIC ANTIBODIES IN CANCER IMMUNOTHERAPY

The cell surface abundance of pMHC complexes for efficient presentation of neoantigens is often a topic of debate (8, 59–61). In general, mAbs are widely used to treat a wide range of diseases, whereas TCRm Abs have not yet been approved for the therapeutic use. This might be a consequence of low-throughput generation of new candidates and their insufficient initial quality that requires laborious downstream refinement.

The development and production of high-affinity, antigen-specific TCRm Abs is highly complex and requires substantial efforts for setting up the manufacturing processes. Provided rather limited number of dominant HLA alleles within a particular ethnic group targeting the p53 (mutant or WT) associated pMHC ligandome leads to an assumption that this therapeutic approach could be implemented as a finite set of the «off-the-shelf» products.

One of the key starting points is selection of the correct antigens (immunogens) that is exposed on the cell surface as pMHCI. Therefore, histocompatible cells expressing such antigens can be used both as immunogens in hybridoma technologies (murine, rat, rabbit) and as a source of antigens for screening the antibody producers.

The APCs can be programmed for expression of pMHC using vector-based approaches (62, 63) or modern CRISPR-based genome-editing techniques (64, 65). Off- target toxicity issues may be resolved by testing in humanized animal models or using cell reprogramming tools to generate different types of tissues for using them as antigen-bearing surrogates or organoids (66). Other options include commercial specificity screening platforms such as developed by Retrogenix Ltd (United Kingdom) for receptor identification, target deconvolution and off-target profiling (67).

Approaches based on TCRm Abs can be broadly grouped into two major categories depending on the antibody isotype: 1) strategies utilizing classical, soluble antibodies, e.g. for delivering a cytotoxic payload or Fc-mediated recruitment of effector cells or other functional molecules; 2) strategies utilizing redirection of cytotoxic cells (e.g. T or NK cells) or their cooperation with APCs (**Figure 3**). The first category TCRm Abs upon binding to pMHCI initiate assembly of the membrane attack complex (MAC), antibody-dependent cell-mediated cytotoxicity (ADCC) or even trigger the apoptosis. The second category TCRm Abs can be engineered to additionally express CARs that combine intracellular TCR signaling domains and extracellular Fv regions of the antibodies to confer target specificity. CARs are formed by single-chain variable fragments

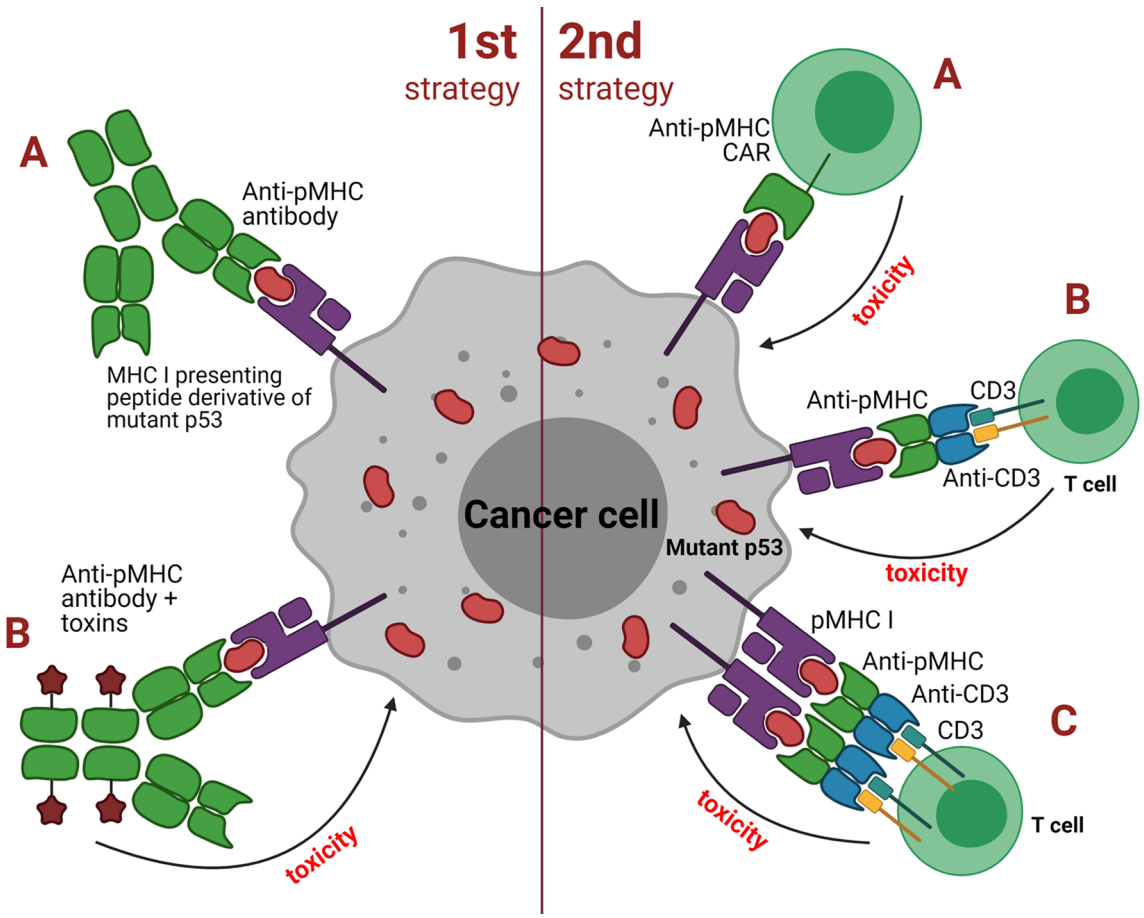

FIGURE 3 | Two strategies employed by TCR mimic antibodies against cancer cells with mutant p53. First strategy: **(A)** classical soluble antibodies for binding to pMHC to induce direct apoptosis or targeted destruction of the tumor cell; **(B)** antibody drug conjugates (ADCs) such as effector molecules, cytokines, toxins or radioactive substances that are coupled to the antibody and upon binding to pMHC result in tumor cell death. Second strategy: **(A)** anti-pMHC CAR to redirect T cells to recognize and lyse tumor cells *via* the scFv fragment derived from a TCR mimic antibody; **(B)** bispecific molecules that bridge cytotoxic T or NK cells with pMHC of the antigen-presenting tumor cell using of the scFv fragment of a TCR mimic antibody; **(C)** similar to B but employs dimeric bispecific T cell-engaging tandem scFv antibodies.

(scFv) capable of redirecting T cells to specifically recognize target antigens and lyse cancer cells. CARs do not directly compete with native TCRs, instead they provide supportive co-stimulation of the cytotoxic signaling cascades. The combination of CAR-T cell therapy with TCR-like antibodies might significantly increase the overall therapeutic potential of this approach.

Alternatively, cytotoxic T cells can be recruited indirectly *via* heterodimeric molecules such as bispecific T cell engagers (BiTEs) that have specificity for pMHC of the target cells and CD3 of T or NK cells. Recent studies reported encouraging data on using this type of immunotherapy against p53-mutant tumors. TCRm Abs specific to pMHC presenting WT and mutant p53 antigens have demonstrated encouraging anti-tumor effects both *in vitro* and *in vivo* in animal models (55, 68).

An interesting example of the BiTE approach is based on bispecific TCRm Ab that recognizes cancer cells expressing the p53(R175H) neoantigen (61). One domain of this antibody recruits TCR and the other binds the pMHC presenting the mutant p53 antigen. In mouse models of multiple myeloma, the BiTEs effectively stimulated T cells to destroy cancer cells bearing mutant p53 without affecting the normal cells with WT p53. Even when the p53 target was presented on the surface of the tumor cells at "extremely low" levels the BiTEs were still able to activate specific T cell-mediated antitumor response. Thus, the employment of TCRm Abs could be potentially useful to target cancers with somatic p53 mutations in addition to other approaches (69).

TCRm Abs were also reported to be designed as bispecific antibodies in single-chain diabody format that demonstrated substantial specificity towards cancer cells expressing neoantigens of the mutant Ras protein (G12V and Q61H/L/R) in mouse models (70). The authors suggested that many TCRm Abs grafted into an optimized BiTE format might be capable of

specifically recognizing and destroying cancer cells bearing low levels of the cognate antigens. This could be a highly attractive approach even compared to CAR-T cell therapy that typically requires up to a few thousands of antigen molecules on a single tumor cell for efficient recognition and cytolysis. Worth noting that as opposed to the conceptually preceding TCR approach the TCRm Ab affinity may reach picomolar levels when developed using animal hybridoma technology.

In addition to the above mentioned, CAR-T cell therapy requires a complex and time consuming manufacturing process which significantly limits its broad availability, whereas TCRm Abs if approved are expected to be much more affordable. Another complication of CAR-T cell therapy is the requirement for lymphodepletion prior the infusion (71). As opposed to CAR-T cell therapy, TCRm Ab was not developed to be a personalized treatment. Instead, TCRm Ab therapies link endogenous T cells to tumor-expressed antigens and activate the cytotoxic potential of a patient's own T cells to eliminate cancer. Also, compared to cell-based immunotherapies antibodies appear much more widely applicable owing to the simplicity of application, reproducibility of results and scalability for mass production. Finally, TCRm Abs can be designed to target both tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) which fit well with the character of p53 expression in the majority of tumors.

In many cases p53 mutations were associated with significant overexpression of immune checkpoint proteins, such as PD-1, which suggests these types of tumors might be amenable for anti–PD-1/PD-L1 immunotherapy in addition to others approaches (72).

CONCLUSION

The p53 protein is an important part of the innate immune and anti-tumor responses. Mutations of p53 often result in loss of its transcriptional activity and therefore inability to regulate anti-tumor and immunomodulatory responses. The peptide neoantigens from a proteolytically processed mutant p53 protein are presented by APCs to B and T cells to activate the immune response. Novel cell-based and humoral immunotherapies will offer previously unavailable levels of medical precision in targeting specific types of tumors. Adoptive T cell-based immunotherapies such as TILs, CAR-T or TCR-T cells may be applied for the treatment of a wide range of tumors. Genome-wide screenings will assist the identification of multiple mutant p53 neoantigens amenable for therapeutic targeting. However, it is important to keep in mind that transgenic TCRs require careful testing for potentially toxic cross-reactivity and might need additional modifications to prevent mispairing with cognate TCRs.

Expanding the target repertoire of therapeutic antibodies to a broad variety of pMHC complexes will offer opportunities for the development of new anticancer strategies and improved treatments. TCR-mimic antibodies can transform the fine cellular specificity of the T cell recognition machinery into a flexible immunotherapeutic approach that fits well in the growing field of personalized medicine. The vast plethora of potential targets represented by a range of mutant p53 neoantigens within the context of the pMHC complexes suggests that TCR-mimic antibodies will find an important place as highly promising immunotherapeutics.

AUTHOR CONTRIBUTIONS

VC, MZ, and EB conceived the idea and coordinated the writing. MZ contributed to section about TCR mimic antibodies. RM and RK contributed to section about antibodies. AV and EZ contributed to section about adoptive cell therapy. EZ and IG prepared the figures and table. VC, AR, and EB contributed to introduction and conclusion. All authors contributed to the article and approved the submitted version.

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REVIEW
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Application of Immunotherapy in Hepatocellular Carcinoma

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Hepatocellular carcinoma is one of the most common malignancies globally. It not only has a hidden onset but also progresses rapidly. Most HCC patients are already in the advanced stage of cancer when they are diagnosed, and have even lost the opportunity for surgical treatment. As an inflammation-related tumor, the immunosuppressive microenvironment of HCC can promote immune tolerance through a variety of mechanisms. Immunotherapy can activate tumor-specific immune responses, which brings a new hope for the treatment of HCC. At the present time, main immunotherapy strategies of HCC include immune checkpoint inhibitors, tumor vaccines, adoptive cell therapy, and so on. This article reviews the application and research progress of immune checkpoint inhibitors, tumor vaccines, and adoptive cell therapy in the treatment of HCC.

Keywords: hepatocellular carcinoma, immunotherapy, immune checkpoint inhibitors, tumor vaccines, adoptive cell therapy

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INTRODUCTION

Primary liver cancer is one of the common malignant tumors, and its main pathological type is hepatocellular carcinoma (HCC). According to the 2018 cancer statistics of the World Health Organization, the incidence of liver cancer ranks 6th and the mortality rate ranks 4th among the most common cancers in the world (stomach cancer ranks third with a slight advantage) (1). The latest cancer statistics in 2020 show that the incidence of liver cancer still ranks sixth among the most common cancers in the world, but its mortality rate has risen from the fourth to the third (significantly exceeding the mortality rate of stomach cancer) (2) The traditional treatment mainly includes surgery, radiotherapy, chemotherapy, radiofrequency ablation (RFA), intervention, and targeted therapy. Multidisciplinary comprehensive treatment is an effective treatment strategy for prolonging the survival time of patients with HCC. However, the current 5-year survival rate of HCC patients after surgery is only about 36.9% (3), and the 5-year recurrence rate is as high as 70% (4). This is closely related to its tumor microenvironment. HCC is a typical inflammatory-related tumor. Its microenvironment contains a large number of macrophages, innate immune cells, and adaptive immune cells, forming a complex immune tolerance microenvironment (5, 6). Besides, the liver itself is a special immune-tolerant organ that can effectively escape the immune response (7). In recent years, immunotherapy has gradually become an important treatment for HCC. Tumor immunotherapy can enhance the immune response of the body, stimulate tumor-specific immunity, reactivate immune cells, and finally achieve the purpose of anti-tumor. Common tumor immunotherapy includes immune checkpoint inhibitors, tumor vaccines, and adoptive cell therapy. This paper reviews the application and research progress of immune checkpoint

inhibitors, tumor vaccines, and adoptive cell therapy in the treatment of HCC. It aims to provide some references for clinicians in the treatment of HCC.

IMMUNOSUPPRESSIVE MECHANISMS AND IMMUNE ESCAPE IN HCC

The liver has a complex immune microenvironment. The liver is continuously exposed to various antigens passing through the portal vein, especially those from intestinal tract. Therefore, the liver microenvironment continues to show immune tolerance, which is to inhibit inappropriate inflammatory reaction and prevent autoimmune liver injury (8, 9). The specific immune system of HCC and tumor cells constitute a special immune tolerance microenvironment, which can protect tumor cells from the attack of their own immune system and promote the immune escape of tumor cells (10). The immunosuppressive mechanism of HCC is not completely clear at present, which may be related to the following mechanisms:

a. The occurrence and progression of HCC are usually accompanied by chronic inflammation (e.g. viral hepatitis B and C) and chronic disease (e.g. liver cirrhosis). Under the action of long-term inflammation, many inhibitory cytokines (e.g. IL-10, IL-35 and TGF-β) are constantly produced, and a large number of immunosuppressive cells, such as regulatory T cells (Tregs), M2 macrophages, and myeloid-derived suppressor cells (MDSCs), are recruited into the liver (11). Furthermore, some immunosuppressive cells of the liver itself are activated or normal cells are transformed into immunosuppressive cells (10). These inhibitory cytokines and immunosuppressive cells together form the immunosuppressive microenvironment of HCC (11).
b. Immunosuppressive cells in tumor tissue can promote HCC tolerance (12, 13). Tumor-associated monocytes, for example, can significantly increase the glycolysis level in the area around the tumor. Activation of glycolysis induced these cells to express PD-L1 (through NF-κB signaling pathway) and decreased the function of cytotoxic T lymphocyte (13).
c. Tumor-associated macrophages (TAMs), as one of the key components constituting the immunosuppressive microenvironment of HCC, not only cannot eliminate tumor cells, instead will promote tumor growth (14, 15).
d. HCC cells can release some cytokines, such as 14-3-3ζ, which can destroy the activation, proliferation and anti-tumor function of tumor-infiltrating T lymphocytes (TILs) (16). Beyond that, overexpression of 14-3-3ζ can also differentiate naive T cells from effector T cells to Tregs (16).
e. The expression of immune checkpoints in HCC tissues is increased (5, 17, 18). The combination of immune checkpoints and their respective ligands will inhibit the activation and proliferation of T cells.
f. Activation or alteration of some genes and signaling pathways may promote immune escape in HCC (19). For example, activation of β-Catenin (20) or mutation of *CTNNB1* (21) may promote immune escape in HCC.
g. Epithelial-to-mesenchymal-transition (EMT) can induce the up-regulation of PD-L1, PD-L2, CD73 and B7-H3; and reversing EMT can inhibit the expression of these markers (22).

In response to these mechanisms, some corresponding measures can be taken to block, inhibit or reverse these mechanisms. For instance, measures can be taken to neutralize inhibitory cytokines or prevent their production. Yang et al. (23) found that reducing the level of IL-35 could reduce the metastasis of HCC and improve overall survival (OS) of HCC patients. HCC patients with high expression of PD-1/PD-L1 can be treated with corresponding immune checkpoint inhibitors (ICIs). Combination therapy based on PD-1/PD-L1 inhibitors can promote the response of antigen-specific CD8+ T cells in HCC (24). For TAMs, some special methods (for example, modulatory miRNA methods and immune checkpoint blockade) can be used to repolarize TAMs to the anti-HCC phenotypes (25). For the moment, these coping strategies mostly stay in theoretical and preclinical studies. With the rapid development of tumor immunotherapy, it has gradually become one of the important methods for the treatment of HCC in recent years. These immunotherapy mainly include ICIs, tumor vaccines and adoptive cell therapy, especially ICIs are used more frequently.pt?>

POSSIBLE RESISTANCE MECHANISMS RELATED TO THE IMMUNOTHERAPY OF HCC

In recent years, unprecedented progress has been made in tumor immunotherapy. Drugs, therapies and strategies related to tumor immunotherapy are also emerging one after another. Nevertheless, the low response rate and the consequent resistance problem have greatly limited the efficacy of immunotherapy (26). These mechanisms of immunotherapy resistance can be divided into the intrinsic mechanisms and the extrinsic mechanisms. The intrinsic mechanisms include (27, 28): a. The activation of MAPK signaling pathway leads to the production of VEGF and IL-8 (inhibiting the recruitment and function of effector T cells) (29); b. Loss of PTEN expression leads to enhancement of PI3K signaling pathway, which is negatively correlated with gene expression of IFNγ and CD8+ T cell infiltration (30, 31); c. The continuous activation of WNT/β-catenin signaling pathway hinders the homing of T cells (32); d. Up-regulation of PD-L1 expression on the surface of tumor cells inhibits the anti-tumor effect of effector T cells; e. Decreased antigen presentation ability (33); f. Decreased T cell function. The extrinsic mechanisms include (27, 28): a. The inhibition of immunosuppressive cells in tumor microenvironment; b. There are other immune checkpoints on the surface of T cells, which will inhibit the function of T cells; c. The influence of gut microbiome (34).

THE IMMUNOTHERAPY OF HCC

ICIs (Immune Checkpoint Inhibitors)

ICI is one of the most rapidly developing immunotherapy strategies nowadays. Immune checkpoints are membrane-

bound molecules that can be expressed not only on the surface of many tumor cells but also on the surface of numerous immune cells (35). These immune checkpoints prevent and inhibit the activation of these cells through a physiological break (27). Immune checkpoints and costimulatory molecules are located on the surface of T cells, but their functions are opposite. Costimulatory molecules can provide activation signals (**Figure 1A**), while immune checkpoints provide inhibitory signals. When immune checkpoints bind to the ligands on the surface of tumor cells, the inhibitory signals transmitted from tumor cells can inhibit the activation and proliferation of T cells. The anti-tumor mechanism of ICIs is that they can block immune checkpoints or their ligands, thereby blocking the transmission of inhibitory signals to T cells (**Figure 1B**). The activation and proliferation of T cells with high expression of programmed cell death 1 (PD-1) are decreased; tumor cells with high expression of programmed cell death 1 ligand 1 (PD-L1)/ programmed cell death 1 ligand 2 (PD-L2) are more likely to escape (36). Researchers analyzed 956 HCC samples and found that about 25% of the samples expressed high levels of PD-1 and PD-L1 (20). Common immune checkpoints include PD-1/PD-L1, cytotoxic T-lymphocyte antigen-4 (CTLA-4), T cell immunoglobulin-3 (TIM3), and Lymphocyte Activation Gene-3 (LAG3). For now, PD-1/PD-L1 and CTLA-4 are the most widely used.

PD-1/PD-L1 Inhibitors

PD-1 is a type I transmembrane glycoprotein expressed on the surface of most immune cells. These immune cells mainly include T cells, NK cells, regulatory cell (Treg), and myeloid-derived suppressor cell (MDSC) (37, 38). The main function of PD-1 is to negatively regulate the immune response. PD-L1 is the ligand of PD-1, which is mainly located on the surface of tumor cells. When PD-1 on the surface of T cells is combined with PD-L1 on the surface of tumor cells, tumor cells will transmit immunosuppressive signals to T cells. These inhibitory signals will inhibit the function of T cells and lead to T cell failure. Currently, the common PD-1 inhibitors include Nivolumab and Pembrolizumab, which are all-human IgG4 monoclonal antibodies. Common PD-L1 inhibitors include Durvalumab and Atezolizumab, which are IgG1 monoclonal antibodies.

In 2017, the United States Food and Drug Administration (FDA) approved Nivolumab for the treatment of HCC (39). In one clinical trial (40), 262 patients with advanced HCC received Nivolumab dose escalation and dose extension therapy. These patients included HCC patients who had previously received sorafenib or had not received sorafenib. For newly-treated patients who did not receive sorafenib, the objective response rate (ORR) after receiving nivolumab monotherapy was 20%~23%, and the media survival time was as long as 28.6 months; after receiving nivolumab monotherapy in patients who had received

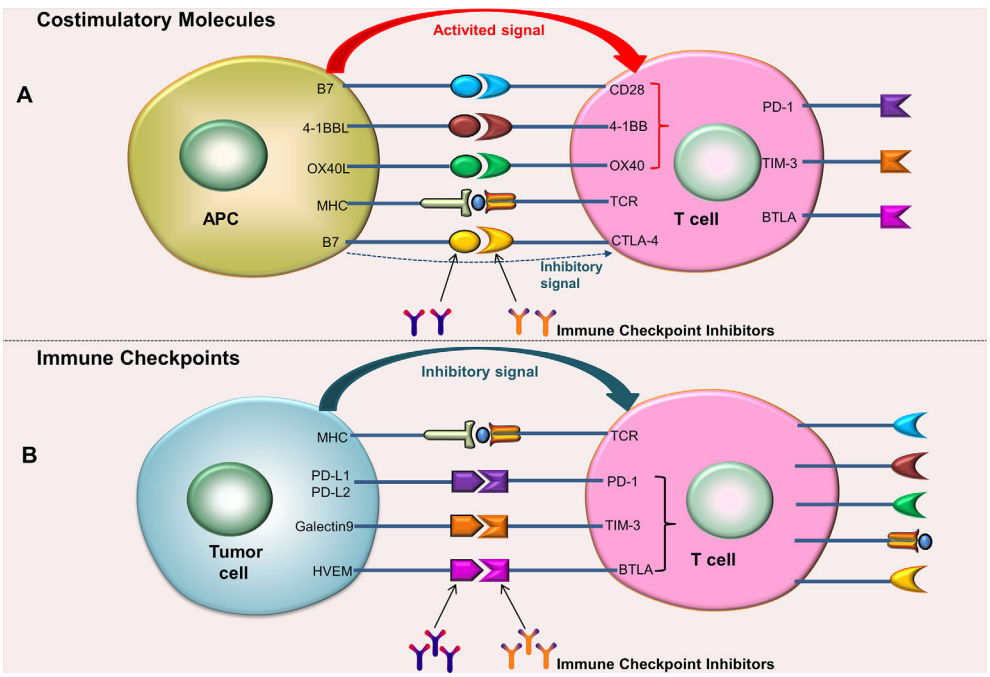

FIGURE 1 | Costimulatory molecules and Immune checkpoints. **(A)** After the costimulatory molecules on the surface of T cells bind to their ligands (located on the surface of the APC), activation signals can be generated, which can promote the activation and proliferation of T cells; the combination of CTLA-4 and B7 produces an inhibitory signals. **(B)** After the immune checkpoints on the surface of T cells bind to their ligands (located on the surface of tumor cells), inhibitory signals can be generated, which can inhibit T cell activation and proliferation. ICIs can block immune checkpoints or their ligands, thereby blocking the transmission of inhibitory signals to T cells.

sorafenib, the ORR was 16%~19%, and the median survival time could reach 15.6 months (40). Another clinical trial showed that compared with sorafenib, patients receiving nivolumab did not achieve the expected overall survival (OS), but the OS rate, objective response rate (ORR), and complete remission (CR) rate were significantly improved (41).

Pembrolizumab is a humanized IgG4/kappa monoclonal antibody against IgG4/K, which can directly inhibit the binding of PD-1 to PD-L1. A phase II clinical trial in 2018 showed that 104 patients with HCC who were intolerant or still progressing after receiving sorafenib treatment were treated with Pembrolizumab (42). The results showed that the objective remission rate was 17%, 1% of the patients achieved complete remission, 44% of the patients were in stable condition, and 33% of the patients had disease progression.

For HCC patients, the high expression of PD-L1 is associated with lower tumor differentiation, a higher level of AFP, more frequent vascular invasion, and worse prognosis (43). PD-L1 inhibitors include Durvalumab and Atezolizumab. Atezolizumab has been used in the treatment of non-small cell lung cancer and triple-negative breast cancer. Presently, these inhibitors are in the evaluation stage of advanced HCC clinical trials.

CTLA-4 Inhibitors

CTLA-4 is a type of protein receptor located on the surface of T cells. CTLA-4 and CD28 receptors have two ligands in common: CD 80 and CD 86. Compared with CD28, CTLA-4 has a higher affinity with both ligands. CTLA-4-CD80 has the highest affinity, while CD28-CD86 has the lowest affinity (44, 45). CTLA-4 can compete with CD 28 for ligand binding, leading to a decrease in the co-stimulatory effect of CD 28 on T cells, and ultimately inhibiting T cell function (46). HCC is sensitive to CTLA-4 inhibitors (47). CTLA-4 inhibitors that have been approved by the FDA include Ipilimumab and Tremelimumab, etc. A phase II clinical trial showed that 20 patients with diagnosed advanced HCC were treated with Tremelimumab, ORR was 17%, disease remission rate (DCR) was 76.4%, and median progress free survival (PFS) was 6.48 months (48).

Combination Therapy of PD-1/PD-L1 Inhibitors and CTLA-4 Inhibitors

PD-1/PD-L1 and CTLA-4 pathways are different in negatively regulating immune activity, but their complementary effects are the same (47, 49). Blocking PD-1 or CTLA-4 can promote cell activation and proliferation, and alleviate immunosuppression mediated by Treg cells (50). Some pre-clinical studies of solid tumors showed that, compared with monotherapy, the combination of PD-1 inhibitor and CTLA-4 inhibitor could produce synergistic effects and enhance their anti-tumor activity (51). The Results of The CheckMate 040 Randomized Clinical Trial in 2020 showed that the ORR and DCR of Nivolumab combined with Ipilimumab in the treatment of advanced HCC were 32% and 27%, respectively (47). This study proved that the combination of two immunosuppressants might have a better therapeutic effect and was also safe for HCC patients. Furthermore, increasing the dose of Ipilimumab might improve the persistent response and prolonged the survival time of patients with advanced HCC. The NCT02519348 study (52) also showed that the combination of tremelimumab and durvalumab was more effective than single drug [ORR: 22.7% *VS* (7.2% and 9.6%)] and had an encouraging safety. ICIs can also be combined with other treatment strategies, these strategies include locoregional treatments, antiangiogenetic therapy, chemotherapy, the mammalian target of rapamycin inhibitor, etc. (53).

Immune checkpoint inhibitors represented by anti-PD-1/PD-L1 and anti-CTLA-4 antibodies have shown good results in the clinical treatment of HCC, providing a new treatment method for HCC patients (**Table 1**). Nevertheless, the safety, efficacy, and prognosis of the combination of 2 ICIs still require extensive clinical studies to verify.

ICIs Combined With Other Therapies for HCC

Although ICIs have achieved certain clinical efficacy, it is necessary to adopt some combination strategies to further improve its efficacy due to the limited response rate of monotherapy. These strategies include combined molecular

TABLE 1 | Partial research results of ICIs for HCC.

Medicine	Time	Case	Test Phase	OS (month)	Median PFS (month)	ORR (%)	DCR (%)	Trial Registration
Anti-PD-1								
Nivolumab	2017	214	I/II	15.1	4	20	64	NCT01658878 (40)
Nivolumab	2021	49	I/II	NO	NO	12	55	NCT01658878 (54)
Pembrolizumab	2018	104	II	12.9	4.9	17	62	NCT02702414 (42)
(Pembrolizumab/placebo)	2020	278/135	III	13.9/10.6	3.0/2.8	18.3/4.4	62.2/53.3	NCT02702401 (55)
Camrelizumab	2020	217	II	13.8	2.1	14.7	44.2	NCT02989922 (56)
Anti-PD-L1								
Durvalumab	2019	39	I/II	13.2	2.7	10.3	33	NCT01693562 (57)
Anti-CTLA-4								
Tremelimumab	2013	20	II	8.2	6.5	17.6	76.4	NCT01008358 (48)
Anti-PD-1/PD-L1 + Anti-CTLA-4								
(Nivolumab + Ipilimumab)	2020	148	I/II	NO	NO	32	27	NCT01658878 (47)
(Durvalumab + Tremelimumab)	2020	332	III	NO	NO	22.7	NO	NCT02519348 (52)
(Ipilimumab + Nivolumab/pembrolizumab)	2021	25	I	NO	NO	16	40	N.F. (58)

OS, overall survival; PFS, progression-free survival; ORR, objective remission rate; DCR, disease control rate.
N.F., related information not found.

targeted drugs, combined chemotherapy, combined radiotherapy, combined TACE and combined ablation.

ICIs Combined With Molecular Targeted Drugs

Molecular targeted therapy is to block or inhibit the key genes or signal pathways in the process of tumor occurrence and development at the molecular level, and finally achieve the purpose of anti-tumor. Sorafenib, a multiple tyrosine kinase inhibitor (TKI), is the first molecular targeted drug approved for the treatment of advanced HCC. TKI mainly achieves the purpose of anti-tumor by inhibiting the tyrosine kinases of several growth factor receptors. It has been proven that sorafenib can only prolong the survival time of HCC patients by several months. Consequently, it is necessary to develop a combined therapy to further improve the clinical efficacy.

Lavatinib, a TKI, has become the first-line treatment for advanced HCC. Finn et al. (59) combined Pembrolizumab (PD-1 inhibitor) with lenvatinib (a multikinase inhibitor) to treat unresectable HCC (uHCC). Test results were evaluated with modified Response Evaluation Criteria In Solid Tumors (mRECIST). The results showed that ORR was 46%, DCR was 88%, median PFS was 9.3 months, median DOR was 8.6 months, and median OS was 22 months. In addition, 67% of patients had treatment-related adverse events (≥Grade 3). The experiment conclusion: Lenvatinib + Pembrolizumab had a good anti-tumor activity against uHCC, and its safety was acceptable. The study by Llovet et al. (60) also showed that Lenvatinib + Pembrolizumab had an encouraging effect for uHCC patients.

In another phase III clinical trial of ICIs combined with targeted drugs to treat uHCC, Finn et al. (61) enrolled a total of 501 uHCC patients. The experimental group (336) was treated with Atezolizumab (PD-L1 inhibitor) + Bevacizumab (anti-angiogenesis); the control group (165) was treated with Sorafenib monotherapy. The experimental results (mRECIST) showed that OS and PFS of the experimental group were significantly better than those of the control group, and the incidence of adverse events between the two groups had no significant difference (98.2% *VS* 98.7%). Currently, Atezolizumab+ Bevacizumab has become the first-line treatment for patients with advanced HCC (62). ICIs combined with molecularly targeted drugs may have a synergistic effect (63, 64) and have promising prospects in the treatment of advanced HCC. These synergistic effects include (65): a. Targeted drugs themselves have anti-tumor effects; b. Targeted drugs can improve DC (dendritic cell) activation and immune cell infiltration; c. Targeted drugs can block the PD-1/PD-L1 pathway; d. Combination therapy can affect Wnt/β-catenin activated mutations.

ICIs Combined With Chemotherapy

Although ICIs are effective in the treatment of many immunogenic tumors, for those cold tumors, ICIs are ineffective in most cases (66). Chemotherapy drugs can inhibit or kill tumor cells, and the destroyed tumor cells can release tumor-related antigens, which can stimulate the body to produce an immune response. Besides, chemotherapy can also consume immunosuppressive cells (such as MDSCs and Tregs) to reduce or relieve the immunosuppressive effect of tumor microenvironment (TME) (67). In the past, chemotherapy was considered to have only immunosuppressive effects, but recently, some new viewpoints suggest that chemotherapy may also have immunostimulatory effects (66) and participate in the active regulation of the immune system (which can transform cold tumors into hot tumors) (68). In a phase II clinical trial evaluating Camrelizumab+FOLFOX4 in the treatment of advanced HCC (69), the researchers included 34 patients with advanced HCC. The experimental results (RECIST) showed that ORR was 26.5%, DCR was 79.4%, and median PFS was 5.5 months. Meanwhile, this combination therapy is tolerable for patients with advanced HCC. ICIs + chemotherapy may provide a promising option for the treatment of patients with advanced HCC.

ICIs Combined With Radiotherapy (RT)

As one of the most important cancer treatment methods, the basic principle of RT is to use high-energy particles to induce DNA damage in tumor cells, which eventually leads to tumor cell death. In recent years, it has been found that RT can not only kill tumor cells directly, but also induce immune-related anti-tumor responses (70). The mechanism mainly includes: a. RT can induce tumor cell death to release large amounts of tumor-associated antigens. These antigens can stimulate the body to produce an immune response; b. RT can up-regulate the expression of major histocompatibility complex class I (MHC-I) molecules, allowing CD8+ T cells to recognize and kill tumor cells (71); c. RT can increase the number of tumor-infiltrating lymphocytes (TILs) in tumor tissue (70); d. RT can improve the immunogenicity of tumor cells, and at the same time, it can also cause immunosuppression. The study of Chew et al. (72) showed that RT could increase the expression of PD-1 and Tim-3 on the surface of CD8+ T cells. Apart from that, RT can also increase the expression of PD-L1 on the surface of tumor cells (73, 74). The role of ICIs is to block these immune checkpoints. Accordingly, the combination of ICIs and RT can produce synergistic effects (70, 75). One preclinical study by Kim et al. (74) showed that compared with anti-PD-L1 therapy or RT alone, the combination of the two methods can significantly improve the anti-tumor ability and the survival rate. Chiang et al. (76) treated 5 uHCC patients with stereotactic body radiotherapy (SBRT) + Nivolumab. The experimental results showed that ORR could reach 100%, 2 patients got complete remission (CR), 3 patients got part remission (PR), and mPFS reached 14.9 months. Additionally, only 1 patient had ≥3 Grade adverse reactions. For now, there are few clinical trial data about ICIs combined with RT in the treatment of HCC. The best combination therapy for HCC still needs to be explored.

ICIs Combined With Transarterial Chemoembolization (TACE)

TACE was first proposed and applied in clinic in 1977. TACE belongs to palliative treatment, and in most cases it cannot achieve radical cure. Its mechanism of action is to deliver chemotherapeutic drugs to the hepatic artery to embolize the artery, causing ischemic necrosis of tumor tissue; in the meanwhile, chemotherapeutic drugs also play an anti-tumor

effect. Due to the rich blood supply of the liver, the portal vein will still supply blood to the tumor tissue after the artery is embolized. As a result, patients with HCC who undergo TACE tend to have a high rate of postoperative recurrence (77). In a phase I clinical trial of ICIs combined with TACE in the treatment of uHCC (78), a total of 9 uHCC patients received Nivolumab + drug eluting bead transarterial chemoembolization (deb-TACE). The results of the study showed that DCR reached 100% (PR was 22%, SD was 78%), and 12-month OS rate was 71%; as an aside, this combination therapy was safe and tolerable. Another phase I clinical trial evaluating Pembromizumab combined with TACE in the treatment of advanced HCC also showed that Pembrolizumab+TACE had tolerable safety with no synergistic toxicity (OS, PFS, ORR and DCR had not been released yet) (79). Up to now, although there are few clinical experimental data of ICIs+TACE in the treatment of HCC, its future development prospect is promising.

ICIs Combined With Ablation

Tumor ablation is one of the main interventional treatments for HCC. It mainly includes radiofrequency ablation (RFA), microwave ablation (MWA) and cryoablation. Both RFA and WMA deliver large amounts of energy to the tumor site, leading to local heating and destroying tumor cells through thermal efficiency. Cryoablation is to freeze tumor tissue at local low temperature, which induces delayed necrosis of tumor cells after injury.

Ablation can also mediate immune regulation (80). The mechanism may include: a. Tumor-associated antigens released after death of tumor cells can activate adaptive immune cells (81); b. Following RFA or MWA, large quantities of (Heat shock proteins-70) HSP-70 are released in serum, which may lead to local inflammation and activation of antigen-presenting cells (APCs) in tumor area, thus inducing anti-tumor response (82). c. RFA increases the infiltration of dendritic cells (DCs) in tumor tissues and significantly enhances the response of CD4+ T cells and CD8+ T cells (83). d. After receiving RFA locally, the number of central memory lymphocytes increased remarkably (84). e. After receiving RFA locally, the expression of inhibitory cytokines decreased and the level of anti-tumor cytokines increased (81). At the present time, there are few studies about the effect of MWA and cryoablation on tumor immunity of HCC patients. The study of Leuchte et al. (85) showed that MWA can enhance the tumor-specific immune response of HCC patients. In a I/II clinical trial, a total of 32 patients with advanced HCC received Tremelimumab + (RFA/chemoablation) (86). The experimental results showed that 5 (26.3%) of the 19 evaluable patients achieved PR; median time to tumor progression (TIP) was 7.4 months; median OS was 12.3 months; 6-month tumor PFS and 12-month tumor PFS were 57.1% and 33.1%, respectively; and no dose limiting toxicity occurred in this trial. As of now, there are few clinical experimental data about ICIs combined with ablation in the treatment of advanced HCC, and a large number of clinical experimental data are still needed to explore the best combination scheme. ICIs and ablation have different anti-tumor mechanisms, which may produce synergistic effects in the combined treatment of tumors. In conclusion, ICIs combined with other therapies is an effective and potential treatment for HCC (**Table 2**).

Tumor Vaccines

The principle of tumor vaccines is to introduce tumor antigens into patients in various forms, so as to overcome the immunosuppression caused by tumor, enhance the immunogenicity of tumor cells, activate the immune system of patients, and eventually achieve the purpose of anti-tumor. For the moment, tumor vaccines used for HCC treatment and research mainly include nucleic acid vaccines, peptide vaccines, oncolytic virus vaccines, and DC vaccines.

Nucleic Acid Vaccines

Nucleic acid vaccine refers to the recombination of a gene (DNA or RNA) encoding a certain tumor antigen with a vector, and then injecting it into the patient. After these nucleic acids enter the host cells, the host cells can express the corresponding polypeptides or proteins, thus inducing the body to produce an immune response against these antigens (**Figure 2A**). DNA vaccines are easy to manufacture, low cost, and stable. Unfortunately, DNA cannot be amplified in transfected cells

TABLE 2 | Partial research results of ICIs combined with other therapies to treat HCC .

Medicine	Time	Case	Test Phase	OS (month)	Median PFS (month)	ORR (%)	DCR (%)	Trial Registration
Anti-PD-L1+ molecular targeted drugs								
(Durvalumab + Ramucirumab)	2020	28	Ia/b	18	4.4	11	61	NCT02572687 (87)
(Atezolizumab + Bevacizumab/Sorafenib)	2020	336/165	III	NO	6.8/4.3	27.3/11.9	73.6/55.3	NCT03434379 (61)
(Avelumab + axitinib)	2019	22	Ib	NO	3.8	31.8	NO	NCT03289533 (88)
(Lenvatinib + pembrolizumab)	2020	104	I	22	9.3	46	88	NCT03006926 (59)
(Lenvatinib + pembrolizumab)	2019	67	I	NO	NO	44.8	82.1	NCT03006926 (60)
Anti-PD-1+ molecular targeted drugs								
(Sintilimab+ IBI305, high-dose/low-dose)	2020	21/29	Ib	NO	NO	33.3/24.1	83.3/N.F.	NCT04072679 (89)
Anti-PD-1+ chemotherapy								
(Camrelizumab+ FOLFOX4)	2019	34	II	NO	5.5	26.5	79.4	NCT03092895 (69)
(Lenvatinib+ FOLFOX4)	2020	24	I	NO	NO	66.7	79.2	N.F. (90)
Anti-PD-1+ RT(Nivolumab+RT)	2019	5	I	NO	14.9	100	100	N.F. (76)
Anti-PD-1+ TACE(Nivolumab+ deb-TACE)	2020	9	I	NO	NO	100	78	NCT03143270 (78)

IBI305, a biosimilar candidate of bevacizumab; N.F., related information not found; FOLFOX4, infusional fluorouracil, leucovorin and oxaliplatin; TACE, transarterial chemoembolization; RT, radiotherapy.

like viral vectors. It needs to enter the nucleus to be translated into the corresponding proteins. Moreover, it is not easily taken up or expressed by DC cells, therefore, it cannot induce the body to produce an effective immune response (91). Once the DNA is integrated into the genome, there may be such a risk, that is, causing the activation of oncogenes or the inactivation of tumor-suppressor genes. In contrast, RNA vaccines are safer than DNA vaccines. RNA can participate in the synthesis of protein only by entering cytoplasm, but cannot be integrated into the genome. The potential carcinogenicity is weaker. Nevertheless, the poor stability and short half-life of RNA vaccine limit its clinical application to a certain extent. Up until now there are few clinical trials of nucleic acid vaccines for HCC, and most of them are still in preclinical research stage.

Peptide Vaccines

Tumor antigen peptides are important components of peptide vaccines. Tumor antigens must be degraded into short peptides and form peptide-MHC-TCR complex in antigen-presenting cells (APCs) to be recognized by T cells and stimulate the corresponding cytotoxic T lymphocyte (CTL) response. The purpose of peptide vaccines is to deliver a high-dose tumor antigen peptides to major histocompatibility complex (MHC) molecules on the surface of APCs, so as to stimulate the specific immune response of the body to tumor cells (**Figure 2B**).

There seem AFP peptide vaccine and GPC3 peptide vaccine which are studied more frequently nowadays. Alpha-fetoprotein (AFP) is one of the most common serum markers in the diagnosis of HCC, and its high expression in hepatocellular carcinoma cells makes it a promising target for vaccine-based therapy (92). AFP peptide vaccine showed good anti-tumor activity in the treatment of HCC (93, 94). One clinical research of glypican-3 (GPC3) peptide vaccine in the treatment of HCC patients revealed that GPC3 peptide vaccine had good tolerance and anti-tumor effect, as well as could prolong the overall survival time of patients (95). Although plenty of tumor antigens have been found in liver cancer, only the vaccines targeting AFP, GPC3 and MRP3 show good tolerance and safety, and the specific T cell response rate of these vaccines exceeds 70% (96).

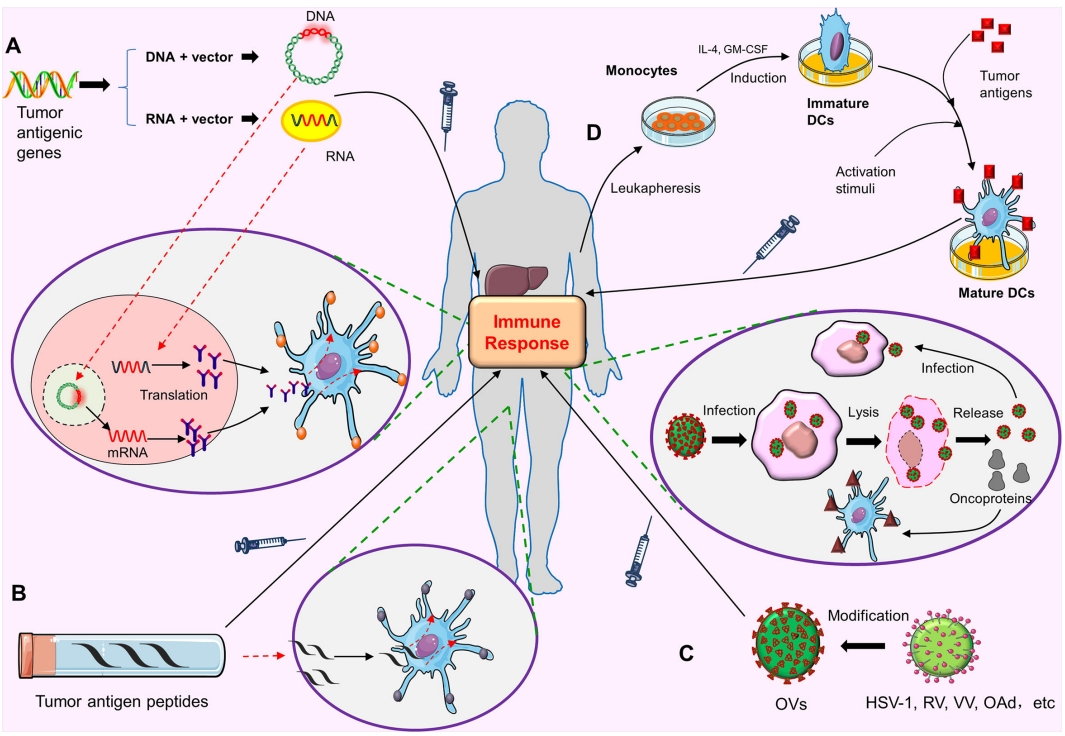

FIGURE 2 | The preparation process and anti-tumor mechanism of tumor vaccines. **(A)** Nucleic acid vaccines. The gene (DNA or RNA) encoding a tumor antigen is recombined with the vector and injected into the patient. DNA needs to enter the nucleus of the host cells, while RNA only needs to enter the cytoplasm of the host cells to be translated into the corresponding proteins. These proteins are secreted out of cell, captured by APCs, and finally activate the human immune response to this tumor antigen. **(B)** Peptide vaccines. High-dose tumor antigen peptides are delivered to the MHC molecules on APC surface, thus stimulating the specific immune response of the body to this tumor antigen. **(C)** Oncolytic viruses(Ovs). OVs can be modified from HSV-1, RV, VV and OAd. OVs can infect tumor cells after entering the body, and proliferate in large amounts in tumor cells, eventually leading to tumor cell lysis and death. Dead tumor cells release OVs virus and tumor proteins. The released OVs virus can continue to infect other tumor cells. Tumor proteins can be captured by APCs and eventually activate the host immune response. **(D)** DC vaccines. Monocytes are extracted from the blood of patients. These monocytes are induced to become Immature DCs under the stimulation of IL-4 and GM-CSF. Then under the action of activation stimuli, the tumor antigens are loaded on the Mature DCs. Finally, these DCs loaded with tumor antigens are injected into the body to cause the body to produce the immune response to tumor cells.

Oncolytic Viruses(OVs)

Oncolytic viruses are a type of viruses that can effectively infect and kill cancer cells. When the virus-infected cancer cells rupture and die, the newly generated virus particles will be released to further infect the surrounding cancer cells. Ovs can not only directly kill tumor cells but also stimulate the immune response of the body and enhance the anti-tumor effect (**Figure 2C**). Most Ovs are modified from herpes simplex virus-1 (HSV-1), reovirus (RV), Vaccinia virus (VV) and oncolytic adenovirus (OAd). OVs can be designed or screened to selectively amplify and kill cancer cells in cancer cells. They can play a role in the primary tumors, as well as in the metastatic tumors (97). A general design principle is to weaken or delete viral virulence factors, and prevent OVs from replicating in normal cells by using tumor-specific distortion of signal pathway in cancer cells, but they can still maintain replication and killing activity in cancer cells (97). JX-59 (pexastimogene devacirepvec, Pexa-Vec) is one of the most commonly used oncolytic viruses (modified from VV) in HCC-related clinical trials. It can be replicated preferentially in cancer cells. The results of one clinical trial showed that it had good safety and could improve the overall survival rate of patients with unresectable HCC (98). Phase I and II clinical trials of advanced HCC related to JX-594 are ongoing (for example, NCT 01636284 and NCT 03071094) at the present time.

DC Vaccines

Dendritic cells (DCs) are the most powerful antigen presenting cells (APCs) so far. They are named because they mature with many dendritic or pseudopod-like protrusions. DCs have the function of immune response and immune tolerance, which is of great significance for maintaining immune balance. There exit many inhibitory cytokines in the tumor microenvironment. These inhibitory cytokines can inhibit the normal function of DCs and promote the escape of tumor cells (99). DC vaccine is the focus of tumor immunotherapy in recent years. The principle is to load tumor antigens on DCs, and then inject these DCs loaded with tumor antigens into the body; these DC cells can promote the proliferation of cytotoxic T lymphocytes (CTL) *in vivo*, and ultimately play the role of anti-tumor (**Figure 2D**). In terms of basic research and clinical application, DC vaccines have shown the application prospects in tumor prevention and tumor treatment (100, 101).

In the phase I and II clinical studies of the DC vaccines in the treatment of patients with advanced HCC, the results showed that the DC vaccines had the tumor-specific immune responses in patients with advanced HCC (102, 103). In a phase II clinical trial, 39 patients with advanced HCC received DC vaccine treatment, of which 25 patients were evaluable with a disease control rate (DCR) of 28% (102). Moreover, all the subjects had no grade 3-4 adverse reactions. The results suggest that the DC vaccine was effective and safe in patients with HCC. Another study (104) showed that patients with HCC were treated with transcatheter arterial embolization (TAE) or TAE combined with DC vaccine, and the results showed that the combined treatment group could more effectively enhance tumor-specific immune response. However, there was no difference in tumor recurrence rates between the two groups, which might be related to the immunosuppressive microenvironment of HCC and the lack of specific HCC target antigens in DC vaccines. This may be related to the immunosuppressive microenvironment of HCC and the lack of specific HCC target antigens in current DC vaccines. About 10% of tumor antigens have immunogenicity, and only a few are tumor rejection antigens, which can trigger immune responses and kill tumors. If these tumor rejection antigens can be fully utilized when constructing DC vaccines, it may further enhance the anti-tumor effect of DC vaccines.

It is also very important for DC vaccine to choose the appropriate route of administration. Presently, the main routes of administration include intravenous, subcutaneous and intradermal routes. Optimizing the route of administration is conducive to more effective vaccination. The intradermal route of DC vaccination shows that the migration level of DCs to the lymph nodes is very low; the ultrasound-guided intra-lymph node vaccination of DC vaccines has the risk of injecting the vaccine into fat rather than a cellular area (105). DCs may not be able to reach the tumor site effectively. Therefore, DCs need to be further improved to increase the ability to migrate to the tumor; or change the way of receiving DC vaccines to increase the number of DCs in tumors. For example, *in situ* DC vaccination, that is, direct intratumoral inoculation of unloaded DCs produced *in vitro*. The unloaded DCs can uptake a variety of TAAs in the tumor, so there is no need to generate TAA vectors *in vivo* and select specific targets (106). Furthermore, the combination of *in situ* DC vaccine and immunogenic cell death (ICD) inducer can further improve the antitumor effect of DCs. Because ICD inducers can not only cause tumor cell death and release TAAs but also increase the secretion of damage-associated molecular patterns (DAMPs) that can activate DCs (107–109).

DC vaccine is a safe and promising anti-tumor therapy (110). Although DC vaccine as an independent therapeutic agent may have limitations, combined with other treatments can improve the effectiveness of treatment. Zhou et al. (111) found that compared with advanced HCC patients who only received sorafenib, dendritic cells and cytokine-induced killers (DC-CIK) combined with sorafenib could increase the tumor response rate and prolong OS of patients without increasing the incidence of adverse events.

Adoptive Cell Therapy (ACT)

ACT is an immunotherapy based on the use of autoimmune cells of cancer patients. The main process is to isolate the immunocompetent cells in the tumor patient, modify these immune cells or stimulate them with some cytokines. These immune cells are amplified and screened *in vitro*, and then they are returned to the patient. ATC achieves the purpose of anti-tumor by enhancing the immune function of patients or targeting to kill tumor cells. Commonly used ACT includes natural killer (NK) cells, cytokine-induced killer (CIK) cells and chimeric antigen receptor (CAR)-T cells, etc.

NK Cells

NK cells are one of the most important immune cells in human innate immunity, which can produce non-specific immune responses without being sensitized by antigens. The activation

state of NK cells is determined by the dynamic balance of the expression of inhibitory receptors and activated receptors on the surface of NK cells. NK cells account for 30% ~ 50% of innate immune cells in the liver (112), which are responsible for presenting cytotoxic granules, secreting effector cytokines, and cooperating with apoptotic receptors to induce apoptosis of target cells.

NK cells in cancer patients are often in a state of functional failure due to the immunosuppressive effect of the tumor microenvironment (TME). If the failure state of these NK cells can be reversed, it is possible to restore their anti-tumor effect. One animal study (113) showed that Sirtuin2 (SIRT2) could reactivate the anti-tumor activity of depleted NK cells in hepatoma mice. SIRT2 could significantly promote the production of cytokines and cytotoxic mediators by activated NK cells. Similarly, NK cells overexpressing SIRT2 showed a stronger antitumor effect on hepatoma cells. Consequently, it is possible to improve the prognosis of HCC patients by adding NK cells with anti-tumor activity to reshape the immune system of the liver.

NK cells come from a wide range of sources, have a broad-spectrum oncolytic effect, and are not restricted by MHC. According to the source of NK cells, NK cell adoptive immunotherapy can be divided into autologous NK cell immunotherapy and allogeneic NK cell immunotherapy. Autologous NK adoptive immune cells are obtained by stimulating the *in vitro* expansion of $CD56^{Birght}$ NK cells in peripheral monocytes with cytokines. NK cells in peripheral blood can proliferate 140 times in a short time by stimulating with some cytokines (114). Meanwhile, these amplified and activated NK cells show strong anti-tumor effects *in vitro* and *in vivo* (114). Nonetheless, the clinical efficacy of autologous NK cells is not significant (115). The high expression of inhibitory killer cell immunoglobulin-like receptor (KIR) on the surface of tumor cells can inhibit NK cell functions after binding to NK cells, which makes tumor cells prone to immune escape. Allogeneic NK cells that do not match the KIR on the surface of tumor cells have better clinical efficacy (116). One clinical study showed that the use of allogeneic NK cells could improve the immune function of HCC patients in a short period of time (117). How to further improve the accuracy and persistence of NK cells in the future is still the focus of research.

Cytokine-Induced Killer (CIK) Cells

CIK cells refers to the heterogeneous cell population mainly consisting of CD3+CD8+ and CD3+CD56+, which are formed by co-culturing peripheral blood mononuclear cells with cytokines (such as IL-1, IL-2 and IFN-γ). Its mechanism of action is to directly kill tumor cells by releasing perforin and granzyme, or indirectly by releasing a variety of cytokines. Similarly, it can also induce tumor cell apoptosis by activating apoptotic genes. CIK cells mainly include NK-like T lymphocytes (NKT cells) and cytotoxic T lymphocytes. Among them, NKT cells are the main effector cells that exert anti-tumor effects.

A retrospective study showed that CIK could significantly improve OS of HCC patients (118). The results of another phase III clinical trial showed that CIK cell therapy could prolong the progression-free survival (PFS) of HCC patients to 44 months (119). The 5-year follow-up results showed that compared with the control group, PFS and OS of HCC patients (receiving CIK treatment group) were significantly prolonged (120). On the contrary, some researchers believed that CIK therapy might not significantly improve OS of HCC patients (121). Additionally, the researchers found that CIK cells could increase the infiltration of immunosuppressive cells in tumors, thus inhibiting their anti-tumor activity (122). Although the long-term curative effect of CIK cell therapy for HCC patients still needs numerous clinical studies to prove, CIK cell therapy is still a promising immunotherapy for HCC patients.

T Cell Receptor (TCR)-T Cell Therapy

TCR is a specific receptor on the surface of T cells. It activates T cells by recognizing and binding antigens presented by MHC, and ultimately promotes the differentiation and proliferation of T cells. The principle of TCR-T cell therapy is to introduce TCR genes that specifically recognize tumor antigens into patients' T cells by gene editing technology, so that these T cells can express corresponding TCR on their surfaces. Then these TCR-T cells are screened and amplified *in vitro*, and finally injected into patients. TCR-T cell therapy can effectively identify and kill tumor cells by enhancing the specific recognition ability of T cells to tumor cells and improving the affinity of T lymphocytes to tumor cells. Compared with CAR-T cells, TCR-T cells can not only recognize antigens on the surface of tumor cells but also intracellular antigens. Currently, some phase I and II clinical trials exploring TCR-T cell treatment of HCC (for example, NCT01967823, NCT03132792, NCT02719782, etc.) are in progress.

(Chimeric Antigen Receptor) CAR-T Cell Therapy

In recent years, CAR-T cell therapy has become a research hotspot in adoptive cell therapy. CAR is mainly composed of four parts, including, single chain variable fragment (scFv); hinge region; transmembrane region (TM); intracellular signal domain (immunoreceptor tyrosine-based activation motif, ITAM). The principle is to construct CAR genes that recognize tumor antigens *in vitro* and combine them with vectors to form recombinant plasmids. Then transfecting these plasmids into patient T cells, which makes these T cells express the corresponding CARs. These CAR-T cells are screened and expanded *in vitro*, and finally returned to the patient. They can target to kill the corresponding tumor cells after entering the body. Compared with TCR-T cells, CAR-T cells do not require antigen processing and MHC presentation.

Since it was put forward by CAR in 1989, it has developed from the first generation to the fifth generation (**Figure 3**). The intracellular domain of the first-generation CARs has only one signal domain (CD3 ζ). Due to the lack of costimulatory molecules, the CAR-T cells can recognize the corresponding tumor cells, but the clinical effect is limited (123). The main reason for this phenomenon is that the first-generation CAR-T cells have poor persistence in the body, and the proliferation of CAR-T cells is low, which eventually leads to CAR-T cells apoptosis.

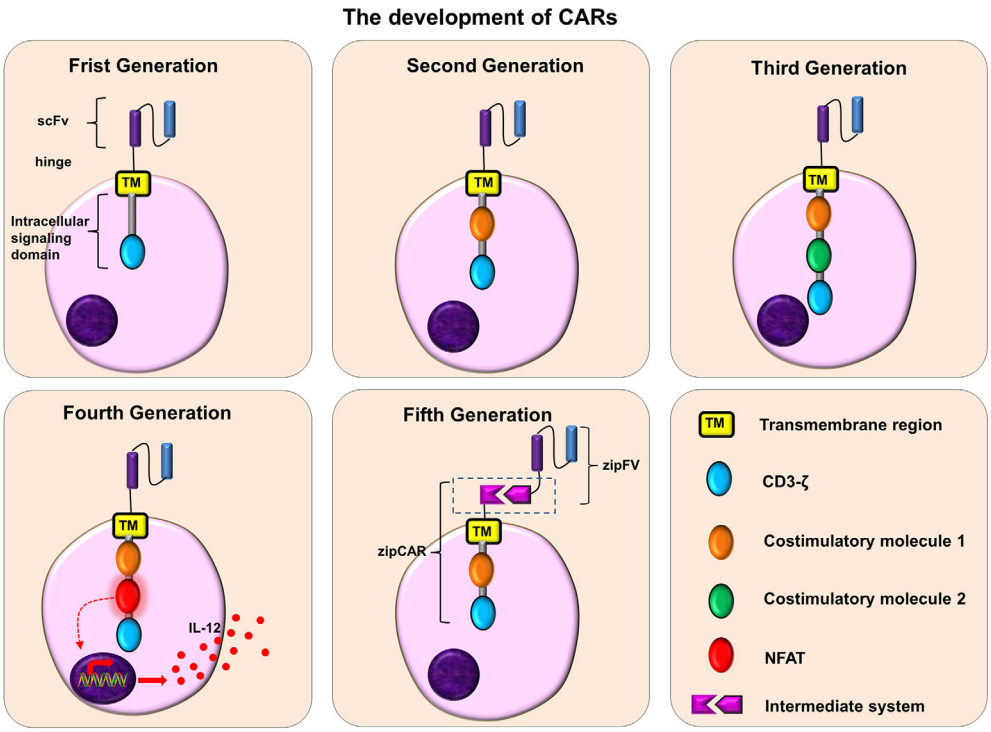

FIGURE 3 | The development of CARs. Frist Generation: scFv + TM + CD3ζ; Second Generation: scFv + TM + one costimulatory molecule + CD3ζ; Third Generation: scFv + TM + two costimulatory molecule + CD3ζ; Fourth Generation: scFv + TM + one/two costimulatory molecule + NFAT + CD3ζ; Fifth Generation: scFv+ intermediate system + one/two costimulatory molecule + CD3ζ.

The second-generation CARs add a costimulatory molecule to the first-generation CARs, such as CD28, 4-1BB (CD137), OX40 (CD134) or ICOS, etc. These costimulatory factors can promote the proliferation of CAR-T cells, the secretion of cytokines, and the secretion of anti-apoptotic proteins, thus improving the persistence of CAR-T cells and their ability to kill tumors. Among them, the CAR with CD 28 or 4-1 BB has a stronger tumor-killing effect (124). Furthermore, compared with CAR-T cells containing CD28, CAR-T cells containing 4-1BB are more durable and may be more resistant to exhaustion (125, 126).

The third-generation CARs contain two costimulatory molecules, which further enhances the activation, proliferation and persistence of T cells, and makes CAR-T cells have a stronger tumor-killing effect. Moreover, the third-generation CARs that contain both CD 28 and 4-1 BB can provide the strongest anti-tumor effect (127).

The fourth-generation CARs, also known as "TRUCKs", are modified by adding nuclear factor of activated T cells (NFAT) on the basis of the second or third-generation CARs. For example, the most common NFAT is the cytokine IL-12. These fourth-generation CAR-T cells (IL-12) can release IL-12 after being activated, which can not only promote T cell activation and regulate immunity, but also recruit other innate immune cells to attack tumor cells (128–131). In order to reduce the toxicity associated with CAR-T cells, some fourth-generation CARs have added suicide genes. When necessary, the suicide gene system can induce CAR-T cell death or shorten its lifespan (132, 133).

The fifth-generation of CARs, are also called "universal CARs". Theoretically, these CARs can target different tumor antigens. In traditional CAR-T cell therapy, one type of CAR-T cells can only target one kind of tumor surface antigens. In order to improve the flexibility and controllability of CARs and expand the scope of antigen recognition, the fifth-generation CAR adopts a "third-party" intermediate system to separate the antigen binding domain of CAR from its T cell signal unit (131, 134, 135). These CAR-T cells can target different tumor antigens to fight against tumor heterogeneity, and can also improve their safety and reduce related toxicity during the treatment of CAR-T cells (135). The common "Third-party" intermediate systems are biotin-binding immune receptors (BBIR) CAR (136) and programmable (SUPRA)CAR (137).

CAR-T cell therapy has made great achievements in the treatment of hematologic malignancies. It also has great potential in the treatment of solid tumors. One of the key factors affecting the curative effect of CAR-T cells is the selection of tumor surface antigens. CAR-T cells are designed for one or more tumor antigens, so they can specifically identify tumor cells expressing these tumor antigens. For CAR-T cells, the best design scheme should be to use tumor-specific antigens (TSAs) to design the corresponding CARs, because these CARs are more targeted and can minimize off-target effects.

TABLE 3 | Some liver cancer-related CAR-T cell therapy targets.

Associated Malignancy	Target Antigens	Co-stimulating Domain	Generation of CAR-T	Author	Reference
	CD133	4-1BB	2^{nd}	Wang et al.	(138)
	NKG2DL	4-1BB	2^{nd}	Sun et al.	(139)
	GPC3	CD28	2^{nd}	Wu et al.; Guo et al.	(140, 141)
		CD28,4-1BB	3^{rd}	Jiang et al.	(142)
		CD28, ICOSL	3^{rd}	Hu et al.	(143)
HCC		CD28, ICOSL,4-1BB	3^{rd}	Hu et al.	(143)
		4-1BB	4^{th}(CXCR2)	Liu et al.	(144)
		CD28	4^{th} (IL12)	Liu et al.	(145)
		CD28,4-1BB	4^{th} (IL15,IL21)	Batra et al.	(146)
	CD147	4-1BB	2^{nd}	Zhang et al.	(147)
	AFP	CD28	2^{nd}	Liu et al.	(148)

Unfortunately, there seem too few TSAs, and most of the common targets are tumor-associated antigens (TAAs). Currently, there are some therapeutic targets of CAR-T cells for HCC research and treatment (some HCC-related CAR-T cell therapy targets, **Table 3**). Among them, phosphatidylinositol proteoglycan 3 (GPC3) is the most widely used. GPC3 is a membrane protein located on the surface of tumor cells. GPC 3 is highly expressed in HCC, which makes it an ideal target for HCC treatment. GPC3-CAR-T cells can effectively kill $GPC3^+$ liver cancer cells, and their anti-tumor effect is proportional to the expression level of GPC3 (149). Batra et al. (146) designed GPC3-CAR-T cells that could co-express IL-15 and IL-21 to treat HCC. These CAR-T cells had superior expansion and persistence *in vitro* and *in vivo*, and the strongest anti-tumor activity *in vivo*. However, some obstacles limit the efficacy of CAR-T cells in the treatment of HCC. These obstacles include the lack of specific targets, homing barriers of CAR-T cells, inhibition of (TME), inhibition of immune checkpoints, etc. With the breakthrough of these obstacles, HCC patients will certainly get more benefits from CAR-T cell therapy.

PROSPECT AND SUMMARY

There remain various therapeutic methods for HCC, among which immunotherapy is playing an increasingly important role. The immunotherapy of HCC is in a rapid development stage. ICIs, tumor vaccines and ACT have great prospects and potential in the treatment of HCC. Combining different immunotherapy or immunotherapy with conventional treatment methods may produce synergistic effects (11). Nevertheless, the current clinical application of immunotherapy is relatively single, and its curative effect is limited. Consequently, it is necessary to strengthen the research of combined treatment mode. Immunotherapy, as an extremely promising therapy, brings new dawn to HCC patients. In the future, immunotherapy may become one of the mainstream methods of HCC.

AUTHOR CONTRIBUTIONS

LM: Writing- Original draft preparation, Investigation, table and figure preparation. ZZ: Investigation and table preparation. ZR: Investigation. YL: Conceptualization, Methodology, Supervision. All authors contributed to the article and approved the submitted version.

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The Right Partner in Crime: Unlocking the Potential of the Anti-EGFR Antibody Cetuximab *via* Combination With Natural Killer Cell Chartering Immunotherapeutic Strategies

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Cetuximab has an established role in the treatment of patients with recurrent/metastatic colorectal cancer and head and neck squamous cell cancer (HNSCC). However, the long-term effectiveness of cetuximab has been limited by the development of acquired resistance, leading to tumor relapse. By contrast, immunotherapies can elicit long-term tumor regression, but the overall response rates are much more limited. In addition to epidermal growth factor (EGFR) inhibition, cetuximab can activate natural killer (NK) cells to induce antibody-dependent cellular cytotoxicity (ADCC). In view of the above, there is an unmet need for the majority of patients that are treated with both monotherapy cetuximab and immunotherapy. Accumulated evidence from (pre-)clinical studies suggests that targeted therapies can have synergistic antitumor effects through combination with immunotherapy. However, further optimizations, aimed towards illuminating the multifaceted interplay, are required to avoid toxicity and to achieve better therapeutic effectiveness. The current review summarizes existing (pre-)clinical evidence to provide a rationale supporting the use of combined cetuximab and immunotherapy approaches in patients with different types of cancer.

Keywords: cetuximab, epidermal growth factor receptor (EGFR), natural killer cells (NK cells), combination therapy, immunotherapy, antibody-mediated cellular cytotoxicity (ADCC)

INTRODUCTION

The field of cancer treatment has significantly advanced, driven primarily through an increased characterization of the molecular biology, the microenvironment, and the involvement of the immune system in several critical mechanisms of cancer. These advances have led to the development and implementation of targeted and immunotherapies. Targeted therapies are aimed at specifically inhibiting oncogenic signaling pathways that control tumor growth and/or

angiogenesis, whereas immunotherapies focus on (re)activating the immune system. Today, both treatment modalities are at the forefront of personalized medicine in cancer treatment.

Several major signaling pathways such as β-catenin, Wnt, phosphatidylinositol 3-kinase (PI3K), and Mitogen-activated protein kinase (MAPK) are recognized for their potentially oncogenic characteristics (1). Among them, the epidermal growth factor receptor (EGFR) is likely the most commonly investigated signaling pathway, renowned for its fundamental role in the tumorigenesis of many cancer types (2). While EGFR expression normally is found between 40 000 to 100 000 receptors/cell (depending on the tissue type), overexpression of EGFR is seen in a majority of cancers, with up to 2 000 000 receptors/cancer cell (3). Thus, downstream signaling of the Ras/Raf/MAPK, PI3K/AKT, JAK/STAT and PLC/PKC pathways is intensified (4), leading to enhanced cellular proliferation, differentiation, survival, migration and motility (5). Inhibition of EGFR has therefore been a compelling topic of research and has led to the development of two classes of anti-EGFR agents: Immunoglobulin G (IgG)-based monoclonal antibodies (mAbs), which competitively bind the ligand-binding site and small-molecule tyrosine kinase inhibitors (TKIs), which compete with adenosine triphosphate (ATP) to bind intracellular EGFR tyrosine kinase domains.

What makes mAbs highly attractive is the ability of IgG1 mAbs to induce antibody-dependent cell-mediated cytotoxicity (ADCC) through Fc receptor-bearing immune cells, increasing tumor immunogenicity and providing a rationale to combine anti-EGFR mAbs with immunotherapies. Cetuximab and necitumumab are the only approved IgG1 mAbs against EGFR (**Table 1**). While cetuximab has been extensively studied in various tumor types (6, 7), literature regarding necitumumab is still limited. Interestingly, similar cytotoxicity has been shown against the DiFi colorectal cancer cell line, due to their affinity for similar EGFR epitopes (8, 9). On the other hand, panitumumab, an IgG2 based anti-EGFR mAb, has similar anti-EGFR activity as cetuximab despite binding different epitopes (10, 11). In monotherapy, the ASPECCT study conducted in chemotherapy-refractory, wild-type *KRAS* metastatic colorectal cancer (mCRC), showed non-inferiority of panitumumab compared to cetuximab (12). Combined treatment of either cetuximab or panitumumab with irinotecan in platinum-refractory mCRC patients similarly suggested non-inferiority (13). Interestingly, studies directly comparing cetuximab and panitumumab in HNSCC have not been conducted. However, while panitumumab failed to improve OS of HNSCC patients in phase II trials in combination with chemoradiotherapy (14, 15) cetuximab, showed clear benefit in both locally advanced and recurrent and metastatic settings and has been granted approval by regulatory authorities herein (16, 17). Therefore, at least in HNSCC, panitumumab, despite having an increased EGFR-affinity, lacks in clinical activity compared to the highly active potential of cetuximab. A possible reason for this may be explained by the differences linked to the IgG backbone.

As evidenced by prior research, chemotherapeutic agents have immunomodulatory effects, causing (in)direct activation of immune cells due to the release of tumor antigens and certain "danger" signals (18, 19). Targeted therapies are similarly able to reshape the tumor immune microenvironment (TIME) and stimulate the induction of an immune response (20). Immunosurveillance, i.e. the recognition and elimination of malignant cells by the immune system (21), is crucial towards cancer prevention and evasion of immunosurveillance is one of the cancer hallmarks. As the immune system is a complex network of humoral and cellular interactions, alterations in many components of the innate and adaptive immunity lie at hand for tumor evasion (22). In addition, selective survival of tumor cells with a decreased immunogenicity contributes to an evasive tumor growth (23). In this regard, the TIME of several cancers has been characterized, showing both dysfunctional immune cells and a suppressive environment as the main reason for an impaired antitumor immunity (24–27). Based on these principles, immunotherapy has now become a major focus of research in oncology and has led to the implementation of immune checkpoint inhibitors, which have the potential to reawaken silenced immune responses. Recently, several immune checkpoint inhibitors have demonstrated durable response rates and gained Food and Drug Administration (FDA) and European Medicines Agency (EMA) approval for use in several oncological indications, including metastatic melanoma, non-small cell lung carcinoma (NSCLC), renal cell carcinoma, head and neck cancer (HNSCC) and colorectal cancer (CRC) (28–30). In the context of EGFR, besides its oncogenic role, EGFR is involved in three main immune-related processes. These include: (1) repression of antigen presentation *via* downregulation of major

Abbreviations: ADCC, Antibody-dependent cellular cytotoxicity; ADP, Adenosine diphosphate; AE, Adverse events; ATP, Adenosine triphosphate; BRCA, Breast cancer susceptibility protein; CAR, Chimeric antigen receptor; CRC, Colorectal cancer; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; DAMP, Damage-associated molecular patterns; DC, Dendritic cell; DDX41, Probable ATP-dependent RNA helicase; DMXAA, Dimethylxanthone acetic acid; EGFR, Epidermal growth factor receptor; EMA, European Medicines Agency; EpCAM, Epithelial cell adhesion molecule; FAS, First apoptosis signal; FDA, Food and drug administration; cGAS, Cyclic GMP-AMP synthase; IFI16, Gamma-interferon-inducible protein Ifi-16; GZMB, Granzyme B; HLA, Human leukocyte antigen; HNSCC, Head and neck squamous cell cancer; IFNγ, Interferon gamma; IL, Interleukins; IRF, Interferon regulatory factor 3; ITAM, Immunoreceptor tyrosine-based activation motif; ITIM, Immunoreceptor tyrosine-based inhibitory motif; KIR, Killer-cell immunoglobulin-like receptors; LAG-3, Lymphocyte-activation gene 3; LILR, Leukocyte immunoglobulin-like receptor B; MHC, Major histocompatibility complex; MIC, MHC class I polypeptide–related sequence A/B; NCR, Natural cytotoxicity receptor; NK, Natural killer; NSCLC, Non-small cell lung cancer; NT5E, 5'-nucleotidase; OS, Overall survival; PAMP, pathogen-associated molecular patterns; PARP, Poly adenosine diphosphate-ribose polymerase; PD-1, Programmed cell death protein; PFS, Progression free survival; PRR, Pattern recognition receptors; PTEN, Phosphatase and tensin homolog; STING, Stimulator of interferon genes; TGF, Transforming growth factor; TIGIT, T cell immunoreceptor with Ig and ITIM domains; TIME, tumor immune microenvironment; TINK, tumor-infiltrating NK cells; TKI, Tyrosine kinase inhibitor;TLR, Toll-like receptors; TME, Tumor microenvironment; TNFR, Tumor necrosis factor-related apoptosis-inducing ligand receptor; TNFRSF9, Tumor necrosis factor receptor superfamily member 9; TRAIL, TNF-related apoptosis-inducing ligand; ULBP, UL16-binding protein; VEGF, Vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis protein; YINM, Tyrosine-based signaling motif; ZAP70, Zeta-chain associated protein kinase.

TABLE 1 | Summary of approved EGFR-targeted mAbs.

Drug (Trade name)	Company	Indication	Approval FDA/EMA	Isotype	Recommended dose	Clinical trials*
Cetuximab (Erbitux)	Bristol-Myers Squibb	HNSCC,CRC	2004	Chimeric IgG1	I.V. 400 mg/m^2 initial, 250 mg/m^2 weekly	NCT00004227 NCT00122460
Panitumumab (Vectibix)	Amgen	CRC	2006/2007	Human IgG2	I.V. 6 mg/kg biweekly	NCT00364013 NCT00115765
Necitumumab (Portrazza)	Eli Lilly and Company	NSCLC	2015/2016	Human IgG1	I.V. 800 mg twice in a 3-week cycle	NCT00981058 NCT01769391

*CRC, colorectal cancer; EGFR, epidermal growth factor receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; HNSCC, head and neck squamous cell carcinoma; I.V., intravenously; NSCLC, non-small cell lung cancer. *Clinical trials upon which approval was based.*

histocompatibility complex (MHC) class I and II expression (31); (2) programmed cell death protein (ligand) 1 (PD-1/PD-L1) pathway activation (32); and (3) secretion of immunosuppressive cytokines, such as vascular endothelial growth factor (VEGF), and interleukins (IL) IL-6 and IL-10 (33, 34). Therefore, the use of anti-EGFR therapeutics, such as cetuximab, is a promising strategy of altering the TIME towards tumor recognition and potentially killing rather than evasion and tumor growth.

Although both targeted and immunotherapies are successfully implemented into clinical practice, they present some limitations. In general, when immunotherapies are successful, they can achieve long-term responses in patients. However, response rates with immunotherapies are typically low. In contrast, targeted therapies can achieve much higher initial responses but are lacking in long-term tumor remission, due to the development of resistance. Therefore, growing evidence suggests that combining targeted therapies with immunotherapies can achieve much greater clinical effectiveness for a larger patient population. However, since tumor types vary greatly in their TIME, the applicability of these combinations is dependent on the tumor type and severity of disease (35, 36). For instance, under healthy conditions, all nucleated cells will express MHC class I "self" antigens as a measure of host and non-threat recognition. However, tumor cells often will decrease the expression of MHC-I to evade T-cell recognition of tumor antigens and also their effector functions (37). Therefore, the applicability of T cell-focused immunotherapies is currently complicated by the inability of T cells to recognize MHC-I^{neg} tumors as well the requirement of neoantigens for the induction of adequate responses. These shortcomings may potentially be circumvented by the innate counterpart of T cells, the natural killer (NK) cells, as they can recognize tumor cells independent of their MHC status and require no presentation of neoantigens. Moreover, NK cell responses can further shape the TIME towards activation of the adaptive immunity, and thus are key effectors of antitumor immunity. In addition, although NK cell infiltration is not equal in all tumor types, the number of tumor-infiltrating NK cells (TINK) has been associated with a significantly better outcome in many tumor types (29, 38–40). Monteverde et al. and others showed that in addition to the number of NK cells, the level of *ex vivo* antibody-dependent cell-mediated cytotoxicity (ADCC) induction can be used as a predictive biomarker for cetuximab treatment in the clinic (41–43). Together, this shows a unique opportunity for NK cell-based immunotherapy together with anti-EGFR targeted therapeutic approaches to re-establish functional NK cell responses, prime the TIME for the adaptive immunity, and generate more durable antitumor responses.

In this review, we will briefly describe the fundamentals of NK cell biology and functionality followed by a comprehensive review of combination strategies involving EGFR targeted therapies together with immunotherapeutic modalities that aim to restore/enhance the antitumor effects of NK cells. We will focus on cetuximab as an anti-EGFR targeted mAb, as its immune activity has been studied extensively both in monotherapy as well as in combination with other molecules. However, the efficacy of anticancer drugs varies significantly among different tumor types. Therefore, similar or possibly improved results could be achieved with other mAb-based immunotherapies following careful examination and characterization of the TIME.

NK CELL BIOLOGY AND ANTITUMOR ACTIVITY

Grouped among the population of lymphocytes, NK cells share the same progenitor as T and B lymphocytes but differentiate themselves through an antigen-independent activation (44). While the effector function of NK cells overlaps with CD8$^+$ T cells, they do respond to different stimuli and thus complement each other in settings where the effectiveness of one is lacking. Therefore, NK cells, as part of the innate immune system, form the first line of defense against cancer and pathogens (45). In humans, NK cells make up roughly 10-15% of all immune cells (46) and are defined as CD3$^-$ CD56$^+$ (47). The two major NK subpopulations are termed CD56^{bright} (high cytokine producers) and CD56^{dim} (high cytotoxicity) NK cells. About 90% of circulating and splenic NK cells are CD56^{dim}, while CD56^{bright} NK cells are mostly present in the secondary lymphoid organs (48). Notably, CD56^{bright} NK cells make up the largest portion of tumor-associated NK cells in several tumor types (48, 49).

Rather than depending on prior antigen presentation, NK cell immunosurveillance is based on a balance between interaction of activating and inhibitory receptors on their surface (50). In this regard, 'the missing self' principle (51) describes activation of NK cells through a decreased expression of MHC class I on tumor cells. However, lack of self-recognition alone does not determine NK cell activation and therefore the 'induced self' hypothesis describes the requirement of tumor antigens or ligands of

activating receptors to be expressed in addition to a reduced self-recognition to establish NK cell activation (**Figure 1A**) (52, 53). A prerequisite for NK cell cytotoxicity is the formation of an immunological synapse, a tight and complex junction formed between an NK cell and its target cell (54). Importantly, FcγRs range in their affinity for human IgGs. The high-affinity FcγRI are therefore able to bind monomeric IgGs while other FcγRs have a low-affinity and are only able to interact with multimeric IgG complexes (55, 56). Following interaction with activating signals, numerous cellular molecules (including receptors, signaling molecules and cellular organelles) will induce cytoskeletal reorganization of NK cells and polarize lytic granules, filled with pore-forming proteins (perforin) and serine proteases (granzymes), towards the synaptic site. Targeted exocytosis of these granules into the synaptic space induces apoptosis in the target cell (57). NK cell activation may occur following interaction with death receptors such as first apoptosis signal (Fas) receptor and tumor necrosis factor (TNF)-related apoptosis-inducing ligand receptor (TRAIL-R) with their ligands, FasL and TRAIL, respectively (**Figure 1B**) (58, 59). In addition, various groups of inhibitory and activating NK cell receptors exist as well, as shown in **Figure 2A**. Inhibitory NK cell receptors that can recognize MHC-I antigens on tumor cells include the killer Ig-like receptors (KIR2DL and KIR3DL), C-type lectins NK cell group 2 (NKG2A/B) subfamily and leukocyte immunoglobulin-like receptors (LILR) (60, 61). In addition, immune checkpoint receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, and the T cell immunoreceptor with Ig and ITIM domains (TIGIT) are present on NK cells as well and prevent sustained activation through inhibitory signaling (62, 63). Interestingly, several of the activating cell surface receptors on NK cells are derived from the same receptor families as their inhibitory counterparts. For example, KIR2DS and KIR3DS belong to the KIR family receptors, while NKG2C/D belong to the C-type lectin family (64). Additionally, the family of natural cytotoxicity receptors (NCR), i.e. NKp46, NKp30, and NKp44, can recognize a broad spectrum of ligands ranging from viral-, parasite- and bacterial-derived to cellular ligands (65). Downstream signaling of NK cell receptors is dependent on the interaction between activating and inhibiting signaling motifs. Activating receptors associated with DNAX-activating protein 10 or 12 (DAP-10/-12) process signals through tyrosine-based signaling motif (YINM) or tyrosine-based activation motif (ITAM) respectively (66). Meanwhile, Inhibitory receptors carry the immunoreceptor tyrosine-based inhibitory motif (ITIM) that overrides DAP-10/-12 signaling and consequently prevents NK cell activation (**Figure 2B**) (67).

Besides direct receptor-ligand interaction, NK cells can become activated by interaction of their Fc receptors (FcγRIIIa/CD16) with the Fc-domain of immunoglobulin G (IgG) antibodies. To achieve subsequent tumor cell killing, the antibody Fab-domain must bind its target on tumor cells to initiate NK cell cytokine and cytotoxic granule secretion, thus inducing ADCC (**Figures 1B** and **Figure 3A**) (68). Interestingly, ADCC dysfunction has been linked to cancer progression and forms an important mechanism of action for therapeutic mAbs (68, 69). Among the IgG subtypes that have

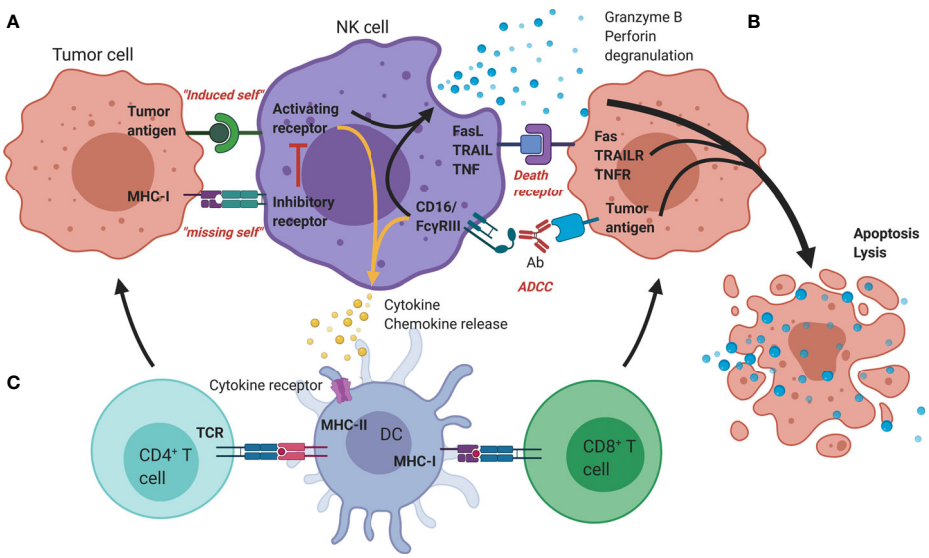

FIGURE 1 | Mechanisms of antitumor functionality of NK cells. **(A)** The represented 'activating' and 'inhibitory' NK cell receptors determine the NK cell activation through interaction with; (i) stress-induced tumor antigens or ligands for activating receptors acting towards an 'induced-self' response or (ii) MHC-I self-antigens or ligands for inhibitory receptors. **(B)** Additional tumor killing can be induced through either death receptors (FAS/TRAILR/TNFR), or antibody-dependent cellular cytotoxicity (Granzyme B/perforin degranulation). **(C)** Additional immune modulation by NK cells occurs through secretion of cyto-/chemokines that promote DC maturation and allow crosstalk with T cells, facilitating the induction of an adaptive immune response. Ab, Antibody; DC, Dendritic cell; FasL, Fas ligand; MHC, Major histocompatibility complex; NK, Natural killer; TCR, T-cell receptor; TNF(R), Tumor necrosis factor (receptor); TRAIL(R), TNF-related apoptosis-inducing ligand (receptor).

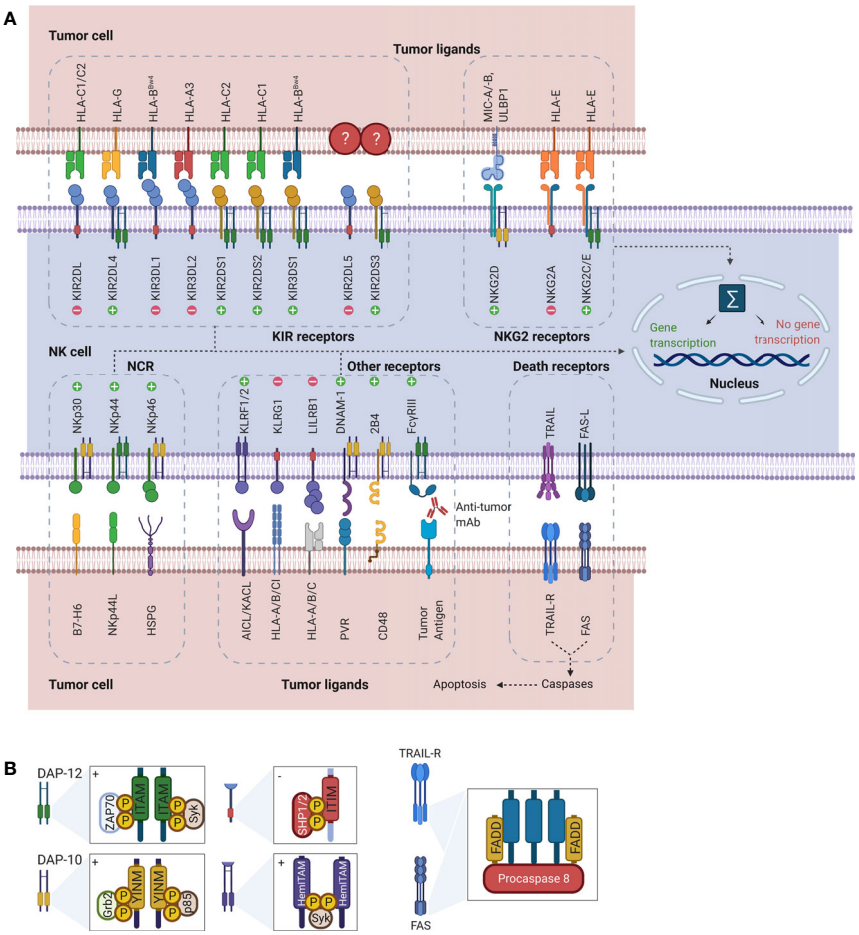

FIGURE 2 | Representation of NK cell receptor-ligand interactions and signaling motifs that enable downstream cell signaling. **(A)** The most common NK cell receptor families are illustrated together with their ligands. While some receptors engage multiple ligands, others such as KIR2DL5 and KIR2DS3/S4/S5 have no known ligands. Interaction of ligands with receptors causes activation of downstream signaling pathways. Depending on the type of receptor, this may cause either activation of gene transcription or suppression. **(B)** Downstream signaling is activated through processing of the receptors-ligand interaction through signaling motifs. Symbols "+" and "-" in the boxes indicate activating and inhibiting signaling. While ITAM and YINM signaling motifs are bound to DAP-10 and -12 adaptor protein respectively, ITIM and HemITAM are present on the receptors and do not require adaptor proteins. The death receptors Fas and TRAIL-R signal through FADD to induce induction of apoptosis in tumor cells. Downstream signaling and gene transcription leading to NK cell activation is dependent on the sum of all activating and inhibiting signals. AICL, Activation-induced C-type lectin; DAP, DNAX-activating protein; DNAM, DNAX accessory molecule; FADD, Fas-associated protein with DD; Grb2, Growth factor receptor-bound protein 2; HemITAM, Hemi-immunoreceptor tyrosine-based activation motif; HLA, Human leukocyte antigen; HSPG, Heparan sulfate proteoglycans; ITAM, Immunoreceptor tyrosine-based activation motif; ITIM, Immunoreceptor tyrosine-based inhibitory motif; KACL; Keratinocyte-associated C-type lectin, KIR, Killer cell immunoglobulin-like receptor; KLRF/G, Killer cell lectin-like receptor F/G; LILRB1, Leukocyte immunoglobulin-like receptor B1 MICA/B, MHC class I polypeptide–related sequence A/B; NCR; Natural cytotoxicity receptors; NK, Natural killer; NKG2, Natural killer group 2; PVR, Poliovirus receptor; SHP1/2, Src homology region 2 domain-containing phosphatase-1; Syk, Spleen tyrosine kinase; TRAIL(R), TNF-related apoptosis-inducing ligand (receptor); ULBP, UL16 binding protein; YINM, Tyrosine-based signaling motif; ZAP70, Zeta-chain associated protein kinase.

been identified and used to generate antitumor therapies, IgG1-based mAbs have the highest potency to bind with CD16 and thus induce the highest ADCC responses (70). This is evident when comparing clinical data of cetuximab (IgG1) with panitumumab (IgG2) indicating that, although both effectively inhibit EGFR signaling, cetuximab mediates a greater extent of immune-related activity (10, 71). Preclinical models in CD16 deficient mice observed similar antitumor responses between cetuximab and panitumumab due to inhibition of EGFR (72, 73). However, CD16 wild-type mice consistently had enhanced antitumor responses with cetuximab which were linked to its IgG1 backbone (73, 74).

Aside from IgG subtypes, other factors related to interindividual heterogeneity rather than the composition of the mAb can affect ADCC by NK cells. First, FcγRIIIa gene polymorphisms are the most well-known factor in this regard. Individuals possessing the 158V/V allotype induce higher ADCC responses in various tumor types compared to individuals with the 158V/F or 158F/F allotypes (42, 75, 76). In vitro, transduction of the human NK-92 cell line with the 158V/V allotype (high-affinity NK; haNK) increased natural cytotoxicity, cetuximab-induced ADCC (77) and cytokine secretion (78). Second, the presence of immunosuppressive cytokines, such as

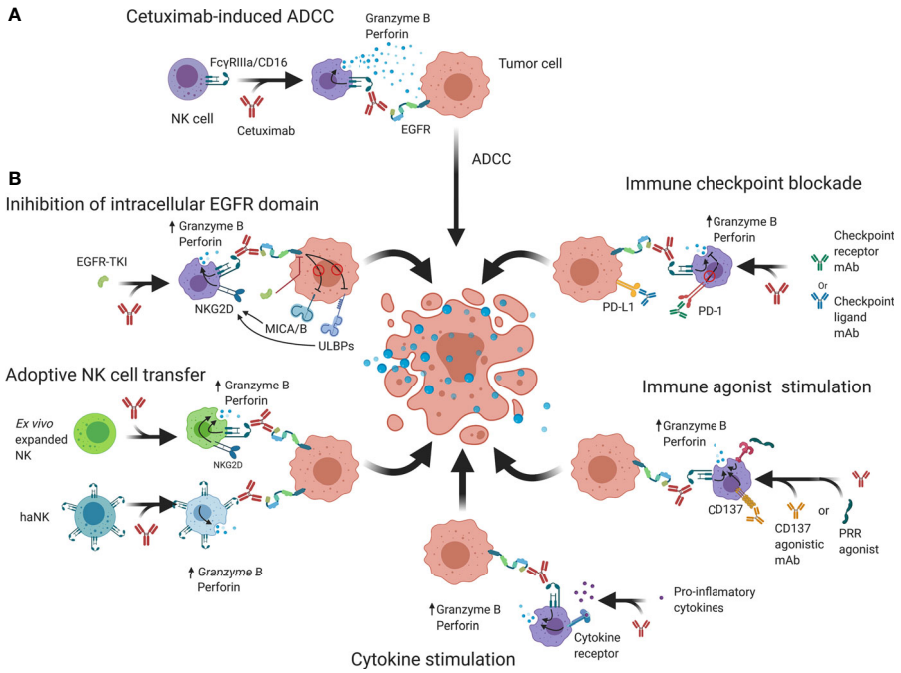

FIGURE 3 | Schematic overview of possible strategies that may be employed to enhance cetuximab-based anticancer NK cell responses. **(A)** Cetuximab is a mAb that interacts with FcγRIIIa/CD16 receptors on NK cells and EGFR on tumor cells to abrogate EGFR signaling and induce granzyme B and perforin release, causing cell death. **(B)** NK cell cytotoxicity may be enhanced by additional binding of intracellular EGFR kinase domains that can regulate expression of NK cell receptors. Genetically engineered NK cells such as haNK or CAR-NK have increased natural cytotoxicity and activating signaling which through adoptive transfer can enhance ADCC. Immune checkpoint blockers prevent suppression of NK cell functions by reducing inhibitory signaling while immune agonists aim to increase activating signals. Cytokine stimulation increases NK cell functions and allows an enhanced ADCC response to take place. ADCC; Antibody-dependent cell-mediated cytotoxicity; CAR, Chimeric antigen receptor; EGFR, Epidermal growth factor receptor; IL-2/12/15/21; Interleukin 2/12/15/21; MICA/B, MHC class I polypeptide–related sequence A/B; NKG2D, Natural killer group 2D; PD-1, Programmed cell death protein 1; PD-L1, Programmed death-ligand 1; PRR; Pathogen recognition receptors; ScFv, Single-chain variable fragment TKI; Tyrosine kinase inhibitor; ULBP, UL16 binding protein.

transforming growth factor β (TGF-β) or IL-10 or increased signaling through inhibitory KIR receptors or NKG2A, provides additional inhibitory signals. This shifts the balance of NK cell activity towards an inhibitory state, preventing the induction of ADCC (79–81). Third, while cetuximab resistance mechanisms limit the effectiveness of anti-EGFR treatments and promote tumor cell survival, they are unable to prevent granzyme B (GZMB)-induced apoptosis by healthy NK cells following cetuximab treatment (82–85). On the other hand, EGFR-independent resistance mechanisms against immune cell-mediated cell death have been described (86). For example, the presence of tumor cells expressing serine protease inhibitor-9 (PI-9), an irreversible inhibitor of GZMB, correlated with a poorer outcome in melanoma patients (87, 88). Overexpression of X-linked inhibitor of apoptosis protein (XIAP), a potent caspase inhibitor, in breast cancer induced resistance to cetuximab-mediated ADCC in both a caspase-dependent and -independent manner (*via* accumulation of reactive oxygen species) (89). Lastly, activation of autophagy under hypoxic conditions showed beclin-1-mediated degradation of NK cell-derived GZMB *in vitro*, which compromised the ability of NK cells to eliminate breast cancer cell lines (90). Notwithstanding these variable factors, the ability for ADCC remains a valuable and promising option in the therapeutic armamentarium, favoring mAbs such as cetuximab.

Next to direct activation, indirect NK cell activation primes NK cells towards activation by increasing the expression of activating receptors, reducing the threshold for activation and reducing the responsiveness to inhibitory signals (91). This can be achieved through interaction with mature dendritic cells (DC) or cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21 and type-I interferons (92, 93). However, vice versa, activated NK cells can cross-talk with DC, promoting their maturation and subsequent $CD8^+$ T cell priming, resulting in the generation of tumor-specific T cells that contribute to the antitumor immune reaction (**Figure 1C**) (94). As such, in addition to tumor elimination, NK cells also modulate and shape antitumor immunity, showing their crucial role to achieve tumor elimination.

STRATEGIES TO ENHANCE CETUXIMAB DRIVEN IMMUNE ACTIVITY

Initial preclinical models showed that efficacy of cetuximab on inhibition of the downstream effectors and interfering with

tumor cell proliferation could be further enhanced through combination with conventional therapies, such as radiotherapy or chemotherapy (16, 95, 96). Later, it became apparent that this enhancement was in part attributable to an immunological response through an enhanced tumor infiltration of immune cells and ADCC (97, 98). However, there is evidence to suggest that TINK cells reside in an impaired state and only induce limited activity (99, 100). Furthermore, NK cell immune evasion by tumor cells has been described to be caused through two main mechanisms: (1) reduction of activating ligands on tumor cells; and (2) a dominance of NK cell inhibitory signals, preventing downstream signaling of activating signals. In addition, additional immunosuppressive mechanisms from bystander regulatory immune cells can further stimulate tumor progression (101). Therefore, in describing NK cell immunosurveillance enhancements, applicability depends on the composition of the TIME of different tumor types. Re-establishing NK cell functionality thus is a topic of great interest, as it could improve the antitumor immune responses observed in the clinic. In this regard, the research mainly focuses on two major approaches: (1) increasing signaling through immunoreceptor tyrosine-based activation motif (ITAM/YINM)-containing receptors; and (2) decreasing signaling and cross-linking of inhibitory motif (ITIM)-containing receptors. Below, we discuss several strategies to potentiate NK cells to elevate cetuximab efficacy to the next level (**Figure 3B**).

Dual Inhibition of EGFR Extracellular and Intracellular Domains

Despite initial promising results observed with anti-EGFR treatments, the most prominent limiting factor of its clinical effectiveness is the presence/development of drug resistance. Research has considerably focused on unraveling mechanisms behind this resistance and results have shown various ways to prevent/overcome EGFR-resistance (102–105). Of these, simultaneous inhibition of extracellular and intracellular domains of EGFR has been suggested to increase the overall antitumor effects. In this regard, the combined use of cetuximab and erlotinib/gefitinib induced synergistic antitumor effects with decreased proliferation and increased apoptosis in various human cell lines (106, 107). Phase I/II trials in NSCLC and CRC using combined treatment of cetuximab with gefitinib or erlotinib reported no additional toxicities with moderately enhanced antitumor effects (107–110). Even better results were obtained with second (afatinib) and third-generation (osimertinib) anti-EGFR TKIs in combination with cetuximab (111, 112). Additionally, the sequential treatment of NSCLC patients in a phase I trial using sequential treatment of afatinib and cetuximab observed improved objective response rates and progression-free survival (PFS) (111).

Besides an improved antitumor effect, it was also suggested that combined targeting of extracellular and intracellular domains of EGFR could improve immunologic responses. While the immunological effects of mAbs are well described, the therapeutic effect of EGFR TKIs has been predominantly attributed to the inhibition of signal transduction. However, current knowledge suggests that TKIs might indirectly be involved in antitumor immune responses. For example, treatment of NSCLC and CRC cells with the anti-EGFR TKI gefitinib or erlotinib increased natural cytotoxicity of NK cells through upregulation of NKG2D ligands ULBP-1/-2 and MHC class I polypeptide–related sequence (MIC)A/B (113–116), and downregulation of PD-L1 expression (117). In contrast, another study reported downregulated MICB and ULBP–2/5/6 expression following treatment with erlotinib (118). This indirect immunomodulatory effect suggests that simultaneous inhibition of extracellular and intracellular EGFR domains could increase antitumoral effects, due to dual targeting of EGFR, and improve immunologic responses as well. Indeed, combined treatment with cetuximab and erlotinib improved NK cell activity in NSCLC cell lines and an NSCLC mouse model through an improved ADCC response (119). This is likely caused by an increased expression of the NKG2D ligands by EGFR-TKI (119), which shifts the balance towards NK cell activity. Together with cetuximab-induced ADCC, this shift increases the overall cytotoxic activity of NK cells. A similar study in ovarian cancer cell lines observed enhanced antitumor responses and increased sensitivity towards cetuximab-induced ADCC following treatment with either erlotinib or gefitinib, even in tumor cells that were either intrinsically or acquired resistant to either TKI treatment (120).

One key consideration is the potential for overlapping toxicities of dual EGFR inhibition. However, most trials observe manageable toxicities, with one trial in particular reporting a similar percentage of grade 3/4 adverse events (AEs) when afatinib was combined with cetuximab simultaneously compared to sequential treatment or either treatment alone. However, the overall incidence of AEs was higher in the combination regimen (121). As clinical doses are based on toxicity and not target inhibition, the tolerable doses of each agent in the combination may be suboptimal. However, further clinical investigation is warranted to compare the observed toxicity profile with the effectiveness of this combination.

Adoptive Transfer Therapy Using (un) Modified NK Cells

Adoptive Transfer of Autologous Expanded NK Cells

As NK cells are often impaired in cancer patients, the use of adoptive NK cellular immunotherapy aims to restore NK cell functionality through supplementation or complete replacement of the NK cell populations with functionally active NK cells. As a result, tumor load, and the immunosuppressive TIME could be reduced. Earliest attempts of adoptive NK cell transfer failed to show meaningful clinical responses using *ex vivo* purified and unstimulated NK cells (122).

Therefore, combination of an NK cellular product with cetuximab could enhance the functionality of these NK cells and achieve overall responses through the induction of ADCC. A phase I trial in CRC administered *ex vivo* expanded patient-derived NK cells following cetuximab treatment (123). Noteworthy, the majority of expanded NK cells showed high expression of NKG2D and CD16, and high lymphocyte-activation gene 3 (LAG-3) and TIGIT expression. Cytotoxic effects toward the tumor remained elevated up to 4 weeks

following NK cell administration, indicating a favor towards NK activation rather than inhibition. Addition of expanded NK cells following cetuximab treatment displayed an increased cytotoxic activity against tumor cell lines and reduced overall tumor size of heavily pretreated cetuximab-resistant patients. Lastly, patients treated with expanded NK cells following cetuximab showed enriched levels of circulating interferon gamma (IFNγ) and reduced Treg frequencies, suggesting an induction of a Th1-type adaptive immune response (123).

Adoptive Transfer of Allogeneic Expanded NK Cells

With the increased understanding of self-regulation in NK cells, a possible alternative for the limited number of patient-derived NK cells has been the use of allogeneic NK cells. This approach may hold several benefits including the ability to obtain NK cells from healthy donors which may retain greater antitumor activity and the development of off-the-shelf application due to easier and greater availability of NK cells (124). Furthermore, several models to predict alloreactivity of NK cells (graft-*versus*-host disease) have been described (125), the 'Receptor–ligand mismatch' model remains one the most established predictive models. Briefly, donor NK cells bearing inhibitory KIR for which the corresponding HLA ligands are missing in the recipient become uninhibited. The presence of (non-HLA-restricted) activating signals can then induce alloreactivity (126, 127).

Sources for alloreactive NK cells include (i) acquiring umbilical cord blood (128),; (ii) partially KIR/HLA matched peripheral blood (126); or (iii) engineered NK cell lines (129). Investigations using the former primary NK cells yielded increased expression of activation markers CD69 and CD16 and strong ADCC responses towards NSCLC and B cell lymphoma *in vitro* and *in mice* (128). Adoptive transfer of the modified NK-92 cell line (haNK) cells with cetuximab harbored the capacity to efficiently kill HNSCC tumor cells in a dose-dependent manner and enhanced ADCC response (130, 131). In a clinical trial in NSCLC, *ex vivo* stimulated KIR/HLA matched healthy donor NK cells were administered together with cetuximab. This combination led to a significantly improved PFS and OS compared to cetuximab alone (132). A phase I trial in gastrointestinal carcinoma used allogeneic IL-2 stimulated NK cells in combination with cetuximab and obtained beneficial clinical responses and a tolerable safety profile (133). Interestingly, while addition of adoptive NK cells increased the number of circulating lymphocytes ($CD8^+$, $CD4^+$, B and NK cells), cetuximab alone, albeit to a lesser degree, was also able to significantly increase lymphocyte levels. This suggests that part of the increased levels may be related to improvement of cellular immunity and prevention of apoptosis of T cells. Indeed, levels of IFNγ and pro-inflammatory cytokines were significantly more present through combination of cetuximab with adoptive NK cell transfer, indicating an enhanced Th1-response (132). These first and promising results of cetuximab stimulating adoptive NK cell therapy in solid tumors are encouraging, since to date clinical effectiveness of adoptive NK cell therapy is only observed in hematological malignancies. Therefore, more research on cetuximab unlocking the potential of adoptive NK cell therapy for solid tumors is warranted.

Chimeric Antigen Receptor (CAR)-Engineered NK Cells

A more recent and promising approach for adoptive NK cell therapy is the use of chimeric antigen receptor-engineered NK (CAR-NK) cells. These can be developed either through lenti-/retroviral transduction of primary adult NK cells or immortalized NK-92 cells to recognize a specific tumor antigen (134). CAR-NK cells have several advantages over CAR-T cells. First, they are more robust as they still maintain their intrinsic target cell recognition. Therefore, a reduction of the target CAR is less likely to be an effective tumor escape mechanism (135). Second, cytokines released by activated NK cells are less associated with the induction of a cytokine release syndrome (136, 137). Third, as NK cells do not clonally expand, the cytokine levels they release is found to be less sufficient to induce a cytokine release syndrome (138, 139). Fourth, NK cells are known to suppress graft-*versus*-host reactions which are induced by T cells due to strict HLA-matching (135, 136, 138).

While CAR-NK therapy research is developing at a rapid pace, combination treatments using CAR-NK together with already established treatments are still limited. Recently, combined treatment of a CRC mouse model with epithelial cell adhesion molecule (EpCAM)-CAR-NK-92 and regorafenib (a sorafenib-related multikinase inhibitor) achieved a synergistic tumor suppression than either treatment alone (140). The basis for this investigation was the observation that regorafenib could modulate the TIME through alteration of Fas and PD-L1 expression in CRC cell lines (140). Similarly, efficacy of cetuximab in HNSCC is also linked to its immunostimulatory activities which include downregulation of PD-L1 expression. Therefore, although not validated yet, this suggests that cetuximab combined with CAR-NK cells against a specific tumor antigen could alter the TIME towards tumor cell killing as a potentially promising treatment strategy. As a proof of concept, CAR-T cells transduced with CD32A or CD16 in combination with cetuximab, achieved a greater cytotoxic response and improved survival of a CRC mouse model bearing EGFR mutations compared to either treatment alone (141, 142). Taken together, although definitive evidence for this regimen is still missing, these early results support the potential strength of cetuximab-based dual-targeting CAR-NK therapy as an adoptive therapy.

A last consideration is that adoptive transfer of (un)modified NK cells in solid tumors is inferior compared to responses observed in hematological malignancies. The most evident cause for this discrepancy is the poor migration of infused NK cells inside the tumor. This may be caused by altered chemokine receptors following *ex vivo* activation. For example, CXCR2/3/4 are important chemokine receptors on immune cells that facilitate migration towards CXCL9/10/12-expressing tumor cells (143–146). Loss of CXCR2/3 following *ex vivo* activation prevented NK cells from migrating towards B16 melanoma tumors (147). Therefore, more recent expansion protocols such as the one described by Somanchi et al. (148) consider the chemokine repertoire in order to achieve efficient expansions of specific NK cell phenotypes that may provide a better invasion in the tumor.

Targeting Negative Immune Checkpoint Molecules Prevents Immune Escape

Discovery of immune checkpoint blockade has played a pivotal role towards integration of immunotherapy into clinical cancer treatment. While initial immune checkpoint inhibitors, such as anti-CTLA-4 (ipilimumab) and anti-PD-1 (pembrolizumab) have focused on reversing the suppressed state of cytotoxic T cells (149), current research is expanding this to other cell types, including NK cells (**Figure 4**). This expanded research also brought with it an increasing number of molecules that are being investigated as possible immune checkpoints and an endless possibility for combinations with checkpoint inhibitors to achieve greater responses.

Programmed Cell Death Protein 1 (PD-1) Pathway

The PD-1/PD-L1 axis has become one of the most studied pathways in cancer immunotherapy, with promising results guiding the approval of several inhibitors (150, 151). Interestingly, early investigations of PD-1 expression on NK cells found 25% of healthy individuals to have PD-1$^+$ NK cells which correlated well with prior human cytomegalovirus infections (152). This prompted the idea that PD-1 expression on NK cells is a result of activation rather than exhaustion, which is the case for T cells following chronic stimulation (153). In cancer patients, peripheral blood NK cells are often found to be PD-1 positive (154–156) and intratumoral NK cells often express high levels of PD-1 (40, 156).

Interestingly, PD-1$^+$ NK cells were found to have downregulated CD16 expression and induce PD-L1 expression on tumor cells *via* IFNγ secretion, thus possibly inhibiting ADCC induction (157). However, inhibition of EGFR-signaling *via* cetuximab is known to interrupt INF-γ signaling and prevent PD-L1 upregulation on tumor cells (117). Thus, combining cetuximab with a PD-1 inhibitor could be viewed as a valuable strategy to prevent CD16 downregulation and PD-1/PD-L1-axis mediated silencing of ADCC. A study in HNSCC found an

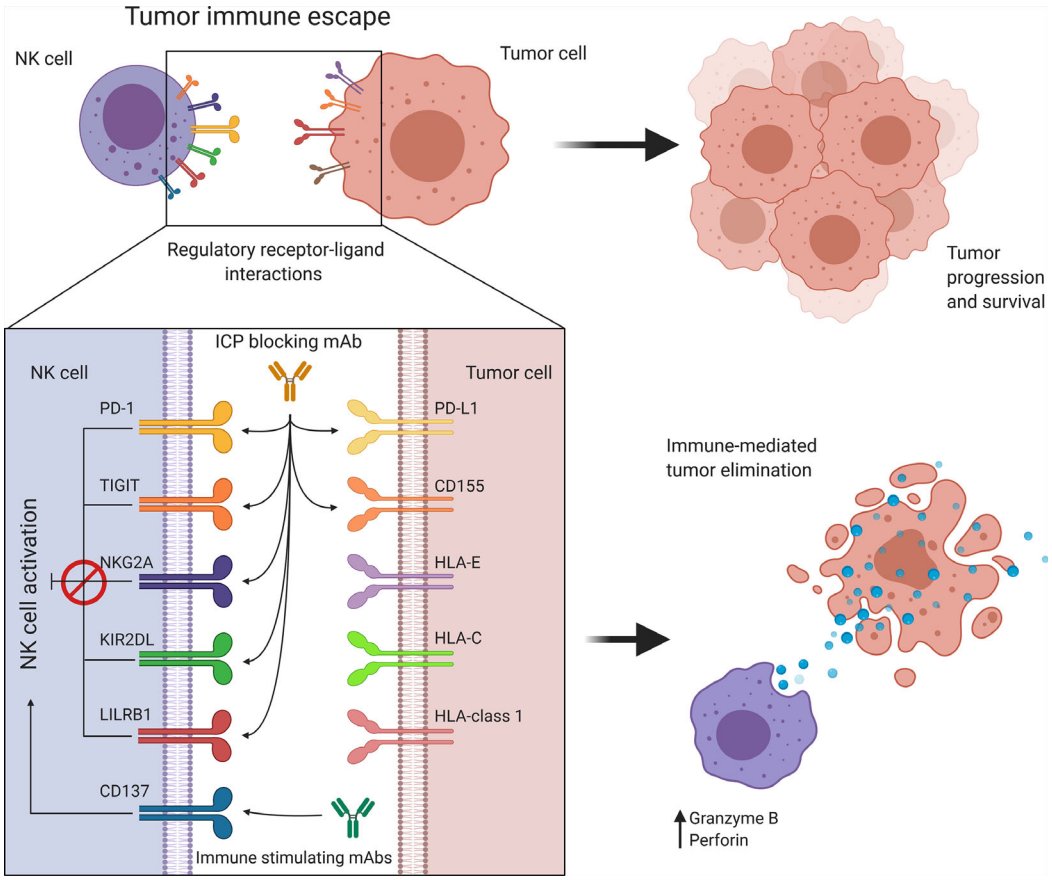

FIGURE 4 | Targeting immune regulatory molecules improves immune effector function against cancer. NK cell activity is regulated by a balance between immune activating and inhibiting interactions. Cancer promotes immune checkpoint expression to suppress NK cell activation allowing tumor immune escape and progression. Antibody-based immunotherapies suppress inhibitory signaling or further activate costimulatory signals to restore and enhance NK cell activity. HLA, Human leukocyte antigen; KIR, Killer cell immunoglobulin-like receptor; LILRB1, Leukocyte immunoglobulin-like receptor B1; NK, Natural killer; NKG2A, Natural killer group 2A; PD-1, Programmed cell death protein 1; PD-L1, Programmed cell death ligand 1; TIGIT, T cell immunoreceptor with Ig and ITIM domains.

increased number of PD-1$^+$ NK cells in patients, which correlated with a diminished NK cell activity, as observed by a downregulated expression of CD16, CD107a and GZMB. In addition, PD-L1 expression correlated with a lack of response to cetuximab alone. Administration of cetuximab in combination with the anti-PD-1 mAb nivolumab successfully reversed NK cell diminishment and enhanced cetuximab-mediated ADCC *in vitro* (157). Early results from a phase I trial in HNSCC patients also reported an increased objective response rate compared to either treatment alone (158). Currently, several trials investigating this combination are ongoing, with preliminary results indicating potentially synergistic effects in advanced solid tumors (159, 160).

T Cell Immunoreceptor With Ig and ITIM Domains (TIGIT) Pathway

Recent years have seen a growing interest in the TIGIT signaling pathway due to its complex immunomodulatory role. Similar to the B7/CD28/CTLA-4 pathway, the TIGIT axis consists of a network of inhibitory receptors (TIGIT, CD96 and CD112R) that compete with the activating receptor (DNAM-1/CD226) for their shared ligands (CD111/NECTIN1, CD112/NECTIN2, CD113/NECTIN3, CD155/PVR) (161, 162). In contrast to DNAM-1, only marginal TIGIT expression is observed on resting NK and T cells while stimulation and tumor infiltration showed upregulated TIGIT expression (161). As a stimulatory receptor, DNAM-1 signaling induces pro-inflammatory cytokine secretion and enhances cytotoxic activity of NK cells. Meanwhile, TIGIT induces an anti-inflammatory, non-proliferative and non-cytotoxic profile in NK cells (163).

Targeting of TIGIT is still in early development but positive early (pre)clinical investigations have enabled further clinical investigations. Interestingly, *in vitro* co-culture and *in vivo* transgenic HNSCC mice models were able to restore the cytotoxic effects of T and NK cells following anti-TIGIT treatment (164). Initial clinical studies in solid tumors demonstrated strong antitumor activity as a single agent (163, 165), that could be further improved when combined with anti-PD1 mAb (NCT03119428, NCT02794571). Furthermore, disruption of the TIGIT/CD155 interaction can also beneficially impact the TIME, in particular by incapacitating myeloid-derived suppressor cells and depleting Tregs. Although not investigated yet, this observation suggests the possible combination of anti-TIGIT-mAbs with cetuximab, thereby reducing the suppressive action of Tregs and targeting specific tumor antigens.

Alternatively to TIGIT, CD155 (PVR), has been suggested as a potential target due to its greater affinity towards TIGIT compared to DNAM-1 and its frequent overexpression in solid tumors (166, 167). However, clinical trials of CD155 are still scarce and preclinical investigations of CD155 in combination with cetuximab are limited as well. However, one study in CRC cell lines reported an improvement of cetuximab-mediated ADCC following effective signaling of DNAM-1/CD155. Blocking this interaction abrogated this effect entirely (168). The same effect was observed by blocking NKG2D/MICA/B signaling. A possible reason for the limited progress in CD155 targeting might be that CD155 inhibition disrupts both TIGIT and DNAM-1 signaling, therefore potentially robbing NK cells from activating signals. However, this concern is not completely warranted, as CD155 under normal circumstances has a greater affinity towards inhibitory receptors, thus preferentially signaling *via* TIGIT even in the presence of DNAM-1 (169). Lastly, administration of anti-CD155 also showed upregulation of DNAM-1 on peripheral blood lymphocytes. As CD155 is not the only ligand capable of binding DNAM-1, this interaction could potentially shift the balance towards increased antitumor immunity (170).

Altogether, this suggests that strategies targeting the TIGIT-axis could reverse immune inhibition through reduced inhibitory signaling and that combinations with cetuximab could enhance ADCC, resulting in an enhanced antitumor response (167).

C-Type Lectin NK Cell Group 2 (NKG2) Subfamily Pathway

Another ITIM-containing signaling pathway expressed on NK and T cells is the NKG2A-HLA-E interaction. Although NKG2A is expressed on a low number of peripheral NK cells, both antigen and cytokine stimulation upregulate its expression (171, 172). While binding of NKG2A to HLA-E is known to inhibit NK cell responses, ovarian cancer cell lines that were treated with the anti-NKG2A mAb monalizumab showed profound antitumor responses and significantly improved cetuximab-mediated ADCC (173, 174). Moreover, monalizumab combined with cetuximab was tested in a phase II trial with recurrent and metastatic HNSCC patients showing promising improvements with an easily manageable safety profile similar to either treatment alone (173). Another trial, where monalizumab was combined with durvalumab (anti-PD-1 mAb) in CRC showed encouraging activity as well (175). Meanwhile, a phase III randomized trial in HNSCC has been announced for this combination (176). Therefore, an anti-NKG2A mAb could be a promising checkpoint inhibitor to enhance antitumor immunity of both T and NK cells.

Killer-Cell Immunoglobulin-Like Receptor (KIR) Pathway

KIRs play a major role in regulating NK cell activity through various inhibitory and activating receptors and are most frequently found on intratumoral CD56^{dim} NK cells (29, 171). Similar to IFNγ, the inhibition of EGFR can increase HLA-C expression through STAT-1 signaling (26, 177). Thus, this could potentially limit NK cell responses through an increased interaction of KIRs with HLA-C. The use of mAbs, such as lirilumab (IPH2102), targeting KIR2DL-1/-2/-3, can mimic the mismatch of KIR with HLA-C and prevent inhibitory signaling. Indeed, various (pre-)clinical reports have described an improved NK cell cytotoxicity following lirilumab treatment (178–180). Furthermore, combination of lirilumab with an anti-CD20 mAb enhanced ADCC against lymphoma cells *in vitro* and *in vivo* (180). Similarly, lirilumab in combination with

cetuximab induced a significantly higher cytotoxic response against HNSCC cell lines in co-culture experiments (149). Hence, despite the lack of extensive literature, investigations of lirilumab in combination with cetuximab suggest that could generate clinical benefit and therefore warrant further investigation. Importantly however, long-term treatment with lirilumab may also hold some drawbacks. To fully develop into functionally mature cells, NK cells undergo a process of 'education' whereby their level of exposure and interaction to 'self' antigens with inhibitory receptors will determine their responsiveness in cases where these antigens are missing (181). Therefore, it is thought that persistent inhibition of KIRs could, besides stimulating the activity of mature NK cells, impede the development of new, functionally competent NK cells (178). In this regard, future clinical trials will have to resolve the optimal scheduling of blockade of inhibitory receptors.

Leukocyte Immunoglobulin-Like Receptor B (LILRB) Pathway

Similar to KIRs although far less understood, leukocyte immunoglobulin-like receptors (LILRs)can regulate immune activity through ligation with MHC class I molecules. However, in contrast to the extensive KIR repertoire being expressed, NK cells predominantly express LILRB1 (182, 183). Interestingly, LILRB1 expression negatively correlated with cetuximab-induced ADCC against breast cancer patients (184). Furthermore, blocking LILRB1 increased both natural cytotoxicity as well as cetuximab-mediated ADCC, especially when both NK cells and cancer cells expressed LILRB1. Interestingly, LILRB1 expression and cetuximab-mediated ADCC were positively correlated in this context, indicating a greater inhibition at higher LILRB1 expression levels. However, LILRB1 research is still limited and factors impacting the regulation of LILRB1 expression should be the focus of future research to assess the potential for clinical implementation of this combination.

Immune Agonists Allow Positive Immune Checkpoint Therapy

Since NK cells are dependent on a balance between positive and negative signals, negative signaling from immune checkpoints is counterbalanced by immune stimulatory molecules that positively enhance antitumor responses. Early attempts of developing potent agonist therapies were met with tremendous clinical toxicities due to selection of CD28, a constitutively expressed 'second signal' receptor on T cells, as a target. Theralizumab, despite the promising preclinical results, induced severe cytokine release syndrome with a high proportion of multiple organ failure in a phase I trial (185). Therefore, cautioned and rational selection of stimulatory molecules is essential to prevent non-discriminatory immune stimulation. Current approaches mostly comprise of selecting inducible targets following stimulation or maturation, rather than constitutive expression by immune cells (186).

Tumor Necrosis Factor Receptor Superfamily Member 9 (CD137/TNFRSF9)

Of interest for the context of this review is the molecule CD137 (4-1BB), expressed on various immune cells following pro-inflammatory stimuli (187). Signaling through CD137 delivers an enhanced tumor-selective cytotoxicity and IFNγ secretion (188). Interestingly, CD137 agonistic mAbs are classified as either strong or weak agonistic Abs. The difference is that strong agonistic Abs (Urelumab) can activate 4-1BB without FcγR-mediated crosslinking, while the weak agonistic Abs (Utomilumab) require FcγR-mediated crosslinking to activate 4-1BB. However, the effects of both classes can still be enhanced through separate FcγR-crosslinking (189). In this regard, although urelumab alone in a breast cancer xenograft model had no effect on tumor size, combined treatment with trastuzumab enhanced trastuzumab-mediated killing significantly (190). Furthermore, urelumab together with cetuximab greatly improved survival of HNSCC patients and elevated DC maturation and T cell cross-presentation together with an increased cytokine secretion (185, 186). Interestingly, TINK but not peripheral blood NK cells substantially increased CD137 expression following treatment with cetuximab. Both urelumab and cetuximab alone also upregulated anti-apoptotic proteins (Bcl-xL and Bcl-2) in NK cells, suggesting an improved survival of activated NK cells, that was further increased following combination treatment (186). These results suggest that urelumab could indeed be combined with cetuximab to enhance immune activity. However, the early clinical observations remain to be investigated in larger cohorts and various tumor types to develop a stronger support for this notion.

Pattern Recognition Receptors (PRR)

A critical role in pathogen recognition is carried out by toll-like receptors (TLRs). As part of the innate immunity, TLRs play a vital role in activating immune responses as well. This is achieved through recognition of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) expressed by microorganisms or released from damaged or dying cells (191). While a total of 11 TLRs have been identified, TLR7/8 are of particular interest in cancer research due to their direct immune stimulatory effect and simultaneous ablation of Treg function (192, 193). Therefore, stimulation of TLR7/8 could be an interesting treatment in tumors that are highly infiltrated with effector and suppressive immune cells. Stimulation through TLR7/8 could potentially polarize the TIME towards tumor killing by producing Th1-polarizing cytokines such as TNF-α, IFNγ and IL-12 (192). In this regard, the use of the TLR8 agonist motolimod, increased peripheral blood mononuclear cell cytotoxicity against HNSCC cell lines, together with a higher production of inflammatory cytokines and chemokines by DCs, monocytes and NK cells (194). Additionally, ADCC was enhanced through combination with cetuximab as well (194, 195), showing a possible way to effectively activate innate and adaptive anticancer immune responses. A phase I trial in HNSCC reported encouraging antitumor activity without dose limiting toxicities when motolimod was combined with

cetuximab. Furthermore, increases in plasma cytokine levels and in frequency and activation of circulating NK cells were observed as well (196). Currently, this combination is being further investigated in a phase II randomized trial (NCT01836029) of chemotherapy plus cetuximab in combination with motolimod in patients with recurrent or metastatic HNSCC.

As part of the PRR family, the stimulator of interferon genes (STING) DNA sensing pathway forms an important part of the innate immunity, as it recognizes cytoplasmic DNA through Cyclic GMP-AMP synthase (cGAS), gamma-interferon-inducible protein 16 (IFI16) and probable ATP-dependent RNA helicase (DDX41) (197). Therefore, STING also recognizes tumor-DNA and induces downstream signaling of NF-κB and interferon regulatory factor 3 (IRF-3). This results in the induction IFNs and inflammatory cytokines such as TNF-α, IL-1β and IL-6 (198). However, STING can also induce mitochondrial apoptosis through Bcl-2-associated X protein (Bax) induction (199). Therefore, the use of STING agonists to induce an inflammatory microenvironment and induce direct tumor apoptosis may be a valuable treatment. However, some reports suggest that STING may play a dual role in cancer, potentially promoting tumor growth in tumors with low antigenicity (200). Therefore, combined treatment of STING agonists with other treatments may achieve a good clinical outcome. Interestingly, EGFR was found to affect IRF-3 phosphorylation, suggesting a possibility for cetuximab to be combined with a STING agonist to enhance IRF-3 signaling and thereby lead to an enhanced antitumor response (201). Indeed, STING activation enhanced cetuximab-mediated ADCC of NK cells against HNSCC cell lines and promoted NK : DC crosstalk, suggesting an important role of STING in effective antitumor immunity (202). A phase I trial of the STING agonist dimethylxanthone acetic acid (DMXAA) (murine STING agonist) plus carboplatin, paclitaxel and cetuximab only demonstrated limited activity due to limited binding to human STING (NCT01031212). However, other clinical trials using human counterparts of STING agonists have provided clinical evidence for its therapeutic effectiveness. However, as no phase III trials have been registered yet, it remains to be seen what the exact clinical benefit of this combination will be. Regardless, the accumulated data so far point towards integration of immune-stimulatory molecules into standardized treatment regimens to induce clinically exploitable systemic responses.

Cytokine-Based Immune Potentiation

Cytokines form a group of small short-lived polypeptides that are involved in growth, differentiation and pro- and/or anti-inflammatory signals depending on the cell type. Although usually secreted in response to a defined stimulus, cytokines such as IL-7, required for immune cell homeostasis, can be constitutively expressed as well (203). Additionally, tumor cells can also secrete cytokines, mostly towards the establishment of an immunosuppressive TIME. Exogeneous administration of immunostimulatory cytokines has long been utilized in several lines of immunological investigations as a means of re-establishing the functionality of the immune system.

Interleukin-2 (IL-2)

Characterization of immunosuppressive factors and their involvement in tumor immune escape mechanisms has prompted researchers to reverse these impaired cytotoxic interactions through implementation of immunostimulatory cytokines. A study in HSNCC patients displayed elevated plasma levels TGF-β1 and soluble MHC I chain-related peptide A (sMICA) to diminish NKG2D expression, TNF-α and IFNγ release by NK cells, suppressing their antitumor responses (204). Interestingly, although NKG2D was downregulated due to high sMICA/TGF-β1 levels, CD16 expression and cetuximab-induced ADCC remained unaltered (204). Furthermore, IL-2 stimulation improved ADCC of sMICA inhibited NK cells resulting in a restored TNF-α and IFNγ secretion (204). Similarly, several other investigations in solid tumors have reported a significantly enhanced antitumor activity with tolerable safety profiles and improved ADCC following combined treatment with IL-2 and cetuximab (133, 205, 206). However, IL-2 administration in patients also causes expansion of $FoxP3^+$ Tregs, which highly express the IL-2α receptor (207). Tumor types with relatively low intratumoral Tregs could potentially still benefit from this combination, as shown by the studies above (133, 208, 209). In contrast, tumors such as HNSCC and melanoma have been characterized as the most Treg infiltrated tumor types, making the use of IL-2 in combination with cetuximab less attractive (29, 210). Therefore, the makeup of the TIME is an important consideration that must be evaluated on a tumor type basis for this combination to be of value.

Interleukin-12 (IL-12)

One of the first alternatives to IL-2 was IL-12, a cytokine produced by DCs and macrophages. IL-12 has anti-bacterial and anti-angiogenic effects and enhances the immune response to Ab-coated tumor cells (211). Stimulation of NK cells with IL-12 leads to secretion of IFNγ and TNF-α, as well as increased levels of chemokines such as MIP-1α, IL-8 and RANTES, further stimulating the infiltration of $CD8^+$ T cells into the tumor. Additionally, IL-12 increases IL-2α expression by NK cells, further enhancing NK cell activity in response to endogenous IL-2 (212). A phase I/II trial of heavily pretreated HNSCC patients investigated the combination of IL-12 with cetuximab and achieved stable disease in 69% of patients, with prolonged PFS. Additionally, ADCC responses were increased together with higher levels of IFNγ, CXCL10 and TNF-α secretion (213). IL-12 was also able to suppress Treg function through downregulation of FoxP3 (207, 214). Thus, in addition to stimulating NK cells, IL-12 administration may also reverse immune tolerance and creates a less suppressive TIME, enhancing antitumor immunity.

Interleukin-15 (IL-15)

IL-15 is a cytokine produced primarily by monocytes and macrophages and stimulates various NK and T cell functions (215). Similar to IL-2, stimulation with IL-15 is able to enhance the antitumor effects of NK cells against various tumor types and

significantly increases cytokine and chemokine secretions (216, 217). Interestingly, besides upregulation of CD16, NKG2D and IFNγ, levels of NKp30 and NKp46 on NK cells of CRC patients were restored following IL-15 stimulation (216). However, IL-15 based therapies face some limitations as well, including a short serum half-life, narrowing down the therapeutic window, and the requirement for IL-15 receptor α-chain (IL-15Rα)-binding prior to activating effector cells, which limits the therapeutic application (218, 219). More recently, the genetically modified IL-15 compound ALT-803, consisting of IL-15 plus the IL-15Rα fused to the Fc portion of IgG1, has been developed in order to address the limitations of IL-15-based therapies. As a result, ALT-803 has higher biological activity and a longer serum half-life compared with free IL-15. Consistently, ALT-803 was able to enhance the ADCC response following cetuximab treatment in HNSCC cell lines to a level similar to or better than IL-15. In mice, while single-agent treatment partially reduced tumor growth, co-administration of cetuximab with ALT-803 showed complete tumor regression and increased secretion of IFNγ, RANTES and IL-8 (218). Early clinical trials with ALT-803 alone have reported promising efficacy and activity, showing an increased expansion of NK and $CD8^+$ T cells (220). Interestingly, combination of ALT-803 with rituximab, another ADCC inducing mAb, gave similar results as ALT-803 plus cetuximab, thus supporting the exploration of ALT-803 to enhance cetuximab therapy (221).

Interleukin-21 (IL-21)

IL-21 belongs to the IL-2 family of cytokines, based on the shared cytokine receptor γ chain (γ_c). In comparison to IL-2 and IL-15, single-agent treatment with IL-21 was shown to be the most potent antitumor cytokine with longer lasting responses and clearing mice from tumors in settings where both IL-2 and IL-15 only showed limited effect (222). Additionally, IL-21 stimulation was also shown to increase levels of IL-2α in addition to IFNγ, perforin and GZMB (223). Interestingly, the combination of IL-21 with cetuximab was also able to enhance the ability of NK cells to recognize and eliminate cetuximab-coated tumor cells (223–225). Clinical trials using IL-21 in combination with cetuximab confirm preclinical findings, reporting increased cytokine secretion, enhanced ADCC and achieving stable disease in patients with different tumor types (225, 226).

Although we have discussed the drawback involved in IL-2 treatment regarding Treg expansion, cytokines also have faced criticism as a potential immunotherapeutic approach, due to additional limitations. These include the relatively short serum half-life, requiring careful exploration of clinical doses that could otherwise lead to severe toxic responses (227). Furthermore, IL-2 and IL-12 induce vascular leaking due to alterations in vascular permeability, which is only minimally present with IL-15 and IL-21 treatment (228, 229). These limitations lie at the basis of the functional properties of cytokines. However, they have not stopped researchers from investigating ways to enhance the effectiveness of cytokines through, for example, genetic engineering. The works of Skrombolas et al. and Berraondo et al. provide a detailed and comprehensive review regarding these strategies (203, 208). Taken together, the combined use of cytokines with cetuximab as an ADCC inducing agent has the ability to restore/enhance cytolytic activity of NK cells. Future research likely will include genetically cytokine engineering or consider the use of cytokine cocktails. These could help provide optimal enhancement of NK cells and prevent the limitations involved with single cytokine administration.

Combinations With Immunomodulatory Drugs

Although various novel compounds targeting tumor or immune antigens are in the developmental pipeline, another class of drugs that is of interest are the immunomodulatory drugs. These are a group of small molecules that were initially developed as treatment for other human diseases than cancer but were eventually recognized and exploited for their positive effects on the immune system.

Poly Adenosine Diphosphate (ADP)-Ribose Polymerase (PARP)

Cancer cells rely on DNA damage repair mechanisms to maintain their survival, making these repair pathways ideal targets for cancer treatment, e.g. poly Adenosine diphosphate (ADP)-ribose polymerase (PARP) (230). PARP enzymes act as DNA damage sensors when single-strand DNA breaks occur. Thus, PARP inhibition can severely inhibit cell survival, trigger cell cycle arrest and apoptosis through accumulation of DNA damage. Interestingly, PARP inhibition also activates the STING DNA sensing pathway, subsequently leading to production of type I IFN and pro-inflammatory cytokines, thus priming an antitumor immune response (231, 232). Therefore, the possibility to combine PARP inhibition with immunotherapy seems highly interesting.

EGFR inhibition with cetuximab diminishes DNA synthesis and double-strand break repair and therefore can increase tumor susceptibility to PARP inhibitors (233, 234). Indeed, combining cetuximab with PARP inhibitors significantly increased ADCC in both Breast cancer susceptibility protein (*BRCA*)-WT and -mutant cell lines (235). Clinically, a phase I study in locally advanced HNSCC patients demonstrated promising responses and tolerable toxicities (236), although results were confounded by continued smoking during treatment of non-responders (237). Thus, this combination warrants further study in a phase II setting to further investigate its effectiveness. The biggest risk involved with PARP inhibition is the potential to develop secondary myelodysplastic syndrome/acute myeloid leukemia due to impaired DNA damage repair. This was limited to patients that additionally received chemotherapy and had germline DNA repair deficiencies, further inducing DNA damage (238).

Thalidomide Derivatives

Despite the severe side effects observed with thalidomide in the 1960s, its mechanisms of action have revealed immunomodulatory

and anti-angiogenic activity. Analogues such as lenalidomide and pomalidomide are more potent immunomodulators and have fewer side effects. Lenalidomide has been approved for treatment of multiple hematological malignancies, as it is known to activate cytokine production, regulate T cell co-stimulation and augment NK cell cytotoxicity (239, 240). Lenalidomide is believed to enhance NK cell functionality in an indirect manner, mainly related to the release of IL-2 by other immune cells (240). Lenalidomide also enhanced ADCC following combination with several IgG1 mAbs, including cetuximab (168, 241). So far, the suggested mechanisms report that this enhancement is likely the result of an increased CD16 expression (168) and partly attributable to an increased presence of IL-2 and/or IL-12 cytokines secreted by T cells or other immune cells (242). On the other hand, lenalidomide-enhanced ADCC was abrogated through blocking of either DNAM-1/CD155 interactions or NKG2D with its ligands, indicating that optimal enhancement of ADCC requires interactions of DNAM-1 and NKG2D (168). Clinical trials investigating the combination of lenalidomide with cetuximab are currently in phase I/II and report a well-tolerated treatment with promising clinical activity in patients with CRC and HNSCC. Moreover, a dose-dependent increase in NK cytotoxic activity was demonstrated, with increasing doses of lenalidomide. This was associated with a significantly increased ADCC activity and an increased number of $CD8^+$ T cells and circulating NK cells (243, 244).

Thus, immunomodulating agents such as PARP inhibitors or lenalidomide combined with EGFR-directed therapies show promising preclinical and early clinical results but remain to be investigated in more detail.

CONCLUSION & FUTURE PERSPECTIVES

Although cetuximab is an established therapeutic agent in HNSCC and CRC, a major roadblock in achieving durable responses is the onset of therapeutic resistance. In contrast, immunotherapy can achieve long-lasting disease control, but only in a small percentage of patients. The TIME plays an important role in cancer-specific drug responses. The recent approval of pembrolizumab as a first-line treatment in HNSCC has sparked an increased interest in the modulation of immune responses to further improve survival of HNSCC patients (245). As increasing evidence points towards immune responses as a major determinant of mAb efficacy, it becomes increasingly difficult not to endorse the rationale of combination therapies. The earliest attempts, for example using IL-2, have indeed enhanced effector functions at the cost of stimulating immunosuppressive cells as well. Current approaches minimize unwanted effects by rational selection of targets such as IL-15. We previously showed that healthy NK cells may overcome cetuximab resistance *in vitro* (68). However, overcoming clinical resistance to cetuximab may require additional immunotherapies to harness the full potential of NK cells. In this review we have discussed several approaches to augment cetuximab-mediated ADCC against solid tumors.

The majority of approaches discussed in this review focus on manipulation of cell surface receptors and cytokines to enhance NK cell activity. These promising early results warrant further research, as there is a window for improvement and a requirement to tailor these strategies to various tumor types. For example, as HNSCC is marked with the highest infiltration of NK cells, effective treatment should focus on enhancing NK cell activity, by reducing inhibitory signaling or increasing activating signals. In contrast, CRC only shows marginal NK cell infiltration and thus the primary objective should be to lure NK cells inside the tumor, either through adoptive transfer or through increased homing. A better understanding of cancer-specific immune interactions will undoubtedly yield stronger scientific and clinical endeavors.

The current era of genomic, transcriptomic and immune profiling analysis will likely improve the tailoring of single-agent or combination therapies towards patient populations, thus entering an era of precision immunotherapy. Key components towards the success of future trials are considerations towards incorporating ADCC, intratumoral persistence and trafficking of NK cells. In this regard, given the clinical results summarized in this review are mostly still under phase I/II investigation, we anticipate future studies to confirm that cetuximab in combination with immune checkpoint inhibitors synergistically enhances the innate and adaptive antitumor immune responses. There are currently at least 109 active trials investigating cetuximab in a combination regimen with various other treatments (clinicaltrials.gov). Of these, at least 19 trials are investigating combinations with immunotherapeutic modalities discussed above (**Table 2**). The potential of cetuximab-based NK cell immunotherapy looks promising and we foresee that NK cells will become appreciated as a natural component in the fight against cancer.

Although we exclusively discussed cetuximab as the primary ADCC-inducing agent in this review, a large portion of these applications could be applied to other IgG1 mAbs (**Table 3**). In this regard, we believe the NK cell-based discussed approaches could also be of interest for other cancer indications employing ADCC-inducing mAbs. Moreover, growing research focuses on the development of engineered mAbs that display enhanced ADCC. These modifications involve altering the mAb Fc portion to increase binding affinity to FcγRIIIa *via* site-directed mutagenesis, editing Fc domain glycosylation and/or removing Fc domain fucosylation. Various Fc-engineered mAbs have shown improved responses compared to unmodified counterparts and have gained approval for clinical use (**Table 3**).

Implementation of any combination treatment requires a strong consideration for potential AEs. Biomarkers for EGFR targeting include EGFR gene amplifications and mutations, but also downstream sarcoma viral oncogene (Ras), PI3K and PTEN activities as well (102, 246). As downstream oncogenic signaling can affect the TIME, it is important to consider immunological biomarkers as well. Besides PD-L1 expression on tumors, factors such as PD-L1 on immune cells and co-expression of

TABLE 2 | ADCC-mediating IgG1 therapeutic antibodies.

Antibody (Trade name)	Company	Approval FDA/EMA*	Indication	Target	IgG1 type	Fc modification	Reference
Unmodified Fc Abs							
Alemtuzumab (Campath)	Ilex Pharmaceuticals	2013	MS	CD52	Humanized	/	(1)
Avelumab (Bavencio)	Merck KGaA and Pfizer	2017	MCC, UC, RCC	PD-L1	Human	/	(2)
Cetuximab (Erbitux)	Bristol-Myers Squibb	2004	HNSCC, CRC	EGFR	Chimeric	/	(3)
Dinutuximab (Unituxin)	United Therapeutics	2015	NB	GD2	Chimeric	/	(4)
Ipilimumab (Yervoy)	Bristol-Myers Squibb	2011	MEL, RCC,	CTLA-4	Human	/	(5)
Necitumumab (Portrazza)	Eli Lilly and Company	2015/2016	NSCLC	EGFR	Human	/	(6)
Ofatumumab (Arzerra)	Genmab	2009/2010	CLL	CD20	Human	/	(7)
Pertuzumab (Perjeta)	Genentech	2012/2013	BCA	HER2/neu	Humanized	/	(8)
Rituximab (Rituxan)	Genentech	1997/1998	NHL, CLL	CD20	Chimeric	/	(9)
Trastuzumab (Herceptin)	Genentech	1998/2000	BCA, GC	HER2/neu	Humanized	/	(10)
Fc modified Abs							
Imgatuzumab	Genentech	/	HNSCC	EGFR	Humanized	Reduced fucosylation	(11)
Margetuximab (Margenza)	MacroGenics	2020/2018	BCA	HER2/neu	Chimeric	Enhanced FcγRIII binding ($F^{243}L$; $R^{292}P$; $Y^{300}L$; $V^{305}I$; $P^{396}L$)	(12)
Mogamulizumab (Poteligeo)	Kyowa Hakko Kirin	2018	CTCL	CCR4	Humanized	Afucosylated	(13)
Obinutuzumab (Gazyva)	Roche	2013/2014	CLL, FL	CD20	Humanized	Afucosylated	(14)
Tafasitamab (Monjuvi)	MorphoSys	2020	DLBCL	CD19	Humanized	Enhanced FcγRIII binding ($S^{239}D$; $I^{332}E$)	(15)
Tomuzotuximab (CetuGEX)	Glycotope	/	NSCLC, CRC, HNSCC, GC	EGFR	Chimeric	Afucosylated	(16)

*BCA, Breast cancer; CCR4, Chemokine receptor 4; CLL, Chronic lymphocytic leukemia; CRC, Colorectal cancer; CTCL, Cutaneous T-cell lymphoma; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; DLBCL, Diffuse large B-cell lymphoma; EGFR, Epidermal growth factor receptor; EMA, European Medicines Agency; FDA, Food and Drug Administration; FL, Follicular lymphoma; GC, Gastric cancer; GD2, Disialoganglioside; HER, Epidermal growth factor receptor 2; HNSCC, Head and neck squamous cell carcinoma; I.V., Intravenously; MCC, Merkel cell carcinoma; MEL, Melanoma; MS, Multiple sclerosis; NB, Neuroblastoma; NSCLC, Non-small cell lung cancer; RCC, Renal cell carcinoma; UC, urothelial carcinoma.*Approval by FDA and EMA within the same year if only a single date is given.*

other inhibitory checkpoints may affect the response to PD-1 targeting (247). Furthermore, consideration of tumor immune infiltration, proportion of immune cell phenotypes and tumor mutational burden have proven to be a better representation for the effectiveness of immunotherapies in solid tumors (40, 248–250).

Importantly, despite the overall success of immune checkpoint inhibitors in various tumor types, meta-analyses often show severe treatment-related AEs that are associated with tumor response. In most patients, these AEs are related to overstimulation of immune reactivity. However, the severity of AEs is dependent on the used inhibitor. For example, CTLA-4 inhibitors have a higher risk of treatment-related AEs compared to PD-1/PD-L1 inhibitors (251). A possible solution might be targeting several biological pathways to induce longer-lasting responses. Interestingly, while the use of dual checkpoint inhibition or combination with TKI increased dose-sensitivity with higher risk of toxicity, mAb combinations, including cetuximab, that aim to elicit higher ADCC responses could be given at their recommended phase II doses without greatly increasing toxicities (252). Nevertheless, future research should always consider the potential for increased AEs in any combination strategy and dose-escalation schemes are greatly useful in that regard.

The next couple of years will undoubtedly bring a more in-depth understanding of the TIME together with the next generation of targets for anticancer treatment. This will allow us to rationally design better combination therapies in order to achieve the most optimal long-term effectiveness. In this era, we believe that cetuximab and many other ADCC-capable mAbs will remain valuable components, as it becomes clear that mAbs can add great benefit to both conventional and immunotherapies. As NK cell activation depends on a balance of stimulatory and inhibitory signals, the combinations that involve stimulation of NK cells through ADCC, together with suppression of inhibitory signals or the attraction of NK cells are of particular interest. As these combinations are currently under (pre)clinical investigation, the knowledge they provide regarding valuable biomarkers will soon guide the next generation of clinical trial measurements and ultimately lead to higher-

TABLE 3 | Active clinical trials evaluating cetuximab in combination with NK cell stimulating immunotherapies.

Clinical trial ID	Study phase	Estimated patients	Initial registration	Indication	Treatment	Primary endpoint	Status
Adoptive NK cell therapy							
NCT03319459	I	100	2018	Advanced Solid Tumors	FATE-NK100 FATE-NK100 + trastuzumab FATE-NK100 + cetuximab	DLT	Active, not recruiting
NCT04872634	I/II	24	2021	LA/M NSCLC	SNK01 (low/high dose) + gemcitabin e SNK01 (low/high) + Cetuximab + gemcitabin e	MTD, AE	Recruiting
Cytokines							
NCT01468896	I/II	23	2011	R/M HNSCC	Recombinant interleukin-12 + cetuximab	DLT, OR	Active, not recruiting
NCT02627274	I	134	2015	Solid tumors	RO6874281 RO6874281 + Trastuzumab RO6874281 + cetuximab	DLT, MTD, OBD	Active, not recruiting
NCT04616196	I/II	78	2020	R/M HNSCC & CRC	Dose Escalation of NKTR-255 + cetuximab Dose expansion of NKTR-255 + cetuximab	AE, ORR	Recruiting
EGFR-TKI							
NCT02716311	II	118	2016	EGFR mutant NSCLC	Afatinib Afatinib + cetuximab	TTF	Active, not recruiting
NCT02979977	II	50	2016	Advanced HNSCC	Afatinib + cetuximab	ORR	Recruiting
NCT03727724	II	37	2018	NSCLC	Afatinib + cetuximab	DCR	Recruiting
NCT04820023	I/II	90	2021	Advance d NSCLC	BBT-176 BBT-176 + cetuximab	AE, DLT, ORR	Recruiti ng
NKG2A							
NCT02643550	I/II	143	2015	R/M HNSCC	Monalizumab + cetuximab monalizumab + cetuximab + anti-PD(L)1	DLT, ORR	Active, not recruitin g
NCT04349267	I/II	308	2020	Advanced Solid Tumors	BMS-986315 BMS-986315 + nivolumab BMS-986315 + cetuximab	AE	Recruiti ng
NCT04590963	III	600	2020	R/M HNSCC	Monalizumab + cetuximab Placebo + cetuximab	OS	Recruiting
PD-1/PD-L1							
NCT02999087	III	707	2016	LA HNSCC	CRT Cetuximab + RT + avelumab	PFS	Active, not recruiting
NCT03174405	II	43	2017	mCRC	Avelumab + cetuximab + FOLFOX	PFS	Active, not recruiting
NCT03494322	II	130	2018	R/M HNSCC	Avelumab Avelumab + cetuximab	DLT, DCR	not recruiting
NCT03498378	I	24	2018	R/M HNSCC	Avelumab + cetuximab + palbociclib	MTD	Recruiting
NCT03608046	II	59	2018	mCRC	Avelumab + cetuximab + irinotecan	ORR	Recruiting
NCT03944252	II	54	2018	LA & R/M SCCAC	Avelumab Avelumab + cetuximab	ORR	Active, not recruiting
NCT04561336	II	77	2018	RAS-WT mCRC	Avelumab + cetuximab	OS	Active, not recruiting

AE, Adverse events, CR, Complete response, CRT, Chemoradiotherapy, CSCC, Cutaneous squamous cell cancer, DCR, Disease control rate, DLT, Dose limiting toxicity, ESqCC, Esophageal squamous cell carcinoma, HNSCC, head and neck squamous cell carcinoma, LA, Locally advanced, mCRC, Metastatic colorectal carcinoma, MTD, Maximum tolerated dose, OBD, Optimal biological dose, OR, Objective response, ORR, Objective response rate, OS, Overall survival, PFS, Progression free survival, R/M, Recurrent and metastatic, RT, radiotherapy, SCCAC, Squamous cell anal carcinoma, TTF, Time to treatment failure, WT, Wild-type.

quality treatments that will provide the most effective benefit to the patient.

AUTHOR CONTRIBUTIONS

HB, JDW, IDP, JBV, and AW conceived the presented idea. The writing of the presented work was supervised by JW, IP, and AW. The original draft was written by HB and reviewed & edited by HB, JW, IP, HZ, MP, JV, FL, and AW. All authors contributed to the article and approved the submitted version. All figures were created using BioRender.com

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Advances in Universal CAR-T Cell Therapy

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Chimeric antigen receptor T (CAR-T) cell therapy achieved extraordinary achievements results in antitumor treatments, especially against hematological malignancies, where it leads to remarkable, long-term antineoplastic effects with higher target specificity. Nevertheless, some limitations persist in autologous CAR-T cell therapy, such as high costs, long manufacturing periods, and restricted cell sources. The development of a universal CAR-T (UCAR-T) cell therapy is an attractive breakthrough point that may overcome most of these drawbacks. Here, we review the progress and challenges in CAR-T cell therapy, especially focusing on comprehensive comparison in UCAR-T cell therapy to original CAR-T cell therapy. Furthermore, we summarize the developments and concerns about the safety and efficiency of UCAR-T cell therapy. Finally, we address other immune cells, which might be promising candidates as a complement for UCAR-T cells. Through a detailed overview, we describe the current landscape and explore the prospect of UCAR-T cell therapy.

Keywords: cellular immunotherapy, chimeric antigen receptor T cell therapy, universal chimeric antigen receptor T cell therapy, gene editing, CRISPR/Cas9

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INTRODUCTION

With the vigorous development of cellular immunotherapy and the blowout of new clinical trials, various emerging cellular drugs have brought about a qualitative leap in the antineoplastic field. Chimeric antigen receptor T (CAR-T) therapy is the most rapid-developed and wide-applicated branch of anticancer cellular immunotherapy. This recent technology rapidly changed the landscape of hematological malignancies and already accounts for more than half of the cell therapies currently under development or in the market. As of March 2020, there were 1,483 anticancer cell therapies under research or on the market worldwide, with an increase of 46.7% compared with 1,011 in 2019. Among these, 858 are CAR-T cell therapies in 2020, a rise of more than 50% compared to the corresponding quarter last year (1).

In a nutshell, this technology is based on T lymphocytes isolated from the circulation, which are then engineered to express chimeric antigen receptors (CARs), enabling modified T lymphocytes to recognize and respond to cancer cells independently of a major histocompatibility complex (MHC) engagement. After proliferation *in vitro*, these cells are reinfused into the patient to drive antitumor immune responses (2). The first generation of CAR used an extracellular antigen-binding domain (usually the single chain variable fragment of an antibody), a transmembrane domain, and an intracellular signaling domain of the CD3ζ chain (**Figure 1A**), simply driving a transient T-cell proliferation and limited cytokine secretion (3). Later, costimulatory molecules such as CD28 or

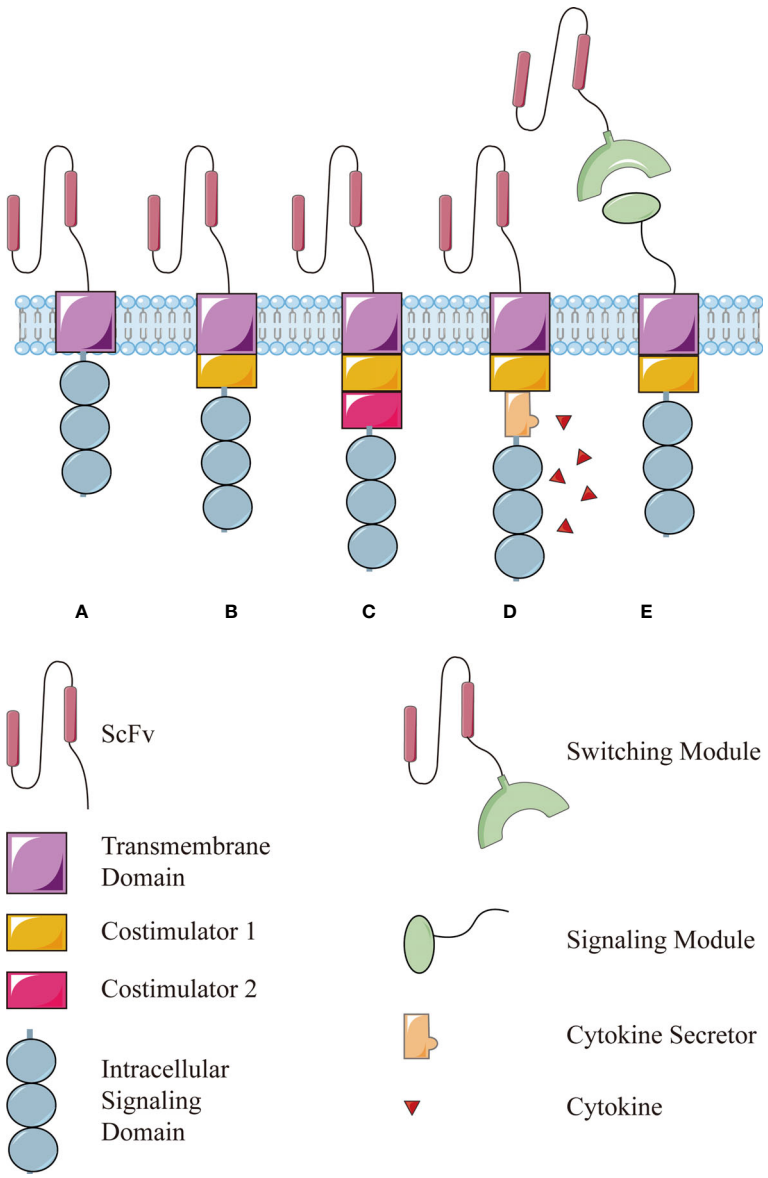

FIGURE 1 | The structure of conventional CAR and modular CAR: **(A)** the first generation of CAR consists of an extracellular antigen-binding domain (usually the single chain variable fragment, scFv), a transmembrane domain, and an intracellular signaling domain of the CD3ζ chain. Then, a costimulator is added in the **(B)** second generation and more in the **(C)** third generation. **(D)** The fourth generation of CAR is modified further to secret a cytokine to enhance the function. **(E)** The modular CAR is split into two interactive parts, the signaling module on T cells and the switching module to recognize targets.

4-1BB were incorporated into CAR structure to promote CAR-T cells survival and functionality *in vivo*, leading to the second generation CAR (**Figure 1B**) (4) and then paired as the third generation of CAR structures (**Figure 1C**) (5). Recently CAR-T cells have been further modified to secreted cytokines such as interleukin (IL)-12, which enhances T-cell viability, recruits and activates other immune cells to enhance potency or safety (**Figure 1D**) (6–8).

The second-generation CAR-T cell is the most effective and widely used. Five CAR-T cell products, namely, Kymriah (tisagenlecleucel, tisa-cel), Yescarta (axicabtagene ciloleucel, Axi-Cel), Tecartus (brexucabtagene autoleucel, KTE-X19), Breyanzi (lisocabtagene maraleucel, liso-cel), and Abecma (idecabtagene vicleucel, Ide-cel), have been approved by the Food and Drug Administration (FDA) for clinical treatment in relapsed or refractory acute B lymphoblastic leukemia, B lymphoid malignancies, and multiple myeloma, respectively. In China, Yescarta was the first approved CAR-T cell product released on the market on June 22, 2021. Relma-Cel, another anti-CD19 CAR-T cell product, is under premarket review as well. In the most recent reports, objective remission rates of Kymriah and Yescarta in the treatment of relapsed or refractory

B non-Hodgkin's lymphoma have reached 52% and 82%, respectively (9, 10).

Nonetheless, some limitations hinder the dissemination and development of CAR-T cell therapy. First, many factors may lead to the failure of CAR-T cell therapy, including the intrinsic factors (such as poor CAR-T cell expansion or short persistence) and extrinsic factors (tumor cells with target deletions or mutations and tumor inhibitory microenvironment) (11). Second, the safety concerns still need to be addressed. CAR-T cells drive tumor clearance but can also lead to potentially lethal toxicity, including cytokine release syndrome (CRS) and neurotoxicity caused by CAR-T cells overactivation, excessive cytokine release, and "on-target/off-tumor effect" due to low specificity of antigen expression (12, 13). In addition, the high cost and the labor-intensive manufacturing process of CAR-T cells still hamper the popularization of CAR-T cell therapy. A one-time infusion of Kymriah costs $475,000, and the total cost for Kymriah or Yescarta treatment is nearly 1 million dollars per patient (14). Furthermore, the current production cycle takes about 2 weeks, during which highly proliferative malignancies continue to progress (15). Moreover, cancer patients frequently suffer from congenital immunodeficiency or lymphocytopenia after repeated chemotherapies, resulting in suboptimal T cells inadequate for CAR-T cell manufacturing. Rarely, but worst of all, if leukemic blasts contaminate isolated lymphocytes and are inadvertently loaded with CAR, they can mask the targets and escape from CAR-T cells. Until now, there was only one reported case where leukemic B cell was unintentionally modified by CD19-CAR, conferring resistance to CD19 CAR-T cells and leading to lethal complications related to progressive leukemia ultimately (16). All of these pitfalls cast a shadow over the development of CAR-T cell therapy (17).

Currently, universal CAR-T (UCAR-T) cell therapy is in the spotlight and expected to break the plight. All existing CAR-T cell products on the market or under testing are autologous (made with same patient-derived T lymphocytes) to avoid severe alloimmune rejection due to a mismatch of MHC between the donor and the recipient. Alternatively, UCAR-T cells would consist of allogeneic CAR-T cells that are taken from healthy donors. Despite sharing the same killing mechanism, UCAR-T cells have distinct manufacturing processes, cost, safety considerations, and applicability (**Table 1**) (18). When customized CAR-T cell therapy can evolve into a universal therapy, many of the flaws that impede CAR-T cell dissemination can be readily addressed. Finally, large-scale production procedures and batch manufacturing could greatly increase the quality and accessibility of CAR-T cell products.

THE EVOLUTION OF UCAR-T CELL THERAPY

The concept of allogeneic CAR-T cell therapy has persisted for a long time. In relapsed patients, successfully treated by allogeneic hematopoietic stem cell transplantation (allo-HSCT), CAR-T cells can be produced from the transplant donors or recipients, but the efficacy and safety of each are still uncertain. In an early study (NCT01087294), 10 persistent patients with B-cell lymphoma or leukemia after allo-HCST and standard donor lymphocyte infusions received transplant donors-derived allogeneic CAR-T cells without lymphodepletion. Three of them showed tumor regression, but none of these patients showed graft versus host disease (GVHD) (19). In another study with longer follow-up, 8 [6 complete responses (CRs) and 2 partial response (PRs)] of 20 patients entered remission, with none developing new-onset acute GVHD and only 2 with mild chronic GVHD after CAR-T cells refusion (20). In contrast, a similar study (NCT01864889) reported grade 2–3 GVHD in two patients 4 weeks after donor-derived CAR-T cells infusions (21). Recently, a retrospective study compared 14 patients receiving allogeneic CAR-T cells (3 donor-derived and 11 recipient-derived) after HSCT with 17 patients receiving autologous CAR-T cells (22). These showed no significant difference between autologous CAR-T cells and recipient-derived allogeneic CAR-T cell therapy on CR rate and long-term survival, but the latter with significantly lower proliferation

TABLE 1 | The comparison of autologous and allogeneic CAR-T cell therapy.

	Autologous CAR-T cell therapy	Universal CAR-T cell therapy
Consistency		
Killing mechanism	MHC-independent	
Gene editing to avoid fratricide	Carried out if needed	
Manufacturing process	T lymphocytes are isolated and transduced with a specific CAR by viral vector, then refused to the patient after amplification	
Difference		
Cell source	Patients themselves	Healthy donors
Activation of the immune system in patients	Hardly	Possible
Manufacturing Line	Customized	Batched
Additional Gene Editing to avoid GVHD and rejection	Unnecessary	Necessary
Cost	High	Much lower
Immediate availability	No	Yes
Application in T-cell malignancies	Restricted	Promising
Main risks	CRS;CRES	CRS;CRES;GVHD
Limitations	Suboptimal quantity and quality of T cells in patients	Lower amplification and shorter persistence *in vivo*

CAR, chimeric antigen receptor; CRS, cytokine release syndrome; CRES, CAR-T cell-associated encephalopathy syndrome; GVHD, graft versus host disease.

and decreased cytokine release reaction. In this study, only two recipient-derived (18.2%) and 1 donor-derived (33.3%) allogeneic CAR-T cells caused acute GVHD (22).

These inconsistent results of GVHD may be explained by chronic hyperactivation, accelerated exhaustion, and activation-induced cell death (AICD), resulting from double stimulation from T-cell receptor (TCR) and CAR on allogeneic CAR-T cells. In a donor-derived allogeneic CAR-T cell mouse model, Arnab Ghosh et al. demonstrated that allogeneic CAR-T cells could be activated by CAR and TCR, respectively; however, activation of one receptor could restrain the function of the other. Hence, GVHD was alleviated when CD19-positive cells activated allogeneic CAR-T cells (via anti-CD19 CAR) before TCR-engagement by alloantigen. Therefore, they recommended that allogeneic CAR-T cells should be transfused only after B lymphocytes recovering from transplantation (23). A contradictory report that only CD19-positive leukemia could drive allogeneic activation of CAR-T cells and mediate acute GVHD. When activated by tumor cells, allogeneic CAR-T cells showed more severe rejection to the alloantigen (24). This discrepancy may be related to the degree of activation of UCAR-T cells. When the stimulation of CAR by target antigen is moderate, allogeneic CAR-T cell is activated but not excessively, driving an effective response to alloantigen. But when CD19 stimulation is overly strong, CAR-T cells become exhausted and unresponsive to allogeneic antigens. This suggests a delicate relationship between CAR and TCR in constant competition and collaboration. Given the complexity of dual signal controlled by TCR and CAR, the elimination of GVHD by disrupting TCR has become a strategy adopted by most allogeneic CAR T-cell researchers.

This strategy of transplant bridging to a recipient or donor-derived CAR-T cell therapy is stranded in one-to-one correspondence, far from the envisaged one-to-many universalization. With the accumulation of experience in allogeneic CAR-T cells, the production of "off-the-shelf" CAR-T cells from third-party healthy donors has been put on the agenda. At the American Society of Hematology (ASH) meeting in December 2017, Cellectis announced the preliminary results of two clinical trials of UCART19, and since then, universal CAR-T cell therapy has officially come into the public sight.

RECENT DEVELOPMENTS IN UCAR-T CELL THERAPY

Targets of UCAR-T Cell Therapy

There have been more than hundreds of preclinical and clinical trials of allogeneic CAR-T cell therapy worldwide (18, 25). The majority of these are applied to hematological malignancies, where the most popular target is CD19, and other classic targets, including CD20, CD22, and BCMA. New developing targets such as CD70, CD7, and CD5 are also included (18, 26). NKG2DL, GD2, and mesothelin for solid tumors are also emerging (**Table 2**) (18, 29, 30).

Allogene Therapeutics was the forerunner in this UCAR-T field with UCART19. Two multicenter phase I clinical trials

TABLE 2 | Summary of targets involved and strategies to improve the efficiency in UCAR-T cell therapy.

Target	UCAR-T product	Improving strategies	Editing tools	Development phase	Reference/NCT number
CD 19	UCART019	TRAC and B2M KO	CRISPR/Cas9	Phase I/II	NCT03166878
	CTX110	TRAC and B2M KO	CRISPR/Cas9	Phase I	NCT04035434
	/	TRAC, B2M and PD-1 KO	CRISPR/Cas9	Preclinical	(27)
	UCART19/ALLO-501	TRAC KO with or without CD52 KO	TALEN	Phase I	NCT02735083; NCT02808442; NCT02746952;
	FT819	TRAC KO and iPSC-derived T cells	CRISPR/Cas9	Phase I	NCT04629729;
BCMA	CTX120	TRAC and B2M KO	CRISPR/Cas9	Phase I	NCT04244656
CD123	UCART123	TRAC KO	TALEN	Phase I	NCT03190278; NCT03203369
CD22	UCART-22	TRAC and CD52 KO	TALEN	Phase I	NCT04150497
CS1	UCARTCS1A	TRAC and CS1 KO	TALEN	Phase I	NCT04142619
CD19/CD20; CD19/CD22	Universal dual specificity CAR-T cells	TRAC KO	CRISPR/Cas9	Phase I/II	NCT03398967
CD5	CT125A	TRAC and CD5 KO	CRISPR/Cas9	Phase I	NCT04767308
CD7	GC027	TRAC and CD7 KO	CRISPR/Cas9	Phase I	(28)
	UCART7	TRAC and CD7 KO	CRISPR/Cas9	Preclinical	(26)
CD70	CTX130	TRAC and B2M KO	CRISPR/Cas9	Phase I	NCT04438083; NCT04502446
Mesothelin	/	TRAC and PD1 KO	CRISPR/Cas9	Phase I	NCT03545815
NKG2D	CYAD-101	TIM peptide of CD3ζ	Retroviral vector	Phase I	NCT03692429
NKG2DL	CTM-N2D	γδ T Cells	/	Phase I	NCT04107142
GD2	/	EBV-CTLs	/	Phase I	NCT00085930

TRAC, T-cell receptor alpha constant chain; B2M, beta-2-microglobulin; PD-1, programmed cell death protein 1; CRISPR/Cas9, clustered regularly interspaced short palindromic repeats/Cas9; TALEN, transcription activator-like effector nuclease; iPSC, induced pluripotent stem cell; BCMA, B-cell maturation protein; TIM peptide, TIM peptide TRAC-inhibitory molecule peptide; EBV-CTLs, Epstein–Barr virus-specific cytotoxic T lymphocytes; KO, knockout.

(NCT02808442 and NCT02746952) aiming to investigate the safety, feasibility, and antileukemic activity of UCART19 in children and adults with relapsed or refractory B-cell acute lymphoblastic leukemia have been conducted. Seven children and 14 adults were enrolled, of which 14 (14/21, 67%) patients had a complete response or complete response with incomplete hematological recovery 28 days after infusion. CRS (19/21, 91%) was the most common adverse side effect, of which 3 (3/21, 14%) were grade 3–4. Other adverse events included mild neurotoxicity (8/21, 38%), grade 4 prolonged cytopenia (6/21, 32%), and grade 1 acute skin GVHD (2/21,10%). Two treatment-related deaths were reported as a result of neutropenic sepsis and pulmonary hemorrhage, respectively (31). Two infants mentioned above acquired molecular remission and bridged to allogeneic HSCT successfully (32). UCART19 is undoubtedly a remarkable step forward in the field of UCAR-T cells, and it offers an opportunity for patients with rapidly progressive diseases who cannot access autologous CAR-T cell therapy.

In addition to CD19, targets of UCAR-T cell products being developed by Allogene Therapeutics include CD123 (NCT03190278, NCT03203369), CD22 (NCT04150497), BCMA (NCT04093596), and CS1 (NCT04142619). Unlike the smooth progress of CD19, the CD123 program has been full of twists and turns. In November 2017, after one death in the clinical trial (NCT04106076), it was announced that UCART123 would continue two phase I clinical trials for acute myeloid leukemia (NCT03190278) and blastic plasmacytoid dendritic cell neoplasm (NCT03203369) subject to agreed clinical regimens with FDA. The detailed results are still unknown.

Most research targeted one specific marker, but UCAR-T cell allows for a CD19/CD20 and CD19/CD22 (NCT03398967) multitarget approach. Recently, Yongxian Hu et al. reported CTA101, a universal CD19/CD22 dual-targeted CAR-T cell that disrupted T-cell receptor alpha chain (TRAC) and CD52 by clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9). This exhibited a CR rate of 83.3% (5/6) without dose-limiting toxicity, GVHD, neurotoxicity, or adverse events related to genome editing (33).

Currently, there are just a few registered UCAR-T cells clinical trials for solid tumors, such as allogeneic NKG2DL-targeting CAR-T cells (NCT04107142) for relapsed or refractory colorectal cancer, breast cancer, and sarcoma. Additionally, allogeneic disialoganglioside 2 (GD2)-targeting CAR-T cells are under test for relapsed or refractory neuroblastoma (NCT01460901) and allogeneic CD70 targeting CAR-T cells for relapsed or refractory renal cell carcinoma (NCT02830724). The latter has been suspended. Based on these clinical trials, it is likely that UCAR-T cell therapy will be first used for hematological malignancies, while for solid tumors, the UCAR-T cell study is still in its infancy with broad prospects for the future.

Gene Editing in UCAR-T Cell Therapy

The CAR-T cell is commonly transduction with viral vectors, mostly lentiviral vectors, which have an advantageous transfection efficiency and stable expression. However, random genome integration raises the risk of insertion mutation and disruption of functional genes (34). Therefore, the development of UCAR-T depends on the progress of gene-editing technology. A variety of gene-editing methods have been applied to improve transduction efficiency, diminish GVHD, and enhance persistence. Zinc-finger nucleases (ZFN) (35), transcription activator-like nucleases (TALENs) (36), and CRISPR/Cas9 (25, 33) can all achieve positional editing in the genome and have been employed in UCAR-T cell therapy. TALENs is most adopted by Allogene Therapeutics, and CRISPR/Cas9 offers even greater flexibility, maneuverability, and relative accuracy, opening the possibility of multiple gene editing (**Figure 2**). Currently, it has been employed in several clinical trials, including UCART019 targeting CD19 (NCT03166878), CTX130 targeting CD70 (NCT04502446, NCT04438083), CTX120 targeting BCMA (NCT04244656), and CT125a targeting CD5 (NCT04767308). For the expression of CAR in check, CD19-specific CAR is knocked into the TRAC locus of T cells, by which its expression is enhanced and unified under the control of the TCR promoter (37, 38). In UCART7 targeting CD7 for T-cell malignancies, TRAC and CD7 are simultaneously knocked down, the former for preventing GVHD and the latter for preventing fratricide of the very effector cells (26).

On the other hand, non-gene editing universal CAR-T therapy has also achieved initial results. Celyad has conducted several clinical trials with the CYAD-101, a non-gene editing natural killer group 2D (NKG2D)-based UCAR-T cell product, in both solid and hematological tumor types. It tampered with or eliminated TCR signals and reduced GVHD by expressing a TRAC-inhibitory molecule (TIM) peptide. The preliminary results of the phase I trial showed no evidence of CYAD-101 causing GVHD in the treatment of metastatic colorectal cancer. Using the short hairpin RNA (shRNA) platform of Horizon, Celyad has developed the next generation non-gene edited allogeneic CYAD-200 series of CAR-T candidates.

Modularization and Logic Gating

Gene editing transforms T cells from third-party healthy donors to a stable and universal cell resource, while the development of CAR structure makes it possible to design a CAR for multiple targets at the same time, the combination of which enables the idea of an upgraded UCAR-T (5).

In 2012, Urbanska et al. proposed a modular CAR design composed of extracellular-modified avidin linked to an intracellular T-cell signaling domain. These modified T cells recognized and bound exclusively to cancer cells pretargeted with specific biotinylated junction molecules, such as biotinylated antibodies (39). Despite the high immunogenicity in humans, this idea opened the door to the modularization of CAR structure (**Figure 1E**). The CAR is split into two parts: (i) the signaling module on T cells, consisting of the extracellular domain that specifically binds to the switching module and the intracellular domain that transmits the activation signals; (ii) the switching module, usually a bispecific antibody or small molecule recognized by the signaling module on T cells and binding to the targets on cancer cells. This split, universal, and

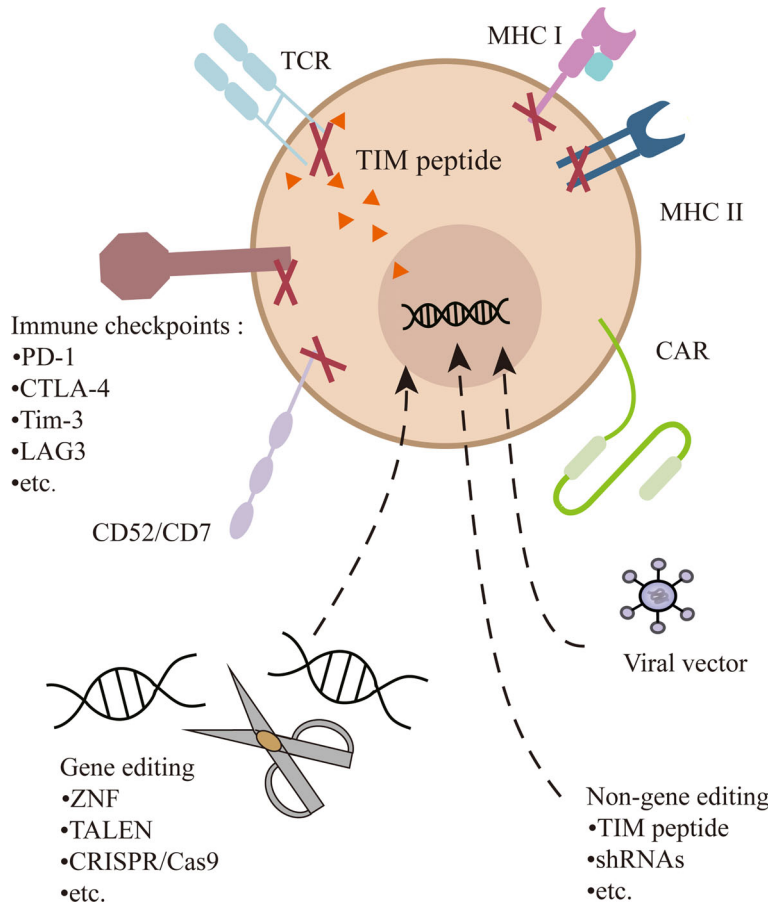

FIGURE 2 | Multiple gene or non-gene editing on UCAR-T cells. In addition to transducing a CAR into T cells, the TCR can be knocked out or inhibited to prevent GVHD. Genetic ablation of MHC-I and/or MHC-II diminish immunogenicity. Destruction of CD52 can make cells resistant to alemtuzumab. CD7 is edited to prevent the fratricide in CD7 UCAR-T cells. In addition, inhibitory checkpoints (e.g., PD-1) can be knocked out to enhance the function of cells.

programmable (SUPRA) CAR system currently adopts a variety of recognition modes including neo-epitopes, SpyTag, biotin, and fluorescein isothiocyanate (FITC) and leucine zippers (40). Clinical trials of SUPRA CAR have been carried out for CD19/CD20 (NCT02776813) and CD123 (NCT04230265). Other targets under development include CD33, prostate stem cell antigen (PSCA), prostate-specific membrane antigen (PSMA), GD2, epidermal growth factor receptor (EGFR), cell-surface-associated mucin 1 (MUC1), and sialyl-Tn (STn) (29). What is more, the CD123-specific targeting module has been further deimmunized to mitigate the potential immunogenicity, which proved its good tolerance and targeting effect in the human body (41).

This flexible CAR structure changes the original rigid structure of CAR to improve security and feasibility. As a bridge between CAR-T cells and tumor cells, the dosage of the switching module can be titrated because it conforms to general pharmacokinetics, and its affinity to target antigens can be regulated by fine-tuning the structure to take control of CAR-T cell activation. Besides, CAR-T cells are held back by blocking agents, which competitively inhibit switching modules when necessary.

Recently, a photo-switchable CAR-T cell with dose-dependent and rapidly terminated cytotoxicity has appeared. Switching modules carrying dual folate and FITC tethered by an ortho-nitrobenzyl ester photocleavable linker (folate-O-FITC), CAR-T cells are turned off under the light of 365 nm, in which switching modules were snipped and activated again by resupplementation with the mediator (42). These make it possible to accurately predict and control the activation of CAR-T cells and the release of cytokines. When various switching modules are injected simultaneously, multitarget CAR-T cell therapy can be easily achieved without altering cells, which is promising in preventing targets mutation (43, 44).

What is even more exhilarating is the logical control of CAR-T cells through multiple switching modules. Existing bispecific CAR T-cell therapy adopts "OR" logic, in which CAR-T cells are activated if the tumor cells express a single target (**Figure 3A**). The modular CAR design can function "AND" and "NOT" gate to promote selective tumor eradication without on-target,

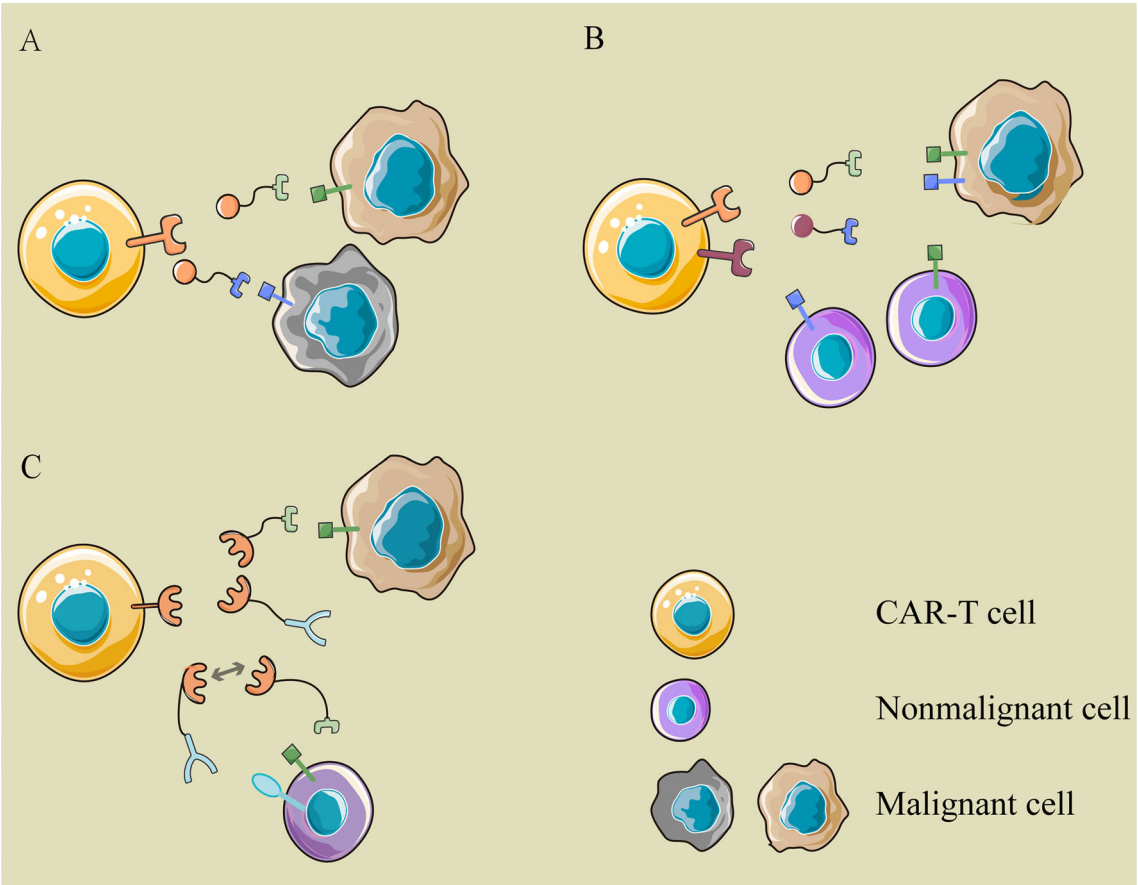

FIGURE 3 | The logic gatings in modular CAR. **(A)** OR logic: the modular CAR-T cell can eliminate different cancer cells with various switching modules, which are recognized by the same CAR-T cell but target different antigens on cancer cells. **(B)** AND logic: the antigen-binding domain and costimulator are separated into two CARs targeting different antigens and cotransduced into T cells. Only when tumor cells express two antigens simultaneously can they be recognized and attacked by these CAR-T cells. **(C)** NOT logic: a tumor-associated antigen is expressed on cancer cells and normal cells simultaneously, while another antigen is expressed on normal cells only. The two modules binding to them are complementary in the site recognized by the signaling module. The extra target works as a safety label to prevent the "on-target, off-tumor" toxicity of CAR-T cells.

off-tumor toxicity (45, 46). For "AND" gating strategies, the antigen-binding domain and costimulatory domain can be separated into two CARs targeting different antigens and cotransduced into T cells (**Figure 3B**). Aiming at two tumor-associated antigens, PSMA and PSCA, Christopher Kloss et al. constructed such an "AND" logic bispecific CAR-T cell, which destroyed tumors that expressed both PSMA and PSCA but did not work on tumors expressing either alone (45). Similarly, the modular CAR system can perform the "NOT" logic to increase tumor specificity through combinatorial antigens (**Figure 3C**). For instance, a SUPRA CAR system targeted cells expressing Her2 only and spared cells expressing Her2 and Axl both. In this design, both Her2- and Axl-positive cells are bound to two switching modules, α-Her2-EE zipFv and α-Axl-SYN2 zipFv, simultaneously. Then, these two modules recognized and combined with each other by zipFv, so they failed to activate CAR-T cells (47). This suggests that when tumor-associated antigens are also expressed on normal cells, an additional target can be combined as a "safety label" to further distinguish normal cells from tumor cells.

When modular CAR is adopted in UCAR-T cells, the ultimate goal of treating different cancers with cells of stable source and CAR of identical structure makes solid progress.

CHALLENGES IN UCAR-T CELL THERAPY

Safety of UCAR-T Cell Therapy

Allogeneic cells and complex gene editing make people more cautious about UCAR-T cell therapy. The existing risks in autologous CAR-T cell therapy, such as CRS and neurotoxicity, cannot be ruled out in UCAR-T cell therapy. However, the GVHD is the first and foremost challenge that hinders the realization of this therapy. It is logical to knock out the TCR on cells and then enrich the TCR-negative UCAR-T

cells for reinfusion. The improvements in gene editing make this technically achievable (48). However, gene editing is not necessarily a complete gospel. Complex genetic manipulation increases the risk of unexpected gene mutations (49). Safe and efficient gene manipulation is still being explored. What is more, higher-dose lymphodepletion chemotherapy in UCAR-T cell therapy is accompanied by the increased risk of opportunistic infection. All of these can be fatal for patients.

Efficiency of UCAR-T Cell Therapy

CAR T cells should undergo a process of proliferation and then persist *in vivo*. Cytokinetics revealed the comparable early expansion but shorter persistence in allogeneic CAR T-cells than autologous CAR-T cells and failure to generate a memory pool (24). In related clinical trials, the failure of UCAR-T cells to expand and maintain sufficient levels in patients remains a major concern. This can be solved by alleviating the host rejection or reducing the immunogenicity of infused cells (**Table 2**).

Increasing clinical practice shows that the lymphodepletion chemotherapy before cell infusion creates a favorable environment for the expansion of CAR-T cells *in vivo*. The commonly used conditioning regimens are fludarabine combined with cyclophosphamide, but more exhaustive lymphodepletion has been applied in UCAR-T cell therapy. In the landmark clinical trial of UCART19 (NCT03939026), T cells were engineered to knock out genes encoding TCRA and CD52, to disrupt the structure of TCR and acquire resistance to anti-CD52 monoclonal antibody alemtuzumab, since CD52 is both positive in T and natural killer (NK) cells, which eliminate the allogeneic CAR-T cells in recipients. The addition of alemtuzumab can further suppress the allogeneic immune rejection in hosts and extend the therapeutic window for the amplification of UCAR-T cells. It was clear that all patients with CR were pretreated with fludarabine, cyclophosphamide, and alemtuzumab (14/17, 82.4%), but none of the four patients without alemtuzumab showed UCART19 expansion or antileukemic activity. The finding illustrated the absolute necessity of a powerful and thorough lymphodepletion for UCAR-T cells amplification (31). Similar gene-modifying and pretreatments were found in CTA101, a CRISPR/Cas9-engineered universal CD19/CD22 dual-targeted CAR-T cells (33).

Except in combination with CD52 knocking out, UCAR-T cells resistant to traditional chemotherapeutics have also been designed. Purine and pyrimidine nucleoside analogues, as common chemotherapeutic agents, such as clofarabine, fludarabine, and cytarabine, take effect only after being metabolized by deoxycytidine kinase (dCK). TCR-negative and chemotherapeutics-resistant UCAR-T cells were obtained by employing TALEN to block the expression of TRAC and dCK, which made it possible to lymphodeplete repeatedly whenever lymphocytes recover without impacting UCAR-T cells unintentionally. Besides, lymphocytes of the recipient might restore by breaking off lymphodepletion and remove overkilling UCAR T-cells to prevent severe toxicity (50).

Like CD52, CD7 is a transmembrane glycoprotein with expression on T cells and NK cells, and it is also a target of great concern in T-cell tumors. In CD7 UCAR-T targeting T-cell malignancies, TCR and CD7 are also knocked out to avoid GVHD and fratricide between effector cells, respectively. In addition to malignant T cells, CD7 UCART can recognize normal T and NK cells as well, resulting in more lasting lymphodepletion. Mathew et al. reported that this UCAR-T cell kept robust antileukemia effect in cell lines and primary T-cell acute lymphoblastic leukemia (T-ALL) blasts *in vitro* and in NSG mice, and no fratricide or GVHD was found (26). Recently, an open-label and single-arm clinical trial of GC027, a CD7 UCAR-T of TCR and CD7 edited by CRISPR, was published in two patients with refractory/relapsed T-ALL after potent lymphodepletion (fludarabine, cyclophosphamide, and prednisone) and a single infusion of GC027. Both patients achieved CR with negative minimal residual disease, and one remained ongoing remission at cutoff (28).

Thoroughly, lymphodepletion is accompanied by serious T-cell aplasia. Different from B-cell aplasia, which can be compensated by periodic infusions of intravenous immunoglobulins, the persistent deficiency of T and NK cells is life threatening. Ideally, one would suppress immunological rejection but retain part of the immune protection. One of the characteristics of alloreactive T and NK cells is the upregulation of 4-1BB on their surface (51, 52). Feiyan Mo et al. engineered an alloimmune defense receptor that identified and attacked 4-1BB upregulated lymphocytes and coexpressed it in allogeneic CAR-T cells. These therapeutic cells could eliminate alloimmune lymphocytes and tumor cells simultaneously but leave resting T and NK cells alone. Later, they found that these CAR-T cells produced sustained tumor eradication without being rejected in mice (53). Although it is still in the preclinical stage, this study can drastically shift the paradigm of prolonging the persistence in UCAR-T cell therapy and broaden its applicability.

Apart from suppressing the immune system in hosts, reducing the immunogenicity of UCAR-T cells is another approach to enhance its persistence. MHC is the major antigen system driving graft rejection. MHC-I is expressed on the surface of almost all living cells in human; therefore, inhibiting the expression of MHC-I can evade the attack of alloreactive T cells in recipients. CRISPR Therapeutics has been taking such an approach, including CT110 targeting CD19, CTX120 targeting BCMA, and CTX130 targeting CD70. Endogenous TCRA and β-2 microglobulin(B2M) genes are disrupted simultaneously by applying CRISPR RNA electroporation to manufacture UCAR-T cells, which are both TCR and MHC-I negative, aiming to evade rejection and deliver antileukemic effects without GVHD, but the results of these studies are still unpublished. Another upgrade study was to generate gene-disrupted allogeneic CAR-T cells deficient in TCR, MHC-I, and PD-1, which demonstrated reduced alloreactivity and enhanced antitumor activity *in vivo* without causing GVHD (27).

Although UCAR-T cells are exempt from alloreactive T cells by B2M knocking out, another militant, the NK cells, are activated in the absence of MHC-I on UCAR-T cells and evolve into the main force in the elimination of UCAR-T cells. Several strategies have been tried to inhibit or clear reactive NK

cells in recipients, but it is not easy to adopt a broad strategy to suppress all NK cells for the heterogeneity of NK cells. Upregulation of human leukocyte antigen (HLA)-E on UCAR-T cells, for example, showed inhibition of a subset of NK cells by binding to NKG2A/B receptors while stimulating another group of NK cells by activating the NKG2C (54), but more studies are needed to achieve the inhibition of activated NK cells.

MHC-II molecule is the subordinate factor to mediate alloimmune rejection by $CD4^+$T cells, and its expression is regulated by regulatory factor X ankyrin repeat-containing protein (RFXANK) and class II MHC transactivator (CIITA) (55, 56). Allogeneic anti-CD19 CAR T cells with B2M, CIITA, and TRAC triple knocking out showed better persistence when cultured with allogenic peripheral blood mononuclear cells (PBMCs) than TRAC and B2M double knocking-out CAR-T cells, without altering the function of T cells (57). Similar engineering in iPSC was conducted to disrupt B2M, CIITA, and CD155 (encoding an activating ligand of NK cells) and transduce HLA-E, serving as a source of CAR-T cells. These hypoimmunogenic CAR-T cells largely escaped rejection by $CD8^+$T cells, $CD4^+$T cells, and NK cells, maintaining antitumor cytotoxicity (58).

Multiple gene editing strategies reduce rejection of UCAR-T cells *in vivo*. On the other hand, increasing accessibility and further ablation of immunogenicity in UCAR-T cells allows for multiple reinjections, making CAR-T cell therapy more like conventional drugs, in which efficacy and side effects can be easily controlled by repeated and transient infusions of cells.

ALTERNATIVE UNIVERSAL CELL THERAPIES

At present, most CAR-T cells are derived from T cells in PBMCs. However, other types of cells may have unique advantages in the process of universalizing the cell therapy, as a supplement or substitutions of UCAR-T cell therapy (59).

Other Subpopulations of T Cells

Certain subsets of T cells with unique superiority in mitigating GVHD are also promising candidates for producing UCAR-T cells. Based on the peptide chain structure of TCR, T cells are divided into αβT cells consisting of α and β chains and γδT cells with γ and δ chains. Despite in lower frequencies, γδT cells play an important role in the innate immune response and anti-infective or antitumor reaction independent of the MHC or antigen-presenting cells (APCs) (60, 61). In antitumor immunity, γδT cells recognize and eliminate tumor cells independent of TCR, which responds to a specific tumor-associated antigen (TAA) (62, 63). These characteristics endow γδT cells with inherent advantages in cellular immunotherapy in solid tumors that lack specific TAAs. Anna Capsomidis et al. reported that GD2-CAR γδT could amplify *in vitro* retaining antigen-presenting function and the GD2-targeting ability (64). A registered clinical research (NCT04107142) based on allogeneic NKG2DL-targeting CAR γδT cells against multiple solid tumors, including colorectal cancer, breast cancer, sarcoma, nasopharyngeal cancer, prostate cancer, and gastric cancer, is still in phase I.

Invariant natural killer T (iNKT) cells are another cell subpopulation that share characteristics of NK and T cells, and they have striking intrinsic antitumor activity for their endogenous TCR, which restrictedly recognizes foreign lipid antigens in the context of CD1d (65, 66). It has been reported that adoptive transferred iNKT cells are able to exert graft versus leukemia (GVL) but suppress GVHD after HSCT in leukemia patients (67). Previous studies have shown that iNKT cells engineered with CAR have equivalent or better cytotoxicity with a better safety profile than conventional CAR-T cells in solid tumors (66, 68, 69). A clinical study of allogeneic CAR19-iNKT cells for hematological malignancy (NCT03774654) is ongoing (70).

In addition, regulatory T cells expressing chimeric antigen receptors (CAR-Tregs) have been tried in autoimmune diseases to induce immune tolerance after organ transplantation (71, 72).

Natural Killer Cells

Compared with CAR-T cell therapy, chimeric antigen receptor NK (CAR-NK) cells focus on natural killer cells, another protagonist in the human immune system, which play an important role in innate and adaptive immunity. Like γδT cells, NK cells take effect without the aid of MHC and are at low risk of GVHD. The activity of NK cells is coregulated by inhibition signals and activation signals. Most of the MHC-I molecules are inhibitory for NK cells and deregulated on tumor cells (73). With these superiorities, NK cells are the rising star of tumor immunotherapy. CAR-NK cells preserve natural killing functions independent of CAR, such as antibody-dependent cell cytotoxicity (ADCC) and cytolysis by secreting granzyme and perforin (74). In addition to PBMC, NK92 cell line, umbilical blood (UCB), CD34 hematopoietic progenitor cells (HPCs), and induced pluripotent stem cells (iPSCs) can also substitute or transform into NK cells. The ongoing clinical trials of CAR-NK cells are mainly based on NK92 cell lines and PBMC (75). Despite limitations such as the tumorigenic risk of the NK92 cell line and the short duration of the CAR-NK cells *in vivo* (73), CAR NK-cell therapy remains a promising direction as off-the-shelf cellular immunotherapy.

Hundreds of preclinical and clinical trials of CAR-NK cell therapy have been launched, with almost evenly splitting between solid tumors and hematological malignancies. In a clinical trial (NCT03056339) in CD19-positive lymphoid tumors, NK cells were transduced to express genes encoding anti-CD19 CAR, interleukin-15, and inducible caspase 9 as a safety switch. Of the 11 treated patients, 8 (73%) had a response, and 7 (64%) had a complete remission. Regarding safety, no cases of CRS or neurotoxicity were observed, neither any obvious increase in inflammatory cytokines nor GVHD with this HLA-mismatched CAR-NK product (nine partial matching at four of six HLA molecules and two non-HLA matched) (76). This preliminary study proves the safety advantages of CAR-NK cells in universal cell therapy.

Induced Pluripotent Stem Cell

Induced pluripotent stem cell (iPSC) is a hotspot of research with unlimited capability to self-renew and differentiate into terminal cells, including T and NK cells with demonstrable antitumor activity. Besides, piles of homogeneous therapeutic cells from iPSC can be prefabricated, inspected, and banked across MHC barriers (77, 78). FT819, an iPSC-derived UCAR-T cell product expressing anti-CD19 CAR and antibody-engaging CD16 Fc receptor and TCR knockout, has shown the efficiency of controlling leukemia progression *in vitro* and *in vivo* in a mouse model, without alloreactivity (79). Maria Themeli et al. reported that iPSC-derived CAR-modifying T cells that resemble the phenotype of congenital γδT cells could effectively inhibit tumor growth in xenotransplantation models (80). Similarly, iPSC-derived CAR-NK cells demonstrated significant tumor inhibition and prolonged survival in the ovarian cancer xenograft model (15, 81). Nevertheless, the immortalization of iPSC also has both risks and opportunities, as the tumorigenic potential of undifferentiated iPSC has not been ruled out yet (48).

Macrophage

Extracellular matrix (ECM) is very important for the development of malignant solid tumors and can act as a physical obstacle to various anticancer treatments, including CAR-T cells. Innate immune cells with phagocytosis activity, such as macrophages, can secrete matrix metalloproteinases (MMPs) to degrade almost all ECM components and penetrate tumors (82). Gene engineering with CARs imparted macrophages a sustained proinflammatory phenotype (M1) and antigen-specific phagocytosis (83). Recently, in two xenograft mouse models, CAR macrophages (CAR-M) targeting the solid tumor antigens mesothelin or HER2 decreased tumor burden and prolonged overall survival, which preliminarily proved its feasibility in solid tumors (84).

CONCLUSIONS AND PROSPECTS

The achievements of CAR-T cell therapy in hematological malignancies have established cellular immunotherapy as a new pillar of antitumor therapy, but a series of limitations, such as high cost, low accessibility, and uncontrolled quality, have restricted its further dissemination and application. UCAR-T cell therapy is a comprehensive upgrade based on the original CAR-T cell therapy, which can remarkably improve accessibility and applicability. The gallop of gene-editing technologies and more plentiful cell sources have given it wings to reality. Many scientific and medical institutions and biotech companies have made initial successful attempts, although the persistence of UCAR-T cells is not as good as that of autologous CAR-T cells so far. In conclusion, the ultimate goal of UCAR-T cell therapy is to develop a conventional, living drug, just like blood transfusion, to provide a powerful booster for convenient, effective, and economical antitumor therapy. Current advances demonstrate that it is not a distant dream.

AUTHOR CONTRIBUTIONS

HL wrote the manuscript and was the primary author. LZ and WM made substantial contributions to designing and revising the article. All authors contributed to the article and approved the submitted version.

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Novel Natural Inhibitors Targeting Enhancer of Zeste Homolog 2: A Comprehensive Structural Biology Research

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The enhancer of zeste homolog 2 (EZH2) is a methylated modification enzyme of Histone H3-Lys 27. The high expression of EZH2 in cells is closely related to the progression, invasion, and metastasis of neoplasm. Therefore, this target is gradually becoming one of the research hot spots of tumor pathogenesis, and the inhibitors of the EZH2 enzyme are expected to become new antitumor drugs. This study used a series of virtual screening technologies to calculate the affinity between the compounds obtained from the ZINC15 database and the target protein EZH2, the stability of the ligand–receptor complex. This experiment also predicted the toxicity and absorption, distribution, metabolism, and excretion (ADME) properties of the candidate drugs in order to obtain compounds with excellent pharmacological properties. Finally, the ligand–receptor complex under *in vivo* situation was estimated by molecular dynamics simulation to observe whether the complex could exist steadily in the body. The experimental results showed that the two natural compounds ZINC000004217536 and ZINC000003938642 could bind tightly to EZH2, and the ligand–receptor complex could exist stably *in vivo*. Moreover, these two compounds were calculated to be nontoxic. They also had a high degree of intestinal absorption and high bioavailability. *In vitro* experiments confirmed that drug ZINC000003938642 could inhibit the proliferation and migration of osteosarcoma, which could serve as potential lead compounds. Therefore, the discovery of these two natural products had broad prospects in the development of EZH2 inhibitors, providing new clues for the treatment or adjuvant treatment of tumors.

Keywords: EZH2, inhibitor, histone methyltransferase, structural biology, virtual screening

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INTRODUCTION

EZH2, namely, enhancer of zeste homolog 2, is a pivotal member of epigenetic regulatory factor Polycomb group (PcG) proteins. PcG proteins can lead to gene suppression through methylation modification (1), which comprises several essential molecules like Polycomb repressive complexes (PRCs). PRCs have inherent histone methyltransferase (HMTase) activity, which can inhibit gene expression through core histone methylation (2). PRC2 is of vital importance in PcG proteins, as it plays a role in the development of cancer (3). PRC2 consists of three subunits: EZH2, SUZ12, and EED, of which EZH2 and chaperone proteins are essential to correctly coordinate differentiation and proliferation of cells (4).

EZH2 has methyltransferase activity and can catalyze the methylation of histone H3-Lys 27 (H3-K27); it is essential for PRC-mediated gene suppression (5). Research had reported that human EZH2 was upregulated in different kinds of tumors like breast cancer, prostate cancer, and osteosarcoma (OS) (6). Cyclin-dependent kinase 1 (CDK1) promotes EZH2 ubiquitination by mediating the phosphorylation of Thr-345 and Thr-487 (T345 and T487) sites of EZH2 (7). And the posttranslational modifications of EZH2 are essential to improve its protein stability that related to the function of tumor cells and tumor metastasis, which could further lead to the accumulation of EZH2 and the occurrence of cancers (8, 9).

In summary, EZH2 is related to different kinds of neoplasms, which was abnormally expressed and could serve as a therapeutic target (10–12). Therefore, inhibition of EZH2 protein could provide new ideas and methods in the treatment of cancers. GSK126 is a new type of competitive inhibitor targeting EZH2, which had begun tests in clinical trials (13). GSK126 significantly reduces the level of H3K27me3 in tumor cells by inhibiting the methyltransferase activity of EZH2, thereby inhibiting the growth of tumor cells such as human tongue squamous cell carcinoma and multiple myeloma cells (14, 15). In addition, the appropriate concentration of GSK126 could also induce tumor cell apoptosis through the mitochondrial pathway (16). Research also reported that EZH2 may promote tumor invasion and metastasis by downregulating downstream targets such as E-cadherin and vascular endothelial growth factor (VEGF)-A (17, 18). VEGF-A is an important cytokine that regulates angiogenesis, which is closely related to tumor metastasis (19). Research on EZH2 inhibitors has become hot spots in recent years, which has changed the treatment scheme as well as ideals dramatically. Nevertheless, novel efficient inhibitors targeting EZH2 still remained less. Consequently, more inhibitors regarding EZH2 were needed to discover from a natural medicine library in order to screen novel natural lead compounds and provide new clues in the discovery of EZH2 inhibitors. Existing studies had confirmed that EZH2 was highly expressed in OS patients and could serve as potential biomarker (11), while research on targeted therapy of OS targeting EZH2 had hardly been reported. Up to now, the research on EZH2 inhibitor GSK126 had made notable progress in different kinds of cancers, including prostate cancer cells and gastric cancer cells (20, 21). Consequently, this study chose GSK126 as the reference compound to compare the pharmacological properties of the candidate compounds in order to discover more potential lead compounds targeting EZH2. Besides, this study aimed to validate whether EZH2 could serve as a therapeutic target in the treatment of OS.

Recently, natural products and natural extracts may be highly available compounds with proper biological activity that has potential medicinal value. They are therefore important sources for discovering, designing, and improving new drug skeletons (22, 23). Extensive investigations have shown that natural products and their derivatives are currently playing an important role in the medical industry. It is already determined that the natural compounds from natural product database have considerable pharmacological potential (24, 25). The second part of this study provides a theoretical basis and guidelines for discovering new inhibitors from natural product repository by screening inhibitory compounds related to EZH2. Besides, absorption, distribution, metabolism, and excretion (ADME) and toxicity prediction, ligand binding research, and molecular dynamics (MD) simulation were carried out on the selected candidate compounds, laying the foundation for the improvement of tumor drugs.

MATERIALS AND METHODS

Discovery Studio Software and Ligand Library

The LibDock module of Discovery Studio 4.5 software (BIOVIA, San Diego, CA, USA) is used to screen better energy-optimized natural products, and the ADMET module can be applied to ADME analysis and the prediction of carcinogenicity and Ames mutagenicity. The CDOCKER module can be used to analyze the binding force between the products and the corresponding target of the protein and analyze the stability of the complex. This experiment selects the natural product database in the ZINC database to screen EZH2 inhibitors. The ZINC15 database is provided by the Irwin and Shoichet Laboratories in the Department of Pharmaceutical Chemistry at the University of California, San Francisco (UCSF), which provides a free virtual screening database of commercially available compounds.

Structure-Based Virtual Screening Using LibDock

The LibDock module was widely used in the drug development process (26). The LibDock module used a grid placed in the binding site and used polar and non-polar probes to calculate protein hot spots, then further used hot spots to arrange the ligands to form favorable interactions. Moreover, the study also used the Smart Minimiser algorithm and the CHARMm force field (Cambridge, MA, USA) to minimize the ligands (27). Then, all ligands' positions were adjusted and ranked according to the calculated ligand scores. The 2.5-Å crystal structure of human EZH2 [Protein Data Bank (PDB) identifier: 5WF7] and the structure of the inhibitor GSK126 were downloaded respectively from the PDB and ZINC15 database, and they were imported into the working environment of LibDock.

Figure 5 shows the chemical structure of EZH2. The protein was prepared through several steps, including removing the crystal water and other surrounding heteroatoms, then adding hydrogen, protonation, ionization, and energy minimization. Among them, the energy minimization was realized by the CHARMM force field and the Smart Minimiser algorithm. In the case that the root mean square gradient tolerance was 0.1, the minimization performed 2,000 steps. After calculation, the binding site of the prepared protein was defined through the "Edit binding site" option. Analyzing the binding site of the ligand (GSK126) to generate the active binding site for docking the ligand with the receptor. Virtual screening was performed by docking the ligand exported from the database with the defined active binding site through the LibDock module. All compounds were grouped and ranked according to their LibDock score.

Prediction of Absorption, Distribution, Metabolism, and Excretion and Toxicity

The ADMET module of DS4.5 was used to estimate the adsorption, distribution, metabolism, and excretion properties of compounds. And the TOPKAT module of DS4.5 was employed to predict the carcinogenicity, Ames mutagenicity, and developmental toxicity potential in rodents. These pharmacological properties are fully considered when screening suitable EZH2 inhibitors to ensure the safety of the drug.

Molecule Docking and the Prediction of Drug Affinity

The CDOCKER module of DS4.5 was used for molecular docking research. CDOCKER is a tool to calculate high-precision docking results based on the CHARMM force field. During the docking process, the structures of the ligands are allowed to bend, while the structure of the receptor remains rigid. The CHARMM energy and interaction energy of each posture generated are calculated to reflect the binding affinity of the ligand and the receptor. Since crystal water molecules may affect the formation of receptor–ligand complexes, fixed water molecules are usually removed during the semi-flexible and rigid docking process, and hydrogen atoms are added to the protein to ensure the accuracy of the experiment (28, 29). The crystal structure of EZH2 was obtained from the protein database, and the three-dimensional structures of ZINC3938642 and ZINC4217536 were obtained from the ZINC15 database. In order to verify the reliability of the results, this experiment also downloaded the reference compound GSK126 from the ZINC15 database. Similarly, the GSK126 was docked with EZH2 to calculate the root mean square deviation (RMSD) of the molecular docking conformation and compared it with the RMSD of the conformations of the ligand–receptor complex that are selected in this experiment. The binding site of EZH2 is defined as an area within a 5-Å radius from the geometric center of the ligand GSK126. In this experiment, the selected ligand was allowed to bind to the protein group residues in the binding site sphere. The identified hit structures were prepared and docked with the binding site of EZH2. Based on the numerical values of CDOCKER interaction energy, the different postures of each ligand–EZH2 receptor complex were generated and analyzed in detail.

Molecular Dynamics Simulation

Among the various postures predicted by the molecular docking program, the best binding conformation of the EZH2–inhibitor complex is selected as the object for MD simulation. The ligand–receptor complex is placed in an orthogonal box and solved with an explicit periodic boundary solvated water model. At the same time, to simulate the physiological environment, sodium chloride with an ionic strength of 0.145 was added to the system. Then, the system is subjected to the CHARMM force field and is relaxed through energy minimization (1,000 steps of steepest descent and 1,000 steps of the conjugated gradient). The reaction system was slowly driven from the initial temperature of 50K to the target temperature of 300K, the driving time was 2 ns, and the equilibrium simulation was performed when the time was 1 ns. The time for MD simulation (production) is 40 ns, and the time step is 2 fs. The simulation adopts the NPT (normal pressure and normal temperature) system at a constant temperature close to 300K, and the results were stored at a frequency of 0.02 ns. The Particle Mesh Ewald algorithm was used to calculate the long-range static electricity, and the linear constraint solver algorithm was used to fix all bonds involving hydrogen. Set the initial complex as the reference object. Use the Discovery Studio 4.5 analyze trajectory protocol to determine the structural properties, RMSD, and potential energy of the trajectory simulated by MD.

Cell Lines and Reagents

Human OS cell lines MG-63 (CL-0157), HOS (CL-0360), and human normal liver cell line LO2 (CL-0111) were purchased from Procell Life Science & Technology Co., Ltd. These cell lines were incubated in high-glucose Dulbecco's modified Eagle's medium (DMEM; Procell, Cat. no. PM150210), containing 10% fetal bovine serum (FBS; Gemini, USA) and 100 units/ml penicillin and 100 mg/ml streptomycin under normal cell culture conditions (37°C and 5% CO_2). Drug ZINC000003938642 was provided by Selleck Chemical Co. (Cat. no. S3668). The drug was dissolved in ultrapure water based on manufacturer-provided instructions to obtain the stock solution. Dimethylsulfoxide (DMSO) was not used to dissolve the drug in this study so that the toxicity effect on cells was negligible. Then, appropriate culture medium was added into the stock solution to configure different concentrations of the drug.

Cell Counting Kit-8 Assay

The standard Cell Counting Kit-8 (CCK-8) assay (provided by ApexBio, USA) was conducted to measure the cellular viability and proliferation of OS cells (HOS and MG-63) and human liver cell (LO2). Cell lines were plated into 96-well culture plates with a density of 3,000 cells/well overnight. Cells were treated with different doses of drug ZINC000003938642 for 24 h. The concentration gradients of each treatment were 0, 5, 10, 20, 40, 80, 160, 320, and 540 μmol/L. Cells were cultured for 1.5 h after addition of 10 μl/well CCK-8, and then the OD value of each well was measured based on the wavelength of 450 nm according to the microplate reader (BioTek Instrument, Synergy H1, USA).

Colony Formation Assay

Colony formation assay (CFA) assay was performed to detect the effects of different doses of drug on proliferation of tumor cells.

HOS and MG-63 cells were incubated into six-well plate with the density of 600 cells/well. After 24 h in culture, we configured cell culture medium with drug concentration of 100, 250 μmol/L; DMSO was not used in this study so the influence of DMSO on cells could be neglected. After 10 days of cultivation, the developed colonies were rinsed with phosphate buffered saline (PBS) twice and fixed in 4% paraformaldehyde, then 0.5% crystal violet solution was used to stain the colonies for half an hour. Lastly, we counted and described colonies according to microscopic examination.

In Vitro Scratch Assay

OS cells (HOS and MG-63) were cultured in six-well plate to assess the effects of drug on the migration of tumor cells. When the degree of fusion reached 90%, a 1-ml pipette tip was used to make a consistent cell-free area. Then, PBS was used to rinse twice to wipe off the cell debris, and serum-free medium was changed to culture, and different concentrations (0, 25, 50, 100, 250 μmol/L) of drug were used to treat cells and observe the scratch width at 0, 6, 12, 24 h. After corresponding time, we captured images of scraped area with phase contrast microscopy and measured the wounds and scratch width. The migration rate of OS cells was calculated as:

$$\text{percentage of wound closure} = \frac{(\textit{scratch area of } 0H - \textit{scratch area of corresponding time})}{\textit{scratch area of } 0H}$$

Western Blotting

OS cell lines (HOS and MG-63) were seeded into T25 culture flask and treated with different doses of drug ZINC000003938642 for 48 h. Then, proteins were extracted by radioimmunoprecipitation assay (RIPA), and bicinchoninic acid (BCA) protein assay was conducted to define protein standard curve and detect the protein concentration of each sample. Ten percent sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins of samples, and then proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Five percent nonfat milk dissolved in Tris-buffered saline and tween 20 (TBST) buffer was used to block the membranes for 2 h, after that, the membranes were incubated with primary antibodies [EZH2, c-Myc from Abcam and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from Proteintech] at 4°C overnight. On the second day, the membranes were washed with Tris-buffered saline and tween 20 (TBST) three times and then horseradish peroxidase-conjugated secondary antibody was added to incubate the membranes for 1 h at room temperature. The membranes were visualized with enhanced chemiluminescence reagents to detect corresponding protein signals. Viber Bio Imaging tools were used to measure the band densities.

Apoptosis Assay

OS cells (HOS and MG-63) in log growth phase were inoculated into six-well plate and were treated with different concentrations of the drugs. After culturing for 24 h, cells were extracted through trypsin (without EDTA) and Annexin-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double staining was performed according to the manufacturer's instructions. Lastly, the stained cells were analyzed by flow cytometry techniques; the apoptosis rates were examined by ACEA NovoCyte flow cytometry.

Pharmacophore Predictions of the Ideal Lead Compounds

After initial validation of the antitumor effects of the selected compounds, this study further analyzed their pharmacophore characteristics. Pharmacophore predictions of compounds were performed according to 3D-QSAR pharmacophore generation module, which generated up to 255 fits per molecule to represent a small molecule, and only fits with energy values within the energy threshold of 10 kcal/mol were finally preserved.

RESULTS

EZH2 Expression in Third-Party Database

To figure out the expression situation of EZH2 in OS, this study analyzed the expression values of EZH2 between normal and OS patients in Gene Expression Omnibus (GEO, https://www.ncbi.nlm.nih.gov/geo/) database. In total, three GSE series were analyzed including GSE14359, GSE33382, and GSE126209. As shown in **Figure 1**, results demonstrated that the expression of EZH2 in OS patients was significantly upregulated compared with that in normal patients ($P < 0.05$, Wilcoxon nonparametric test).

Fast Virtual Screening of Potential Inhibitors of EZH2

The SAL/SET domain of EZH2 protein is regarded as an important regulatory site for its enzymatic activity. Inhibitors bind to the SAL/SET domain of EZH2 by inserting into the ligand pocket of EZH2 and exerts the function of inhibiting the activity of EZH2: The small molecules binding to this site can prevent S-adenosyl methionine (SAM) from providing EZH2 with the methyl group needed to methylate H3K27me3, thereby reducing the enzymatic activity of EZH2. After SAM loses its methyl group, it is metabolized and hydrolyzed into intermediate products including S-adenosyl-L-homocysteine (SAH) and adenosine. S-adenosylmethionine is a methyl donor for one-carbon unit metabolism in organisms, and by moderately promoting the metabolic level of SAM, the activity of EZH2 can be inhibited (30). Based on this mechanism, inhibitors of enzyme activity against EZH2 could be identified. Therefore, this domain was chosen as the docking site for screening. The crystal structure of EZH2 was displayed in **Figure 2**, which contained the binding site sphere for docking, as well as the Ramachandran diagram of the protein, to check the rationality of EZH2 structure. Firstly, LibDock module of DS4.5 was performed to virtually screen small molecules that functioned in binding with the receptor protein EZH2. Downloading commercially available natural compounds from the ZINC15 database, a total of 13,537 ligands were generated by virtual screening. At the same time, the effective selective inhibitor GSK126, which could inhibit the activity of EZH2, was selected as the reference compound. After

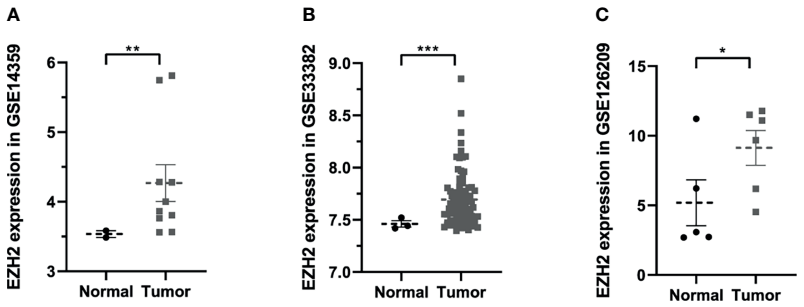

FIGURE 1 | The expression situation of Enhancer of Zeste Homolog 2 (EZH2) between osteosarcoma and normal patients in Gene Expression Omnibus (GEO) database: **(A)** GSE14359, **(B)** GSE33382, **(C)** GSE126209. Data were represented as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.0001; the same below.

screening, 669 compounds were found with higher LibDock scores than GSK126 (LibDock score: 132.143). The top 20 compounds were listed in **Table 1**.

Absorption, Distribution, Metabolism, and Excretion Characteristics and Toxicity Prediction

By using the ADME and TOPKAT prediction module, we obtained the candidate 20 kinds of ligands and GSK126's pharmacological properties, including penetration of the blood–brain barrier, degree of human intestinal absorption, water solubility level, inhibitory effect on cytochrome P450 2D6, hepatotoxicity, and plasma protein binding properties (**Table 2**). The water solubility prediction showed that 18 compounds could be dissolved in water relatively well. Among them, 10 compounds had a high solubility level (scores >2), which were greater than the reference compound GSK126 (moderate solubility, score: 1). For the degree of human intestinal absorption, 19 compounds had a good absorption effect, the same as GSK126, and ZINC000085826837 had a medium absorption level. Besides, seven compounds and GSK126 could be bound strongly by plasma proteins, while the remaining compounds did not have tight binding affinity and strong interactions with plasma proteins. Cytochrome P4502D6 (CYP2D6) was a key enzyme in the process of drug metabolism. Compounds involved in the screening had no inhibitory effect on CYP2D6. GSK126 was also predicted to be a non-inhibitor of CYP2D6. For liver toxicity, 12 compounds were predicted to be nontoxic drugs, while the remaining compounds and GSK126 were toxic to the liver.

Subsequently, this experiment also calculated the safety properties of the candidate compounds and GSK126 through the TOPKAT module, including Ames (Ames mutagenicity), developmental toxicity potential (DTP), and rodent carcinogenicity [based on the United States National Toxicology Program (NTP) data set]. Experimental results displayed that those 12 compounds were non-mutagenic in long-term effect. It was predicted that four compounds were non-carcinogens and three compounds had no developmental toxicity potential. In addition, the reference compound GSK126 also predicted with pretty characteristics on Ames and NTP carcinogenicity, while it was computed with probability of DTP. The detailed information of the indicators among compounds and GSK126 were shown in **Table 3**. Based on the above data, ZINC000004217536 and ZINC00000 3938642 were neither CYP2D6 inhibitors nor hepatotoxicity drugs. Moreover, they were predicted to be free of Ames mutagenicity and rodent carcinogenicity. Consequently, ZINC000004217536 and ZINC000003938642 were analyzed to be candidate drugs with

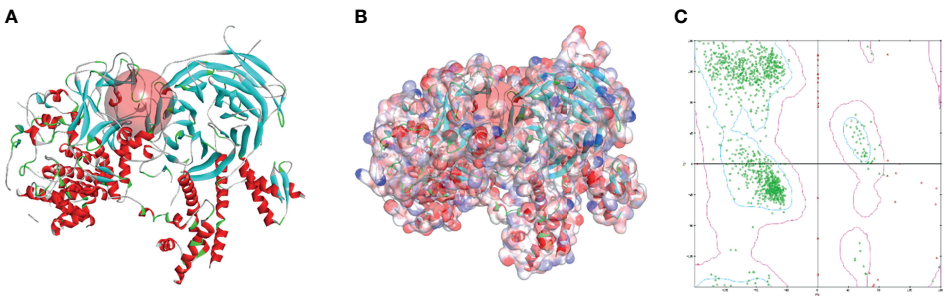

FIGURE 2 | The molecular structure of Enhancer of Zeste Homolog 2 (EZH2). **(A)** Initial molecule structure and added active binding sphere, the active binding sphere was shown as red region. **(B)** Surface of binding region added. Blue represented positive charge, and red represented negative charge. **(C)** The Ramachandran diagrams of EZH2 protein.

TABLE 1 | Top 20 ranked compounds with higher Libdock scores than GSK126.

Number	Compounds	Libdock Score
1	ZINC000085545908	207.393
2	ZINC000085544839	207.175
3	ZINC000004096059	198.67
4	ZINC000004099069	194.59
5	ZINC000008552069	193.20
6	ZINC000056897657	191.551
7	ZINC000004217536	191.439
8	ZINC000095620524	189.085
9	ZINC000004096684	187.339
10	ZINC000062238222	184.582
11	ZINC000100084136	183.874
12	ZINC000150338786	182.677
13	ZINC000014951658	182.425
14	ZINC000003938642	181.651
15	ZINC000004096878	181.45
16	ZINC000004099068	180.745
17	ZINC000004096653	180.432
18	ZINC000085826837	178.464
19	ZINC000049784088	178.376
20	ZINC000008220033	177.227

high safety and were selected for further study. The detailed chemical and crystal structures of these compounds were shown in **Figure 3**.

Ligand–EZH2 Binding Analysis

In order to study the binding mechanisms between the ligand and receptor EZH2, CDOCKER module was conducted to dock ZINC000004217536, ZINC000003938642, and GSK126 at the regulatory site of EZH2, and the corresponding CDOCKER potential energy of these complexes was calculated, as shown in **Table 4**. Hydrogen bonds and π–π interactions between EZH2 and these compounds were analyzed (**Figures 4**, **5**). Results visualized that ZINC000004217536 formed four pairs of hydrogen bonds with EZH2: O27 with TYR855:HN of EZH2, H68 with TYR855:O of EZH2, and H70 and H79 with VAL853:O of EZH2. ZINC000003938642 formed eight pairs of hydrogen bonds with EZH2: H62 with ARG304:NH1 of EZH2, H62 with LYS852:O of EZH2, O11 with ASN851:ND2 of EZH2, H74 with ASN880:OD1 of EZH2, H89 with TYR826:OH of EZH2, H90 with SER876:O of EZH2, H91 with ILE879:O of EZH2, and H86 with ARG877:O of EZH2. Two pairs of hydrogen bonds were formed between the reference compound GSK126 and EZH2: O12 with ARG304:N of EZH2 and O23 with TYR809:N of EZH2. Additionally, these three compounds formed two pairs, one pair each of π–π interactions with EZH2. The detailed chemical bond interactions were displayed in **Table 5**.

Molecular Dynamics Simulation

The best binding conformations of each compound–EZH2 complexes (ZINC000004217536-EZH2 and ZINC000003938642-EZH2) were obtained from precise docking program CDOCKER and applied for the following experiment. In this study, the stability of the ligand–EZH2 complex under *in vivo* circumstance was predicted by MD simulation module. The predicted results were shown in **Figure 6**, including energy values (**Figures 6A, D**) and RMSD curve (**Figures 6B, E**) diagram of each complex. The orbitals of all complexes reached equilibrium after 100ps. The complexes' RMSD and energy values like total energy, potential energy, and electrostatic energy all got stabilized over time. Results suggested that hydrogen bonds formed by the compound and EZH2 and the π-π-related interactions contributed a great effect on the stability of these complexes. Furthermore, chemical bonds heatmap after MD also illustrated that these chemical bonds, which contributed largely to the stability of complexes, could still exist steadily with the progression of MD in the body (**Figures 6C, F**). Based on the above evaluation indicators, the complexes formed by

TABLE 2 | Adsorption, distribution, metabolism, and excretion (ADME) properties of compounds.

Number	Compound	Solubility level	BBB level	CYP2D6	Hepatotoxicity	Absorption level
1	ZINC000085545908	3	4	FALSE	FALSE	3
2	ZINC000085544839	3	4	FALSE	TRUE	3
3	ZINC000004096059	1	4	FALSE	TRUE	3
4	ZINC000004099069	3	4	FALSE	FALSE	3
5	ZINC000056897657	1	4	FALSE	TRUE	3
6	ZINC000004217536	3	4	FALSE	FALSE	3
7	ZINC000095620524	4	4	FALSE	TRUE	3
8	ZINC000008552069	4	4	FALSE	TRUE	3
9	ZINC000004096684	1	4	FALSE	FALSE	3
10	ZINC000062238222	3	4	FALSE	TRUE	3
11	ZINC000100084136	1	4	FALSE	FALSE	3
12	ZINC000150338786	1	4	FALSE	TRUE	3
13	ZINC000014951658	3	4	FALSE	FALSE	3
14	ZINC000003938642	0	4	FALSE	FALSE	3
15	ZINC000004096878	1	4	FALSE	TRUE	3
16	ZINC000004099068	3	4	FALSE	FALSE	3
17	ZINC000004096653	1	4	FALSE	FALSE	3
18	ZINC000085826837	2	4	FALSE	FALSE	2
19	ZINC000049784088	4	4	FALSE	FALSE	3
20	ZINC000008220033	0	4	FALSE	FALSE	3
21	Reference ligand	1	4	FALSE	TRUE	3

TABLE 3 | Toxicities of compounds.

Number	Compounds	NTP: Mouse		NTP: Rat		Ames	DTP
		Female	Male	Female	Male		
1	ZINC000085545908	1	0	0	0	0	1
2	ZINC000085544839	0	0.851	0	0.954	0.001	1
3	ZINC000004096059	0	0	0	1	0	1
4	ZINC000004099069	0	0	0	0	0.002	0.846
5	ZINC000056897657	0	0.982	1	0	1	1
6	ZINC000004217536	0	0	0	0	0	1
7	ZINC000095620524	1	0	1	0.999	0	1
8	ZINC000008552069	0.015	0	0	0.997	1	1
9	ZINC000004096684	0	1	1	0	1	1
10	ZINC000062238222	0	0	0	0.969	0.989	1
11	ZINC000100084136	0	0	0	1	0	0
12	ZINC000150338786	0	1	0	0	1	1
13	ZINC000014951658	1	0	1	0	0	1
14	ZINC000003938642	0	0	0	0	0	0
15	ZINC000004096878	0	1	0	0	1	1
16	ZINC000004099068	0	0	0	0	0.002	0.864
17	ZINC000004096653	0	1	1	0	1	1
18	ZINC000085826837	0.186	1	1	0.998	0	1
19	ZINC000049784088	0.995	0	0	0.008	1	1
20	ZINC000008220033	0	1	1	0	0	0
21	GSK126	1.000	0.003	0.000	0.340	0.009	0.924

NTP, National Toxicology Program.

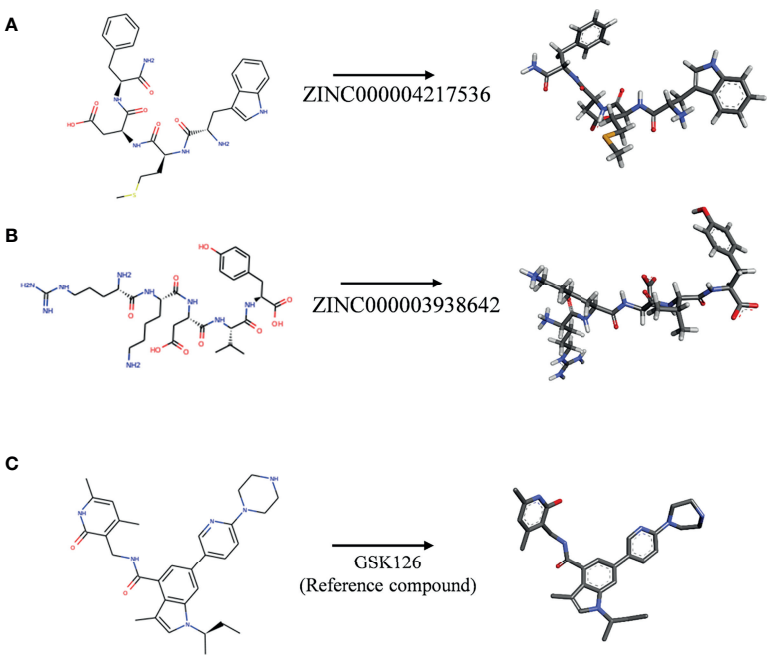

FIGURE 3 | The 2D structures of novel compounds selected from virtual screening and the reference compound GSK126 by chemdraw. **(A)** ZINC000004217536, **(B)** ZINC000003938642, and **(C)** GSK126.

ZINC000004217536 and ZINC000003938642 with EZH2 could exist stably in the internal environment. Consequently, these two compounds could interact with EZH2; they also had a regulatory effect on EZH2 like the reference compound GSK126 did.

ZINC000003938642 Reduced Proliferation of Osteosarcoma Cells

To test the antitumor effects of compounds screened in this study, we purchased one of the two compounds, ZINC000003938642, for

TABLE 4 | CDOCKER interaction energy of selected compounds with Enhancer of Zeste Homolog 2 (EZH2).

Compound	CDOCKER potential energy (Kcal/mol)
ZINC000004217536	-58.3934
ZINC000003938642	-52.6615
GSK126	-46.7202

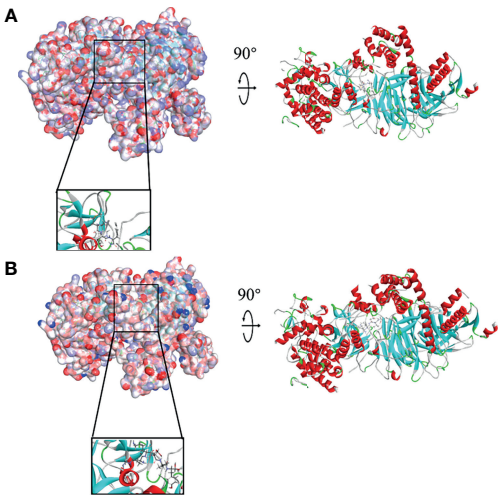

FIGURE 4 | Schematic drawing of interactions between ligands and Enhancer of Zeste Homolog 2 (EZH2). The surface of binding area was added; blue represented positive charge, red represented negative charge; and ligands were shown in sticks; the structures around the ligand–receptor junction were shown in thinner sticks. **(A)** ZINC000004217536–EZH2 complex. **(B)** ZINC000003938642–EZH2 complex.

further *in vitro* experiments, aiming to evaluate the effects on OS cells. To assess the proliferation ability of OS cells in the presence of drug ZINC000003938642, the survival of cells after drug treatment was calculated by CCK-8, and growing ability of OS cells was assessed by CFA. The OS cells were treated with different concentrations of drug for 24 h (0, 5, 10, 20, 40, 80, 160, 320, 540 μmol/L). Results indicated that the cellular viability of both HOS and MG-63 cells were declined with the increase of drug concentration (**Figures 7A, B**). Subsequently, to validate the toxicity of drug to liver cells, LO2 cell line was conducted and measured by CCK-8. Results revealed that drug ZINC00000 3938642 did not inhibit the proliferation of human normal liver cells in a dose-dependent manner and time-dependent manner, which still had a high cellular viability even when subjected to the highest dose (**Figure 7C**).

We then performed CFA to further determine the antitumor effects of drug in OS cells. As shown in **Figure 7D**, after 10 days of cultivation with different drug concentrations (100 and 250 μmol/L), both HOS and MG-63 cells showed fewer and smaller clonogenicities in Petri dishes with drug group than with the control group. The numbers of clone formation in drug groups were significantly lower than those in control groups ($P < 0.05$) (**Figures 7E, F**).

ZINC000003938642 Inhibited Migration of Osteosarcoma Cells

To analyze the effects of drug ZINC000003938642 on OS cell migration, scratch assay was further performed. The width of scratched areas was measured at 0, 6, 12, and 24 h of scratch, and the scratch width represented the migration capacity of OS cells (**Figures 8A, D**). As shown in **Figures 8B, C**, results exposed that with the extension of time and increase of drug concentration, the migration rate of OS cells to the scratch area decreased significantly ($P < 0.05$).

ZINC000003938642 Induced Apoptosis in Osteosarcoma Cells

Flow cytometry and Annexin-FITC/PI double staining were used for apoptosis assay to detect the effects of drugs on programmed cell death. The apoptotic rates of HOS and MG-63 cells were detected after being treated with concentrations of ZINC000003938642 (0, 50, 100, and 250 μM) for 24 h. As shown in **Figure 9A**, results illustrated that the apoptotic rates increased with the increase of drug concentration both in HOS and MG-63 cells. Consequently, live cells were predominant in the control (0 μM) groups, while apoptotic cells were predominant in the drug-treated groups (**Figure 9B**; $P < 0.05$).

ZINC000003938642 Reduced the Expression of EZH2 and Its Downstream Gene C-Myc

The expression of EZH2 was detected by Western blot analysis. As shown in **Figures 10A, C**, the expression level of EZH2 was inhibited by ZINC000003938642 in both HOS and MG-63 cells, and its inhibitory effect displayed a dose-dependent manner ($P < 0.05$; **Figures 10B, D**). c-Myc was the downstream target gene of EZH2, and as an oncogene, the expression levels of c-Myc were also reduced by ZINC000003938642 in both HOS and MG-63 cells (**Figures 10A, C**), and its inhibitory effects also demonstrated a dose-dependent manner (**Figures 10B, D**).

Ligand Pharmacophore Predictions

After initially verifying the antitumor effects of the candidate compounds, this study further analyzed the pharmacophore characteristics of these two compounds in order to observe the potential modification site on compounds. As shown in **Figures 11A, B**, computational results illustrated that there were 51 features in ZINC000004217536 and 69 features in ZINC000003938642, among which, ZINC000004217536 possessed 18 hydrogen bond acceptors, 21 hydrogen bond donors, five hydrophobic centers, one ionizable positive, and one ring aromatic. As for ZINC000003938642, it possessed 22 hydrogen bond acceptors, 38 hydrogen bond donors, two hydrophobic centers, five ionizable positive, and two ring aromatics.

DISCUSSION

Statistics published in these years show that malignant tumors are still the main causes of death among residents in many countries

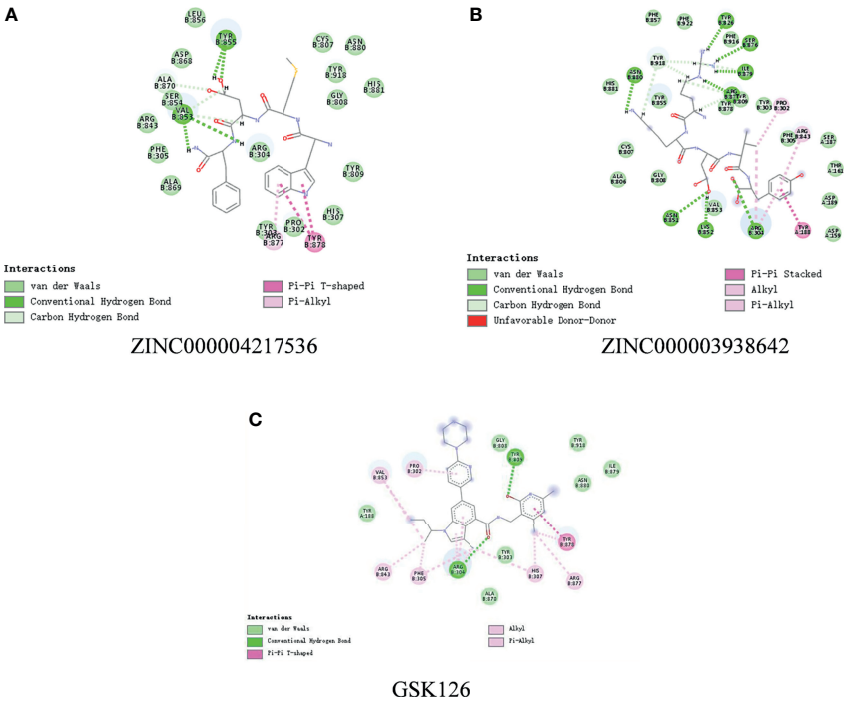

FIGURE 5 | The detailed intermolecular interaction of the predicted binding modes of **(A)** ZINC000004217536, **(B)** ZINC000003938642, and **(C)** GSK126 to Enhancer of Zeste Homolog 2 (EZH2).

TABLE 5 | Hydrogen Bond Interaction Parameters for Each Compound with EZH2.

Receptor	Compound	Donor Atom	Receptor Atom	Distances (Å)
EZH2	ZINC000004217536	B:TYR855:HN	ZINC000004217536:O27	2.02
		ZINC000004217536:H68	B:TYR855:O	3.10
		ZINC000004217536:H70	B:VAL853:O	2.76
		ZINC000004217536:H79	B:VAL853:O	2.46
	ZINC000003938642	B:ARG304:NH1	ZINC000003938642:O47	3.05
		ZINC000003938642:H62	B:LYS852:O	2.27
		B:ASN851:ND2	ZINC000003938642:O11	3.39
		ZINC000003938642:H74	B:ASN880:OD1	2.19
		ZINC000003938642:H89	B:TYR826:OH	2.24
		ZINC000003938642:H90	B:SER876:O	2.38
		ZINC000003938642:H91	B:ILE879:O	2.05
		ZINC000003938642:H86	B:ARG877:O	1.89
	GSK126	B:ARG304:N	K:A9G8009:O12	3.00
		B:TYR809:N	K:A9G8009:O23	2.89

(31). OS is one of the most common malignant tumors of mesenchymal tissues, occurring mostly in the metaphysis of long bones in adolescents; it is a differentiation-defective disease caused by dysdifferentiation of osteoblasts and/or epigenetic changes (32). The harm of malignant tumors to humans is not only a threat to the lives of patients but also the physical pain, mental pressure, and the economic burden they bring to patients (33). EZH2, an essential component of the epigenetic regulatory factor PcG and a catalytic subunit of PRC2, is involved in regulating the methylation of lysine 27 (H3K27) of histone H3 and is highly expressed in a variety of tumors. It plays an important role in regulating gene transcription and gene silencing and participates in the growth, proliferation, and metastasis of tumor cells.

In recent years, EZH2 has become a popular target for cancer therapeutics, and the research of EZH2 inhibitors and their combined application with other antitumor drugs in clinical practice has broad prospects (34). However, relatively few inhibitors of EZH2 had been discovered and analyzed. Existing research had reported the high expression of EZH2 in OS patients. Currently, few studies have been conducted on the efficacy of EZH2 inhibitors in OS. GSK126, which is relatively a mature EZH2 inhibitor, was applied in this study to analyze the

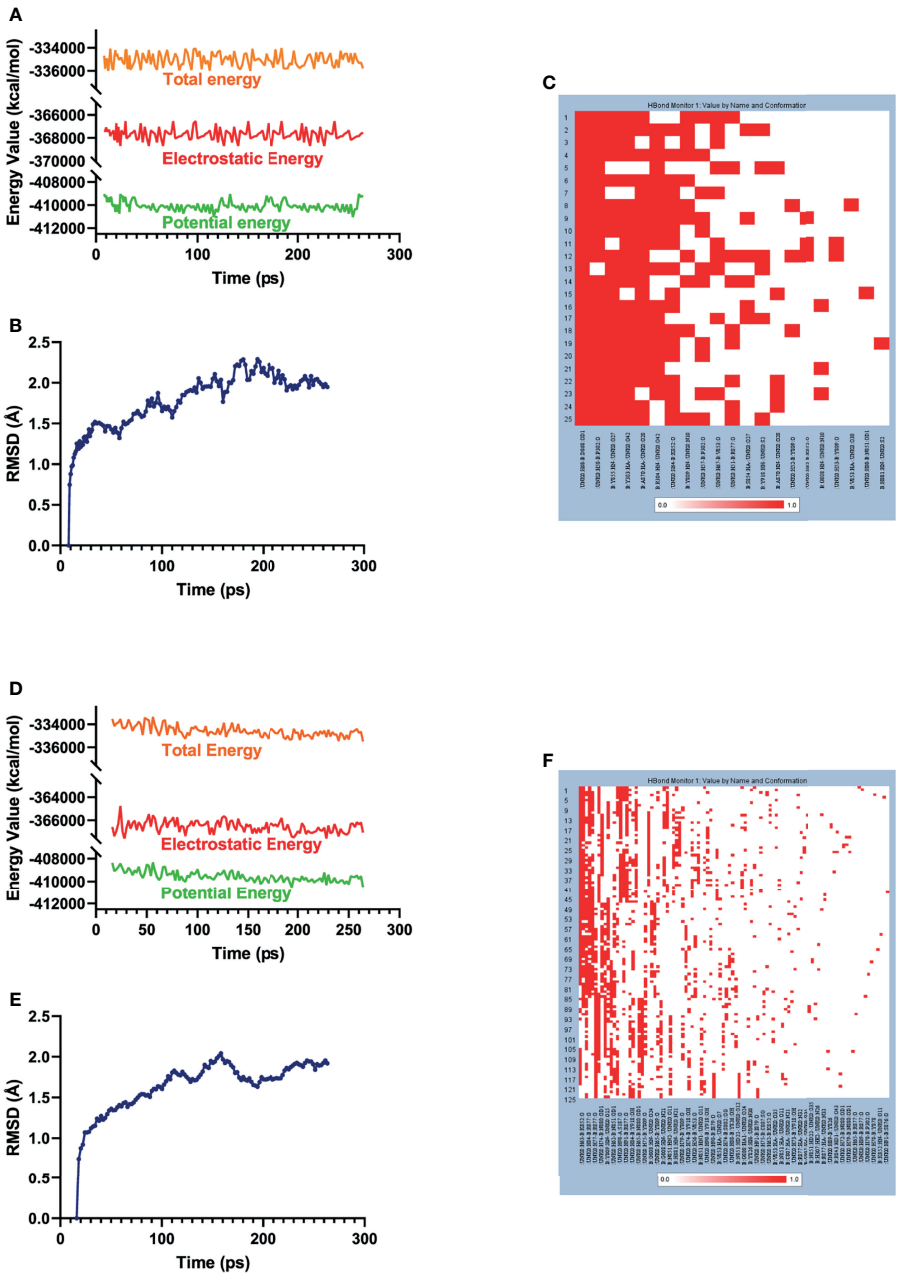

FIGURE 6 | Results of MD simulation of two these complexes. **(A)** Energy values of ZINC000004217536-EZH2 complex during the MD process. EZH2, Enhancer of Zeste Homolog 2; MD, molecular dynamics. **(B)** Average backbone RMSD of ZINC000004217536–EZH2 complex. RMSD, root mean square deviation. **(C)** Chemical bonds heatmap of ZINC000004217536–EZH2 complex in the progression of MD. **(D)** Energy values of ZINC000003938642–EZH2 complex during the MD process. **(E)** Average backbone RMSD of ZINC000003938642–EZH2 complex. **(F)** Chemical bonds heatmap of ZINC000003938642–EZH2 complex in the progression of MD.

antitumor effect and molecular mechanism on OS, and it was regarded as the reference compound to compare pharmacologic properties with novel ligands.

Although GSK126 has certain antitumor functions, it still has some limitations. Relevant studies have shown that EZH2 can produce drug resistance through allelic mutations and protein conformation changes (35, 36). For the purpose of overcoming the drug resistance of EZH2, it is necessary to develop new inhibitors. Furthermore, GSK126 had low solubility and rapid plasma clearance, resulting in low bioavailability, and GSK126 had hepatotoxicity, which leads to unsatisfactory effects of high-dose GSK126 in the process of inhibiting EZH2.

In this study, we aimed to discover more potential lead compounds of EZH2. The available natural compound

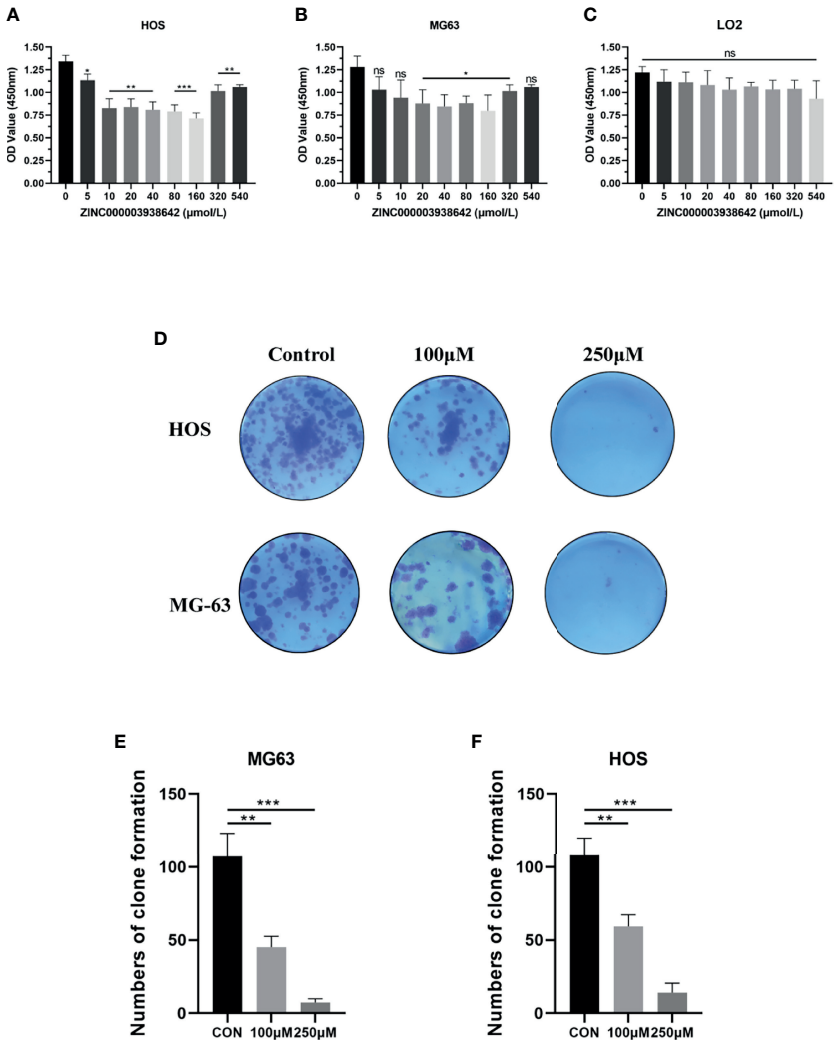

FIGURE 7 | **(A, B)** Cellular viability of osteosarcoma (OS) cell lines (HOS and MG-63) and **(C)** human liver cell line (LO2) treated with different doses of drug ZINC000003938642. **(D)** Clonogenicities in Petri dishes with different doses of drug. **(E, F)** Numbers of clone formation in HOS and MG-63 cell lines. *P < 0.05; **P < 0.01; ***P < 0.0001; ns, none significance.

structures were downloaded from the ZINC15 database for virtual screening, then ADME, TOPKAT, CDOCKER, and other modules were applied to perform ADME prediction, rodent carcinogenicity and Ames mutation prediction, ligand–receptor binding studies, and MD simulations. The LibDock scores suggested the degree of energy optimization and conformational stability between compound and receptor. Compounds with higher LibDock scores illustrated better energy optimization and more stable conformation than compounds with lower scores. Calculation results of the LibDock module showed that a total of 13,537 compounds could be stably combined with EZH2 after fast docking method. Among these ligands, 669 compounds had higher LibDock scores than the reference compound GSK126 (LibDock score: 132.143), suggesting that the stability and energy optimization effect of the complex substances formed by these 669 compounds with EZH2 were more stable than GSK126–EZH2 complex. Based on ranking of the LibDock scores, the top 20 compounds with the highest scores were screened out and tested in next steps.

ADME and toxicity prediction were performed to evaluate the pharmacological properties of these selected compounds. After analysis, results elucidated that ZINC000004217536 and ZINC000003938642 had satisfactory intestinal absorption capacity, and these two compounds had no obvious inhibitory effect on CYP2D6, no hepatotoxicity, and low binding affinity property with plasma protein, which suggested the good selectivity of these drugs; they could avoid rapid clearance by plasma so as to behave the best pesticide effect. Furthermore, compared with other compounds, ZINC000004217536 and ZINC000003938642 were predicted not to have Ames mutagenicity and rodent carcinogenicity, and they had less

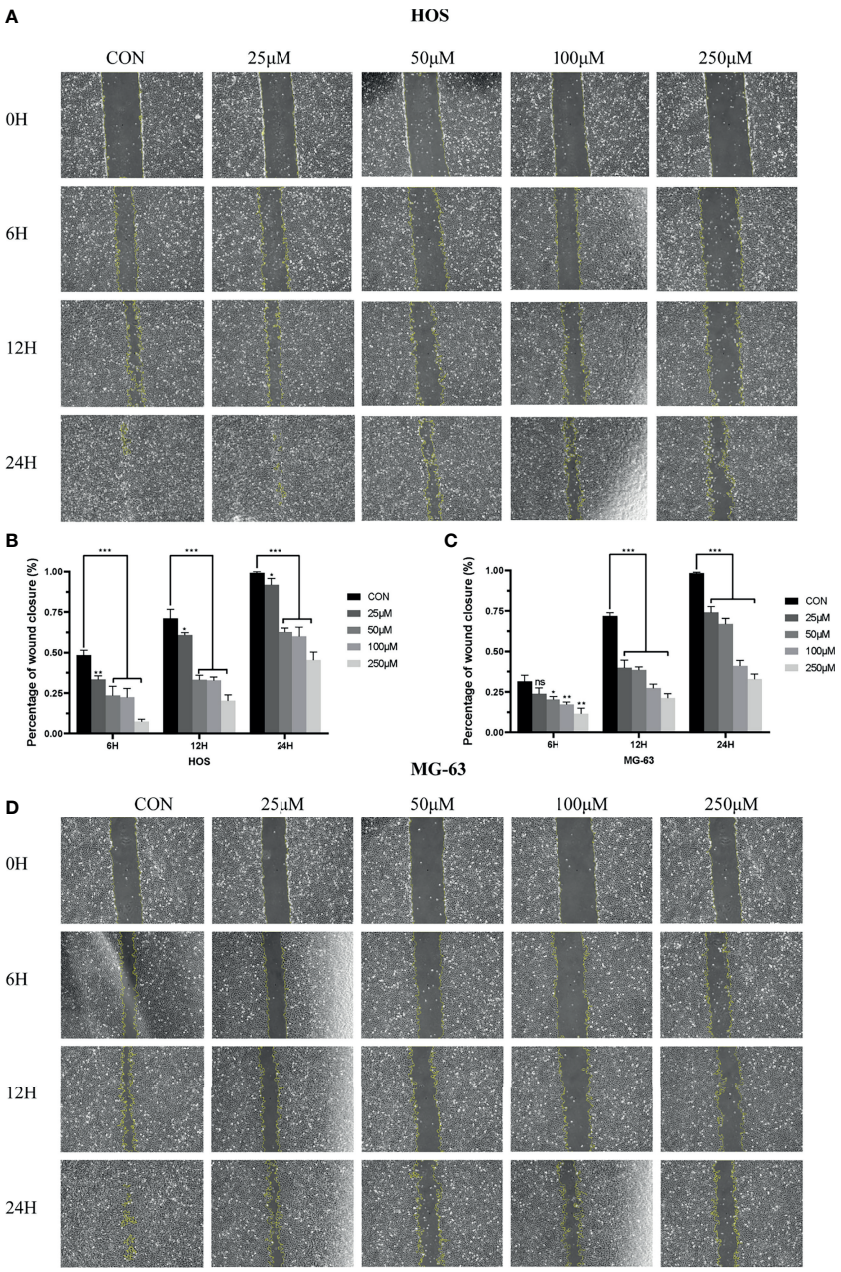

FIGURE 8 | **(A)** Scratch assay of HOS cell line in control and different drug groups at 0, 6, 12, 24 h. **(B)** Percentage of wound closure of HOS cell line in control and different drug groups at 0, 6, 12, and 24 h. **(C)** Scratch assay of MG-63 cell line in control and different drug groups at 0, 6, 12, and 24 h. **(D)** Percentage of wound closure of MG-63 cell line in control and different drug groups at 0, 6, 12, and 24 h. *P < 0.05; **P < 0.01; ***P < 0.0001; ns, none significance.

developmental toxicity potential. Consequently, they were considered as ideal candidate compounds with pharmacologic properties and higher security in the body; these characteristics were enough to be considered as the most potential lead compounds. Based on the above results, ZINC000004217536 and ZINC000003938642 were reasonably recognized as high-quality medicinal materials; these two compounds had broad application prospects in drug development and design. Although other drugs on the list had certain negative effects such as developmental toxicity and Ames mutagenicity, other pharmacological properties were relatively moderate, so they also had a certain potential in drug improvement, which could be achieved by adding or deleting specific functional groups or atoms to reduce their negative effects. In summary, it was determined that ZINC000004217536 and ZINC000003938642 were the most potential lead compounds, and more analyses were further performed.

Subsequently, we analyzed chemical bonds and the binding mechanisms between candidate compounds, GSK126 and

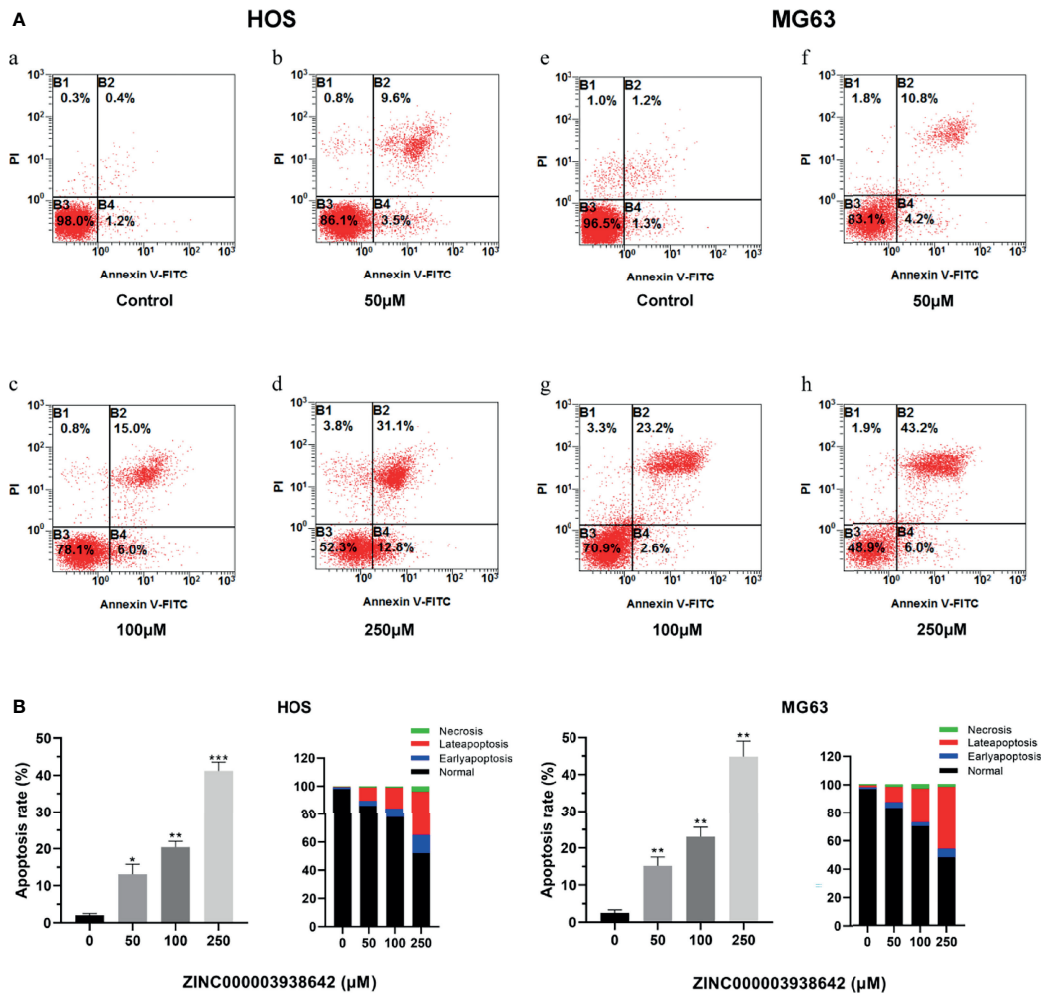

FIGURE 9 | **(A)** The distribution in apoptosis with different concentrations in HOS and MG-63 cells. a–d: drug treatment with 0, 50, 100, and 250μM for 24 h in HOS cells; e–h: drug treatment with 0, 50, 100, and 250 μM for 24 h in MG-63 cells. **(B)** Apoptotic rates and percentage with different concentrations in HOS and MG-63 cells. *P < 0.05; **P < 0.01; ***P < 0.0001.

EZH2. Precise docking method CDOCKER module was conducted; results showed that the CDOCKER interaction energy of ZINC000004217536 and ZINC000003938642 was significantly lower than that of the reference ligand GSK126, proving that the affinity of these two compounds with EZH2 was higher than GSK126–EZH2 in real situations. After that, through molecular detection analysis of their chemical structures among these complexes, results illustrated that the chemical bond and interaction force of the complex formed by EZH2 and the two candidate compounds were stronger, which further explained that they may have a competitive inhibitory effect on the regulatory site of EZH2 and thus inhibit the activity of EZH2, finally producing antitumor effects.

Ultimately, MD simulations were performed to predict the stability of the complexes formed by the candidate compounds and EZH2 in the internal environment. By calculating the RMSD and energy values of these ligand–EZH2 complexes, the RMSD curve and energy curve were drawn. Results showed that the trajectories of the complexes reached equilibrium after 100ps, and the RMSD and energy values of these complexes tended to be stable over time, indicating that the two complexes could exist stably in the internal environment. Furthermore, chemical bonds heatmap elucidated that these chemical bonds, which contributed remarkably to the stability of the complex, could keep steady with the progression of the MD. Consequently, the compounds selected in this study bonded tightly to EZH2, and they were capable of existing stably in the body, thereby exerting corresponding pharmacological functions. Therefore, they have great potential in the development of EZH2 inhibitors. It is noteworthy that the reference compound GSK126 chosen in this study served as a known effective synthetic EZH2 inhibitor; the effects of natural compounds were hardly better than GSK126 *in vitro* or *in vivo*. The role GSK126 played in this process was to provide a primitive crystal complex for us to compare the binding mode and give us an initial active binding sphere, and

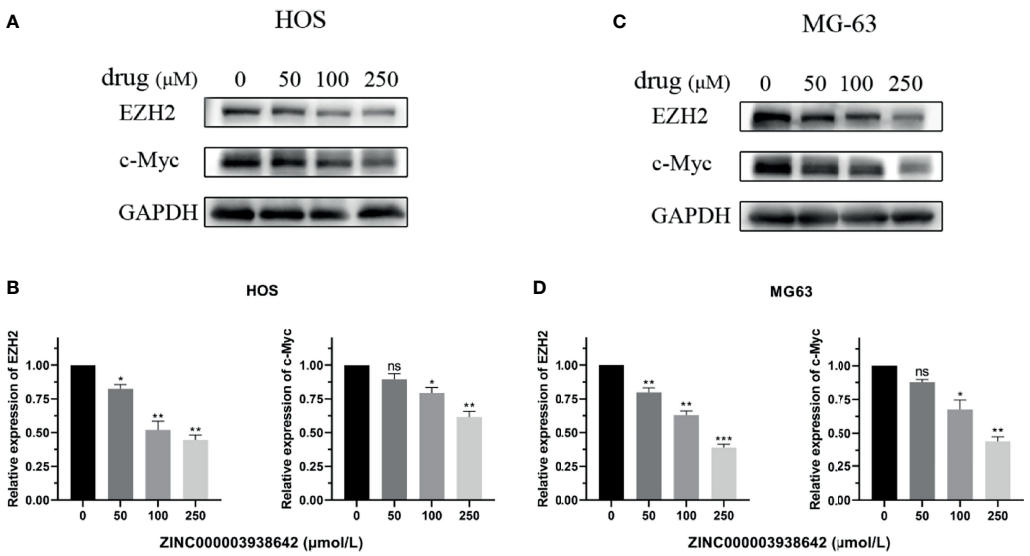

FIGURE 10 | **(A, B)** The expression of Enhancer of Zeste Homolog 2 (EZH2) and its downstream gene c-Myc when treated with different doses of drug in HOS cells. **(C, D)** The expression of EZH2 and its downstream gene c-Myc when treated with different doses of drug in MG-63 cells. *P < 0.05; **P < 0.01; ***P < 0.0001; ns, none significance.

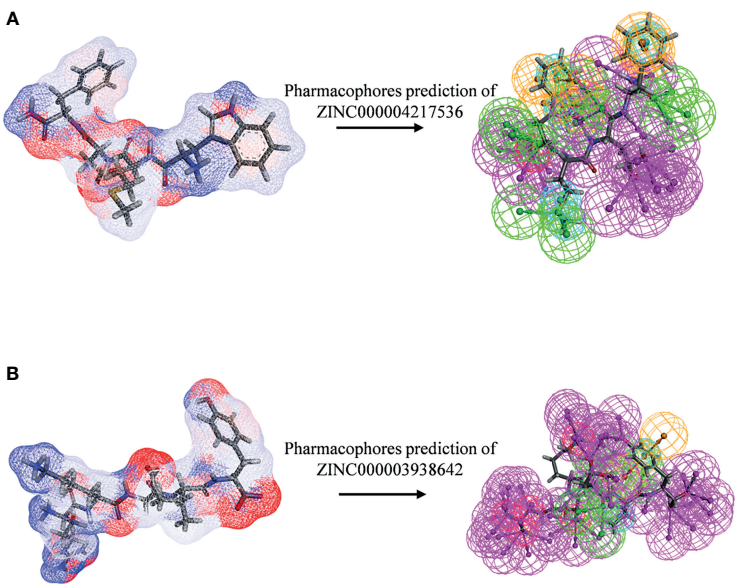

FIGURE 11 | Pharmacophore predictions of **(A)** ZINC000004217536 and **(B)** ZINC000003938642 using 3D-QSAR. Green represents hydrogen acceptor, blue represents hydrophobic center, purple represents hydrogen donor, and orange represents aromatic ring.

it did not provide any guiding significance for the comparison between GSK126 and ZINC000003938642 in antitumor aspects.

Currently, existing studies pointed out EZH2 could serve as a therapeutic target regarding OS (11), while few studies focused on targeted therapy of OS targeting EZH2. Consequently, this study preliminarily discussed the effects of newly found compounds against OS. To prove the pesticide effects of our newly found compounds against OS and the reliability of the screening method in this study, we selected one of the candidate compounds, ZINC000003938642, and performed a series of *in vitro* experiments including CCK-8, CFA, scratch assay, Western blot, and apoptosis assay. In CCK-8 assay, results pointed that the cellular viability in OS cells had a dose-dependent decrease when treated with drug ZINC000003938642,

while the drug was relatively well tolerated for human liver cells LO2. This finding implied that this drug was relatively nontoxic in term of hepatotoxicity, which was also consistent with our predictions in structural biology part that ZINC000003938642 was a nontoxic drug. In CFA, the numbers and size of clonogenicities in drug group were significantly less than those in control group in both HOS and MG-63 cell lines, which was consistent with results that the proliferation of OS cells was reduced by drug in CCK-8 assay and that the effects were dose-dependent. Scratch assay revealed that the wound area in control group decreased more sharply than that in drug group with time. As for apoptosis assay, flow cytometry results visualized that the percentage of apoptotic cells increased with the drug increasing, the apoptotic rates of HOS and MG-63 cells treated with high drug dose groups were significantly higher than those of control (0 μM) group ($P < 0.05$). Western blot analysis revealed that EZH2 expression decreased with increasing drug concentrations. Since c-Myc is the downstream target of EZH2, and as an oncogene (37–39), the expression level of c-Myc could also reflect the inhibitory effects of the drug on EZH2. Results displayed that the downstream oncogene c-Myc was also inhibited by the drug in a dose-dependent manner, implying that drug ZINC000003938642 could serve as a potential EZH2 inhibitor. These experiments suggested the ability of drug to inhibit the proliferation, migration, and EZH2 and c-Myc expression of OS cells, which indicated that drug ZINC00000 3938642 found in this study was an effective inhibitor regarding OS, and EZH2 was a therapeutic target against OS.

The screening of ideal lead compounds is a key step in drug design and development. Regarding the pharmacophore predictions of ZINC000004217536 and ZINC000003938642, they possessed a number of pharmacophores, which elucidated that based on these skeletons of these two compounds, the modification and refinement of the drug could be conducted to further make a whole new design. The natural compounds discovered in this study are of great significance in the development of EZH2 inhibitors. This study provided evidence for the targeted treatment of OS regarding EZH2 and may have the potential to provide better methods for tumor treatment. Besides, in the field of pharmacology, more research could be studied like modifying the molecular structure of the drugs to reduce the toxicity and mutation to continuously improve the pharmacological effect of the inhibitor.

CONCLUSIONS

This study used a series of virtual screening techniques and discovered two natural compounds, ZINC000004217536 and ZINC000003938642, which have the function of inhibiting the active subunit EZH2 of PRC2. These two compounds bind tightly to the target protein. Additionally, they have no carcinogenicity and toxicity, so they can be regarded as potential EZH2 inhibitors. *In vitro* experiments confirmed that drug ZINC000003938642 could inhibit the proliferation and migration of OS, which could serve as potential lead compounds. This study not only provided the pharmacological properties of candidate drugs but also provided meaningful materials for further research of EZH2-targeted inhibitors.

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

This study was completed with teamwork. Conceived the idea: MY, LW, CY, and WL. Wrote the main article: WL, ZD, and YLZ. Used the software: WL, YLZ, YJZ, HZ, RX, and YY. Downloaded and collected data: WL, ZD, MC, YLZ, DW, and SZ. Analyzed the data: WL, ZD, YLZ, MJ, KL, LW, and CY. Prepared figures: YLZ, WL, ZD, YJZ, MJ, KL, HZ, and SZ. Redressed the article: all authors. Reviewed the article: MY, LW, CY, and WL. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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A Novel Type of PD-L1 Inhibitor rU1 snRNPA From Human-Derived Protein Scaffolds Library

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Three marketed anti-PD-L1 antibodies almost have severe immune-mediated side effects. The therapeutic effects of anti-PD-L1 chemical inhibitors are not satisfied in the clinical trials. Here we constructed human-derived protein scaffolds library and screened scaffolds with a shape complementary to the PD-1 binding domain of PD-L1. The RNA binding domain of U1 snRNPA was selected as one of potential binders because it had the most favorable binding energies with PD-L1 and conformed to pre-established biological criteria for the screening of candidates. The recombinant U1 snRNPA (rU1 snRNPA) in *Escherichia coli* exhibits anti-cancer activity in melanoma and breast cancer by reactivating tumor-suppressed T cells *in vitro* and anti-melanoma activity *in vivo*. Considering hydrophobic and electrostatic interactions, three residues were mutated on the interface of U1 snRNPA and PD-L1 complex, and the ranked variants by PatchDock and A32D showed an increased active phenotype. The screening of human-derived protein scaffolds may become the potential development of therapeutic agents.

Keywords: PD-L1, inhibitor, human-derived, scaffold, melanoma, breast cancer

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INTRODUCTION

Recently, immune checkpoint inhibitors have developed rapidly and become the most promising cancer immunotherapy strategy with notable clinical benefits (1). The PD-1/PD-L1 axis is one of the most typical immune checkpoint axes, and its inhibitors were acknowledged as the fourth major cancer therapy (2). Emerging evidence shows that the use of antibodies to block the interaction between PD-L1 and its receptors can strengthen the cytotoxic activity of anti-tumor T cells and alleviate PD-L1-dependent immunosuppressive effects *in vitro* (3). Six monoclonal antibodies (mAbs) have been approved by the Food and Drug Administration (FDA) for use in cancer immunotherapy, including durvalumab, cemiplimab, nivolumab, pembrolizumab, avelumab, and atezolizumab (4). However, antibodies have intrinsic disadvantages that limit their application—for example, high manufacturing costs, low instability, low tissue penetration, and immunogenicity (5). Therefore, drug discovery studies relating to the PD-1/PD-L1 axis have increasingly focused on low-molecular-weight inhibitors such as single-chain antibodies, chemical inhibitors, peptides, and peptidomimetics (6). However, the binding interface of PD-1 and PD-L1 is large and flat and lacks deep pockets; some chemical inhibitors are prone to off-targeting (7). The current development of

chemical inhibitors is focused on inducing PD-L1 dimerization rather than directly blocking, but so far, only publications and patents of PD-1/PD-L1 chemical inhibitors have been disclosed; there are no FDA-approved inhibitors for clinical use, and some chemical inhibitors failed to reactivate T cells and were cytotoxic (8). Therefore, it is still an important proposition to find the direction of a novel molecular structure for therapeutic use. Because immunogenicity is an important issue to develop therapeutic agents, soluble human-derived protein scaffolds are an ideal research and development direction.

However, due to the limitations in computational resources and other aspects, it is still very difficult to predict the interactions between macromolecules in batches. Molecular docking is a computer-aided drug design method based on receptors, starting from ligand–receptor binding, and theoretically calculating and analyzing the interaction modes between ligand and receptor (9). Molecular docking in drug screening mainly focuses on virtual screening and activation prediction with small molecules as ligands (10), while it is less used in drug screening with protein ligands (11). PatchDock uses object recognition and image segmentation techniques similar to those used in computer vision. The surface of a given molecule can be divided into multiple small patches according to the shape by PatchDock. Once the complementary structure is identified, it can be superimposed using a shape-matching algorithm and finally ranked by shape complementarity score (12). Protein scaffolds originally represent a category of affinity proteins that complement the immunoglobulins and antibody derivatives (13). Non-immunoglobulin-based protein scaffolds have been reported as promising alternatives to traditional monoclonal antibodies in recent years (13). The idea of using protein scaffolds as PD-L1 inhibitors originally came from the basic mechanism through which antibodies are produced against antigens. The amino acids in CDRs act as protein scaffolds which can produce diverse structures and form the complementary shape to recognize specific epitopes (14). The process of ligands or inhibitors binding to target proteins is similar to the binding of antigen and antibody, so protein scaffolds, especially human-derived protein scaffolds like the amino acids in complementarity-determining regions, whose shape is complementary to the target protein, have a significant potential to be ideal inhibitors.

In this study, we used rigid molecular docking server PatchDock to screen PD-L1 inhibitors from a human-derived protein scaffolds library (**Scheme 1**). The RNA binding domain of U1 snRNPA was selected as a protein binder to the PD-1 binding domain of PD-L1. Recombinant full-length U1 snRNPA in *Escherichia coli* was proven to inhibit PD-1/PD-L1 interaction directly. The results were demonstrated by T cell reactivation assay and anti-cancer efficacy assay *in vitro*/*in vivo*. The rU1 snRNPA is considered a lead compound; we further mutated its residues on the interface of U1 snRNPA and PD-L1 complex, and the variant A32D showed an active phenotype. Our results suggested that the application of a human-derived protein scaffolds library and rigid molecular docking is a highly efficient and rapid tool for designing novel therapeutic protein drugs.

MATERIALS AND METHODS

Establishment of a Human-Derived Protein Scaffolds Library

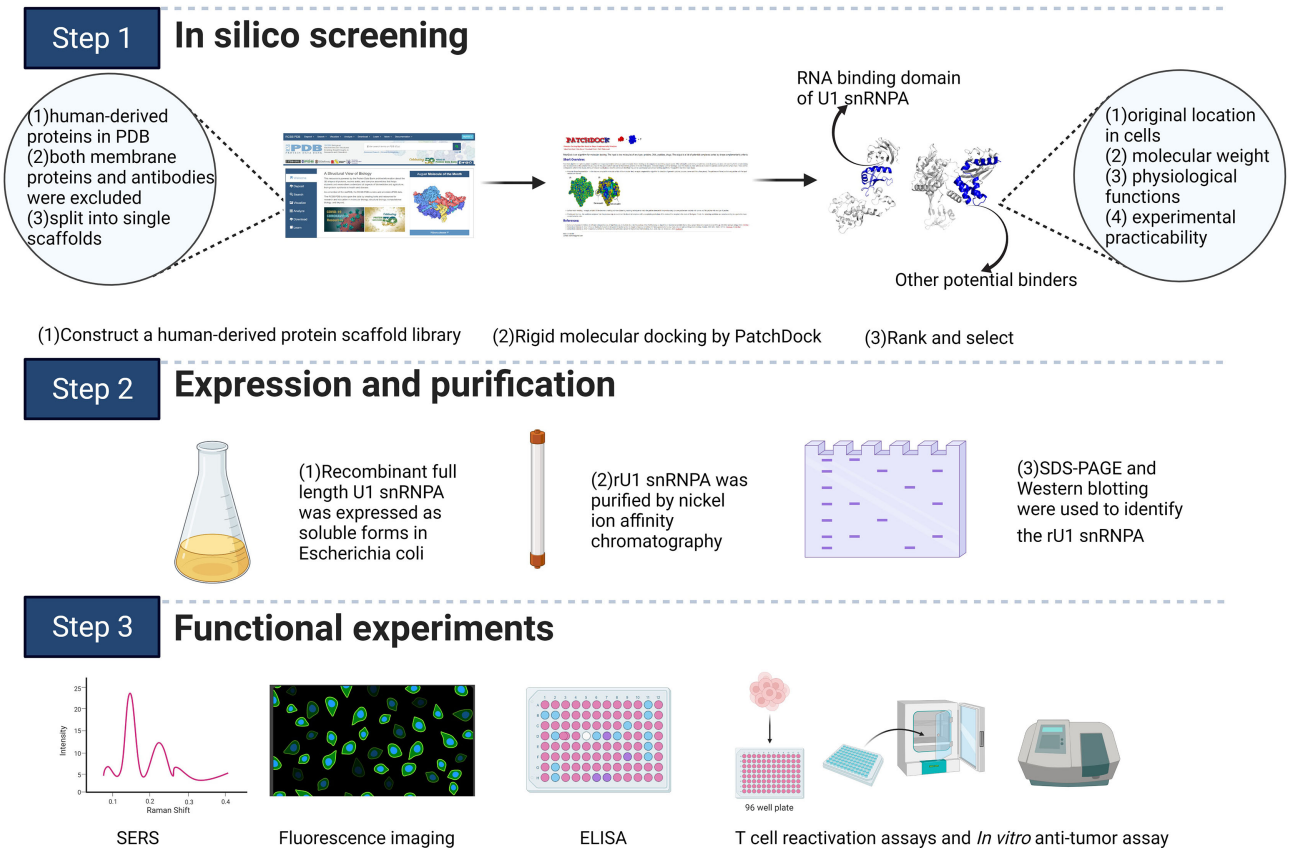
A human-derived protein scaffolds library was established by the following three steps. First, all human-derived proteins in the RCSB Protein Data Bank (PDB; https://www.rcsb.org/) (15) were selected using the category "Organisms: *Homo sapiens*". Second, both membrane proteins and antibodies were excluded using the annotation "transmembrane proteins" and searching for the keyword "antibody" in the PDB, respectively (16). Third, the selected structures were split into single scaffolds using "END" in the PDB file. Considering the available computing ability and the huge number of samples, 1,863 scaffolds were randomly selected and used as a library for screening.

Screening of PD-L1 Binding Scaffolds by Rigid Molecular Docking

The PD-1 binding domain from the human PD-L1 structure (PDB: 5C3T) and the constructed library were defined as the corresponding docking analytes. PatchDock was chosen as the molecular docking program (http://bioinfo3d.cs.tau.ac.il/PatchDock/) (17). PatchDock is a geometry-based molecular docking algorithm. The algorithm has three major stages: molecular shape representation, surface patch matching, and filtering and scoring. The parameters in PatchDock were all set to default values. The PatchDock score of PD-L1 and durvalumab, which has a proven high affinity for PD-L1, was set as a standard value. Only scaffolds with scores close to or higher than this standard value were selected in the first round of screening. For the second-round screening, the top 10 docking models for each scaffold–PD-L1 docking result according to the PatchDock score were downloaded, followed by analysis and assessment of their binding modes and conformations. Scaffolds with similar paratopes to PD-1/PD-L1 were selected (18). The geometric shape complementarity scores of these scaffolds were recorded and ranked. In the third round, the scaffolds were screened further based on their original location, molecular weight, physiological function, and experimental practicability in the laboratory. The RNA-binding domain of the U1 small nuclear ribonucleoprotein A (PDB: 1U6B) was selected for the next step of the research. The key residues on the interaction surfaces of PD-L1 and U1 snRNPA were analyzed using InterProSurf (19).

Inhibitory Effect on PD-1/PD-L1 Detected by SERS

A SERS-based PD-1/PD-L1 inhibitor detection platform was used to detect the inhibitory effect. When AgNPs@PD-1@4-ABP and MNs@PD-L1 bind normally, a high SERS characteristic peak would be produced; if the added substance can inhibit the binding between them, the characteristic peak would decrease. Therefore, the inhibitory effect on the PD-1/PD-L1 signaling pathway can be detected by observing whether the substance caused a reduction of the SERS characteristic peak. The feasibility of this method was preliminarily verified through experiments with existing inhibitors (durvalumab and BMS-202; see the supplementary materials for details).



SCHEME 1 | Schematic illustration of the study. The scheme was created with BioRender.com.

T Cell Reactivation Assays

Levels of IFN-γ or TNF-α production were evaluated to determine whether tumor-suppressed T cells were reactivated. Human CD4+ T lymphocytes were co-cultured with A375 cells, MDA-MB-231 cells, PD-L1-negative HEK293T cells, or HEK293T cells with hPD-L1 expression (HEK293T-hPD-L1) in 96-well plates. A density of 10,000 cells per well was plated and adhered for 24 h; then, 20,000 reactivated CD4+ T lymphocytes per well were added to the plates with different inhibitors, followed by co-culturing at 37°C, 5% CO_2 for 48 h. The level of IFN-γ or TNF-α production in culture supernatants was detected by Human IFN-γ ELISA kit (BD Biosciences) and Human TNF-α ELISA kit (BD Biosciences) (for the expression and purification of rU1 snRNPA, binding ELISA, and competitive ELISA, *in vitro* anti-cancer assay, *in vivo* anti-melanoma assay, see the supplementary materials for details).

RESULTS

U1 snRNPA Was Screened From Human-Derived Protein Scaffolds Library by Rigid Molecular Docking

The whole screening process is illustrated in **Figure 1A**. A library containing 1,863 scaffolds of human-derived proteins was successfully constructed and screened for potential PD-L1 binding ability. The standard value for molecular docking was 16,172, which was the PatchDock score of PD-L1 and durvalumab. By two-round screening, the top 20 binders (**Supplementary Table S1**) were screened to exclude enzymes and their analogues. Finally, the remaining binders were screened based on their original location in the cells, molecular weight, physiological functions, and experimental practicability. The RNA-binding domain of the U1 small nuclear ribonucleoprotein A (U1 snRNPA) (PDB: 1U6B) was selected for the subsequent study.

The key residues of the interaction surfaces between U1 snRNPA and PD-L1 were analyzed by InterProSurf. As shown in **Figure 1B**, the α-helix of U1 snRNPA interacted with the loop of PD-L1. The binding sites were as follows: Ala32, Ile33, Gln36, Ser71, Phe75, and Pro76 in U1 snRNPA and Asp61, Arg113, and Tyr123 in PD-L1.

According to the *in silico* results, recombinant full-length U1 snRNPA was expressed as soluble forms in the *Escherichia coli* (E. coli) expression system. As the sequence of the RNA-binding domain of the U1 snRNPA (PDB: 1U6B) was too short to form the correct tertiary structure, the whole sequence of U1 snRNPA was synthesized and expressed for subsequent experiments, as it had the same binding epitope with PD-L1. After expression, rU1

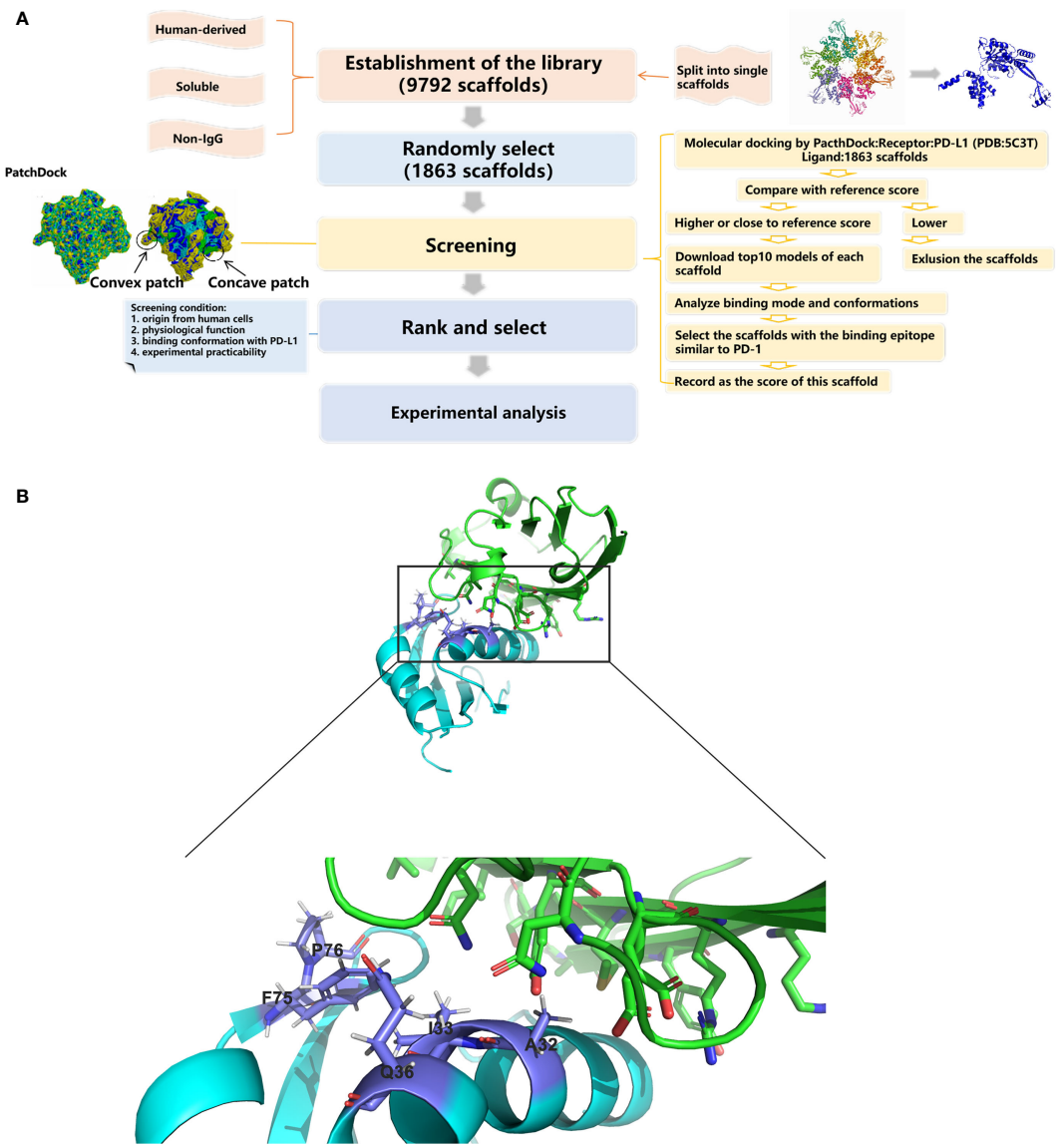


FIGURE 1 | Schematic drawings of the screening processes and interactions of U1 snRNPA/PD-L1. **(A)** Flow chart of database construction and screening processes. **(B)** Cartoon representations for the identified interface of U1 snRNPA/PD-L1 complex. Cyan, U1 snRNPA (PDB: 1U6B); green, PD-L1. Stick representations show the key residues.

snRNPA was purified successfully by nickel-ion-affinity chromatography (**Supplementary Figures S1A**, **S2**).

rU1 snRNPA Inhibit PD-1/PD-L1 Interaction by Binding PD-L1

Based on the results of SERS, we have preliminarily verified the inhibition efficacy of rU1 snRNPA on PD-1/PD-L1 interaction in practice. The schematic illustration of the establishment of the SERS-based PD-1/PD-L1 inhibitor detection platform is shown in **Scheme S1**, and the results are shown in **Supplementary Figure S4**. The intensity of the characteristic peak of 4-amnobiphenyl (4-ABP) had significantly decreased compared to the control when rU1 snRNPA was added (**Figure 2A**), which indicated that rU1 snRNPA inhibited the interaction of AgNPs@PD-1@4-ABP and MNs@PD-L1. The results of ELISA further proved that rU1 snRNPA had the ideal binding ability to PD-L1 in competition with PD-1 (**Figures 2B, C**). The EC50 of rU1 snRNPA was 55.17 nM. The IC50 of rU1 snRNPA was 18.06 nM. Although the EC50 and IC50 of durvalumab (16.05 and 8.326 nM) were lower than those of rU1 snRNPA, these results still proved the enormous research potential of rU1 snRNPA as a primitive PD-L1 binder. SERS was used to analyze the protein–protein interaction in this study. Combined with the results of ELISA, it can be experimentally proved that rU1 snRNPA has the

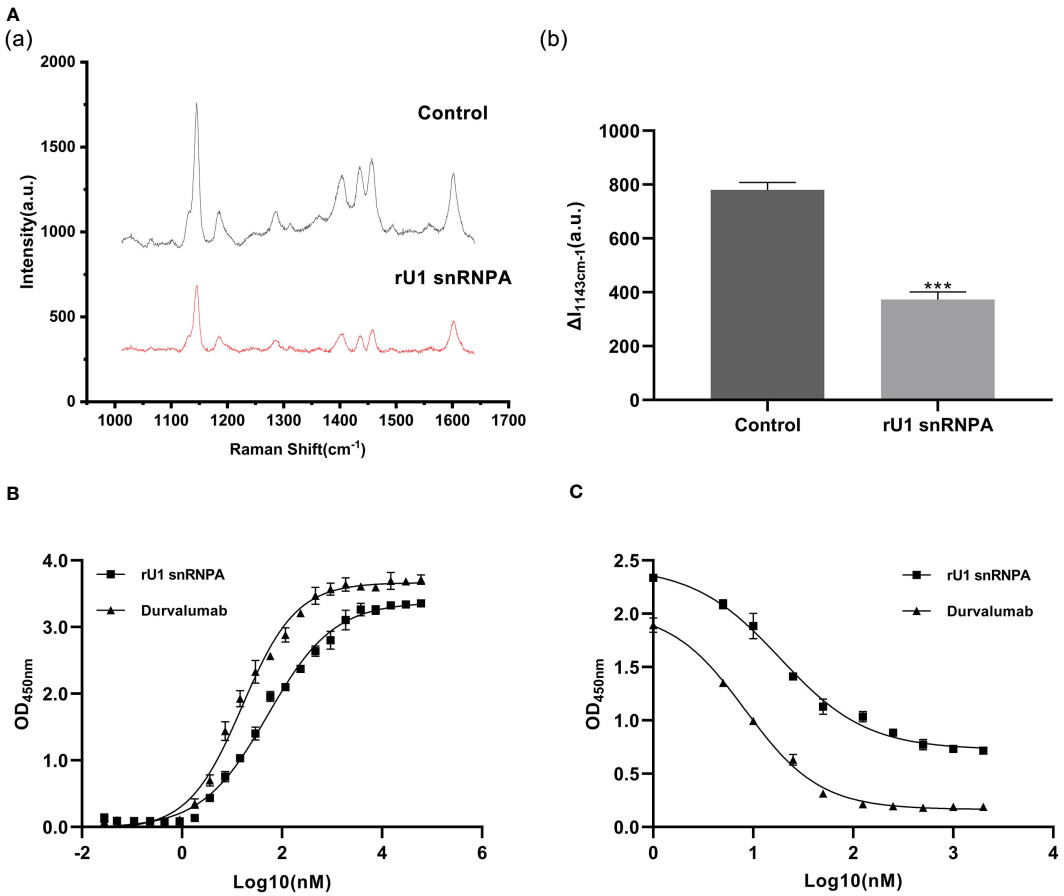


FIGURE 2 | Results of binding ability to PD-L1 and inhibition effect on PD-1/PD-L1 interaction. **(A)** Comparison of SERS spectra of rU1 snRNPA (1.5 μM) and control. (a) Representative SERS spectra. λ_{ex} = 632.8 nm, t = 5 s, and accumulation times = 1. (b) Intensity of the characteristic peak of 4-amnobiphenyl with the results of three repeated experiments. Data are shown as mean ± SD. ***P < 0.001. **(B)** Binding curves of rU1 snRNPA and durvalumab. Curve fitting was performed by using GraphPad Prism 8.0. Error bars denote SD. The experiment was performed in double for each sample group. **(C)** Inhibition curves of rU1 snRNPA and durvalumab. Curve fitting was performed by using GraphPad Prism 8.0. Error bars denote SD. The experiment was performed in double for each sample group.

ability to inhibit the interaction of PD-1/PD-L1 by binding PD-L1, which further proved the effectiveness of the screening method in this study.

rU1 snRNPA Reactivate Tumor-Suppressed T Cells and Had Anti-cancer Efficacy *In Vitro* and *In Vivo*

PD-1/PD-L1 inhibitors work by reactivating tumor-suppressed T cells rather than directly killing tumor cells. In this study, the function of T cell reactivation of rU1 snRNPA was evaluated by measuring the secretion of cytokines IFN-γ and TNF-α in CD4+ T cells. In the T cell–tumor co-cultured assay, A375 cells, MDA-MB-231 cells, and HEK293T-hPD-L1 cells expressed human PD-L1, binding to PD-1 on CD4+ T cells to suppress the function of T cells. The verification of PD-L1 expression on cells by flow cytometry and high-content imaging of A375 cells is shown in **Figure 3**. When rU1 snRNPA was added, the levels of IFN-γ and TNF-α showed a dose-dependent increase similar with durvalumab (**Figure 4A**). Although some groups showed no significant differences at lower concentrations, the levels of IFN-γ and TNF-α were still higher than those of the control group. At the concentration of 15 μM, both IFN-γ and TNF-α secretion elevated significantly compared to the control group. PD-L1-negative HEK293T cells treated with rU1 snRNPA were used to confirm whether the observations were dependent on blocking the PD-1/PD-L1 interaction. The results showed that rU1 snRNPA did not significantly increase the IFN-γ and TNF-α secretion of PD-L1-negative HEK293T cells co-cultured with CD4+ T cells (**Supplementary Figure S5A**). These results indicated that rU1 snRNPA had T cell reactivation function by blocking the PD-1/PD-L1 interaction.

CCK-8 assay was used to evaluate the anti-melanoma and anti-breast cancer efficacy and killing activity of rU1 snRNPA in A375 cells, MDA-MB-231 cells, and HEK293T-hPD-L1 cells. After treatment with rU1 snRNPA or durvalumab mixed with CD4+ T cells, the cells were killed in a concentration-dependent manner, and all three concentrations showed a significant difference compared to the control group. Since 15 μM showed

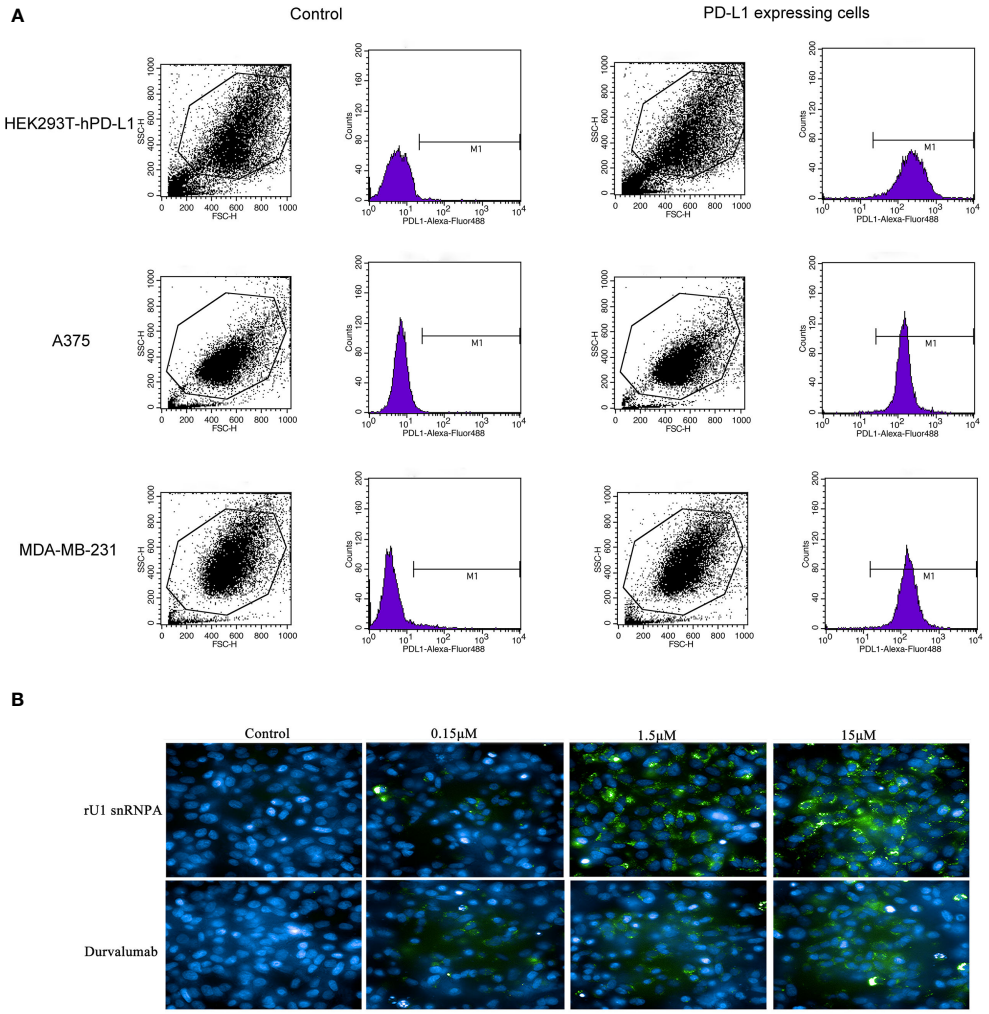


FIGURE 3 | **(A)** Flow cytometry results of the expression of hPD-L1 on cells. **(B)** High content imaging of A375 cells with different concentrations of rU1 snRNPA or durvalumab. Blue, nucleus by Hoechst33342; green, rU1 snRNPA or durvalumab conjugated with Alexa Fluor® 488 NHS Ester.

a little difference to 1.5 μM, the latter was considered as the ideal concentration for further development (**Figure 4B**). rU1 snRNPA showed little killing activity in A375 cells, MDA-MB-231 cells, or HEK293T-hPD-L1 cells without CD4+ T cells or PD-L1-negative HEK293T cells with CD4+ T cells (**Supplementary Figure S5B**), which proved that rU1 snRNPA only kills cells with CD4+ T cells and were not toxic to cells. rU1 snRNPA had *in vitro* anti-cancer efficacy.

Given the promising results of *in vitro* anti-melanoma efficacy, a melanoma–human immune system immunodeficiency mouse model was used to further evaluate the *in vivo* anti-melanoma efficacy of rU1 snRNPA. In this mouse model, PBMCs supplied T cells, which reacted with melanoma A375 cells, and immune responses were reactivated to inhibit tumor growth. As shown in **Figure 5A**, rU1 snRNPA resulted in a similar survival period as that of durvalumab and both inhibited tumor growth (based on volume) (**Figure 5B**). The dose 5 mg/kg was the most effective concentration of rU1 snRNPA in this study. Plasma concentration was monitored and measured by ELISA (**Figure S6A**). The peak plasma concentrations of rU1 snRNPA occurred at 8 h. The absorption and distribution time of rU1 snRNPA *in vivo* were similar to those of durvalumab, but the excretion time was much shorter, and it could hardly be detected in blood after 24 h. This was possibly due to the smaller molecular weight of rU1 snRNPA compared with durvalumab. The organ weight and H&E staining results showed that the effects of rU1 snRNPA treatment on important organs (heart, liver, spleen, lung, and kidney) were similar with those of durvalumab, with no increase in damage to the histology of these organs (**Supplementary Figures S6B**, **S7**). Collectively, these results indicated that rU1 snRNPA possessed ideal *in vivo* anti-melanoma efficacy.

Key Residues on the Interface of U1 snRNPA/PD-L1 Complex

To further verify that U1 snRNPA worked by binding to PD-L1 and find the key residues for binding, we designed three single-

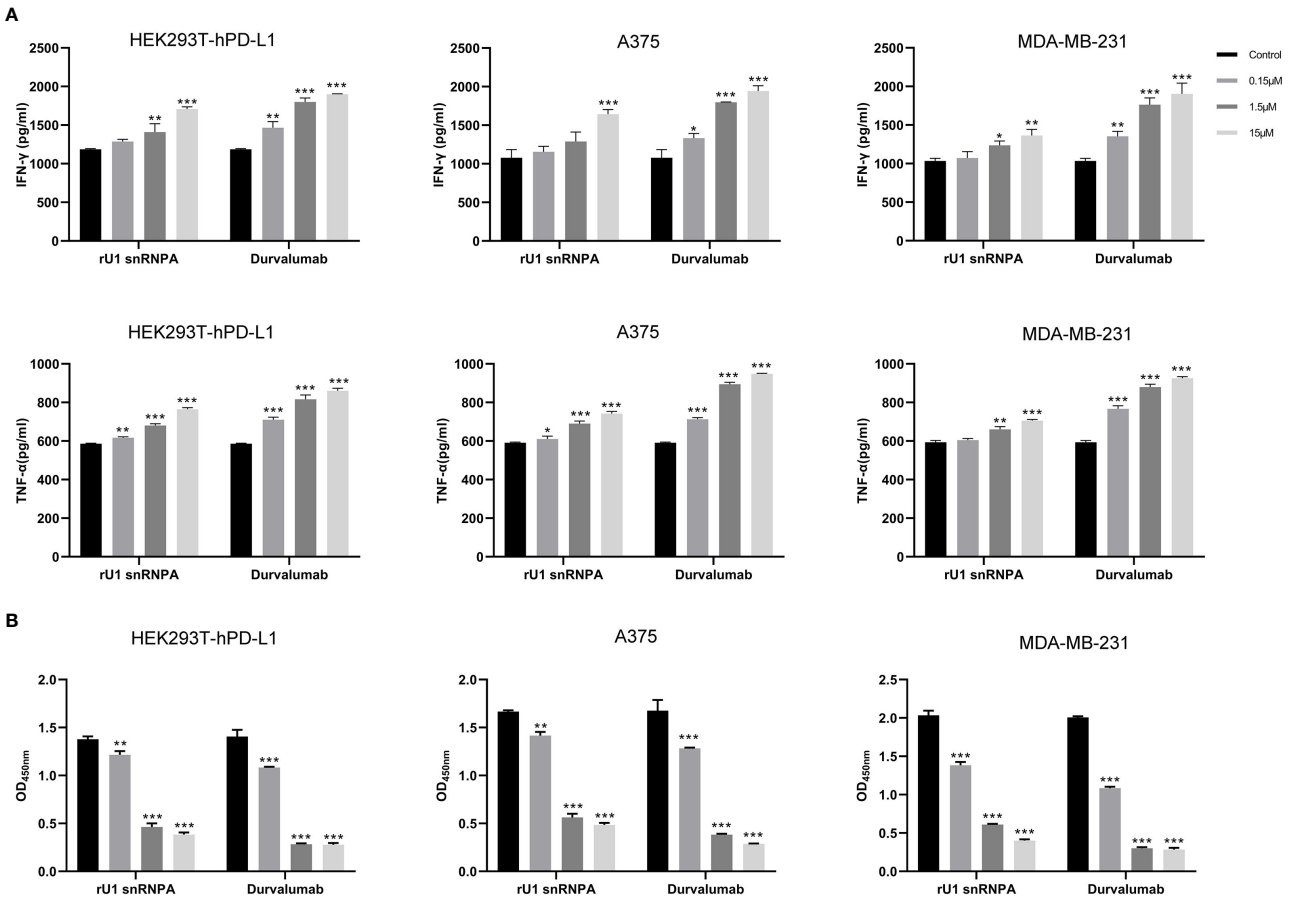


FIGURE 4 | Effect of rU1 snRNPA on T cell reactivation function and anti-cancer efficacy. **(A)** Effect of rU1 snRNPA or durvalumab on IFN-γ and TNF-α secretion from CD4+ T cells co-cultured with HEK293T-hPD-L1 cells or A375 cells or MDA-MB-231 cells. **(B)** Anti-cancer efficacy of rU1 snRNPA or durvalumab mixed with CD4+ T cells in HEK293T-hPD-L1 cells or A375 cells or MDA-MB-231 cells. Data are shown as mean ± SD.*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

point variants based on the structure of the U1 snRNPA/PD-L1 complex (**Figure 6**). According to the hydrophobic and electrostatic interaction, several potential residues were selected and mutated by Pymol; then, the complexes of variants and PD-L1 were scored by PatchDock. Unlike the previous docking in screening, in this docking, there were certain restrictions on the binding position, and full-length U1 snRNPA was used, so the scores were a little different to the scores of screening. Compared with the scores of wild types, we finally designed the variants of A32D, I33F, and Q36R. Among them, A32D raised the score; others lowered the scores in PatchDock (**Supplementary Table S2**).

Three variants were expressed and purified in the same way as the wild types and were used for subsequent experiments (**Supplementary Figure S2**). The binding ability to PD-L1 and the PD-1/PD-L1 inhibition efficacy of the variants were evaluated by the same methods as those of the wild types. The results of SERS showed that all variants lowered the characteristic peaks of 4-ABP compared to the control group, but the degree of reductions had no significant difference between the variants, suggesting that SERS may not be suitable for detecting small changes to the PD-1/PD-L1 interaction (**Figure 7B**). The results of ELISA proved that the variants had changed binding ability or inhibition efficacy, compared to wild types, corresponding to theoretical predictions (**Figure 7A**). Among the variants, the EC50 of A32D (51.36 nM) was lower than that in the wild types, which implied that it had better binding ability to PD-L1. The EC50 of others were higher than those in the wild types, which implied that they had worse binding ability to PD-L1. In the results of competitive ELISA, A32D (14.28 nM) had lower IC50, while the others had higher IC50. As the lower IC50 had better inhibitory effects to PD-1/PD-L1 interaction, A32D had better inhibition efficacy than the wild types. Moreover, the results showed that the EC50 and IC50 were not corresponding completely, suggesting that better binding ability to PD-L1 did not mean better inhibition efficacy to the PD-1/PD-L1 interaction. This feature should be emphasized in follow-up research and development. The results mentioned above verified the key residues of U1 snRNPA for binding to PD-L1 and inhibiting the PD-1/PD-L1 interaction. The study of key

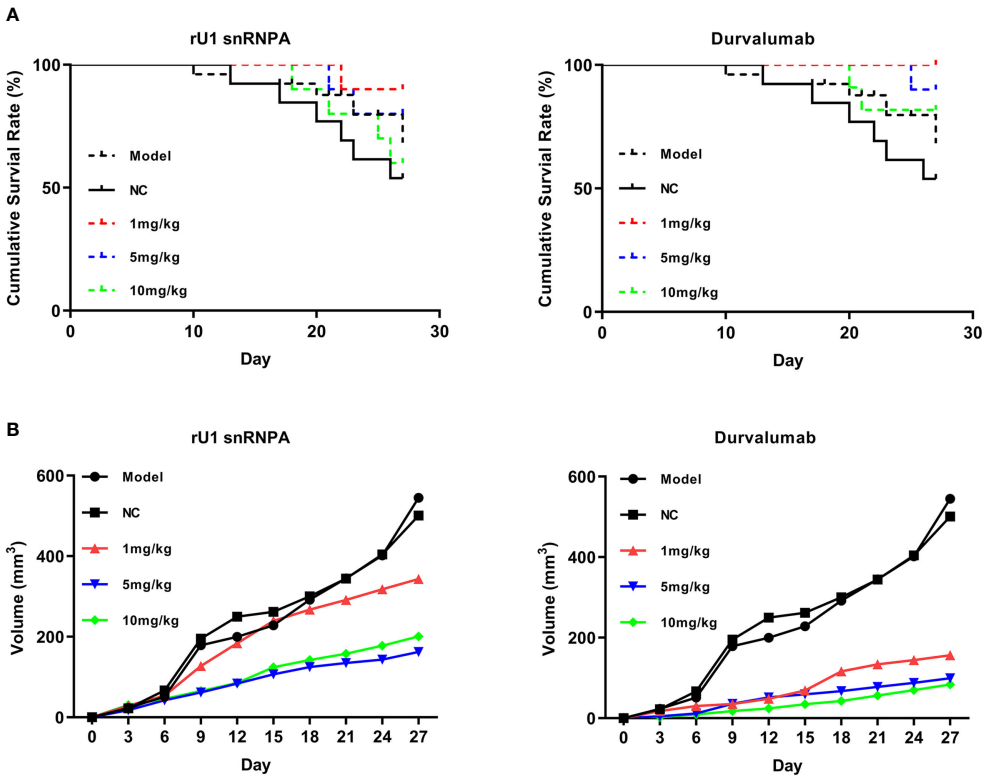


FIGURE 5 | Anti-melanoma efficacy of rU1 snRNPA in a melanoma–human immune system immunodeficiency mouse model. **(A)** Survival rates of rU1 snRNPA or durvalumab. **(B)** Tumor volume changes measured over time in different groups (n = 10).

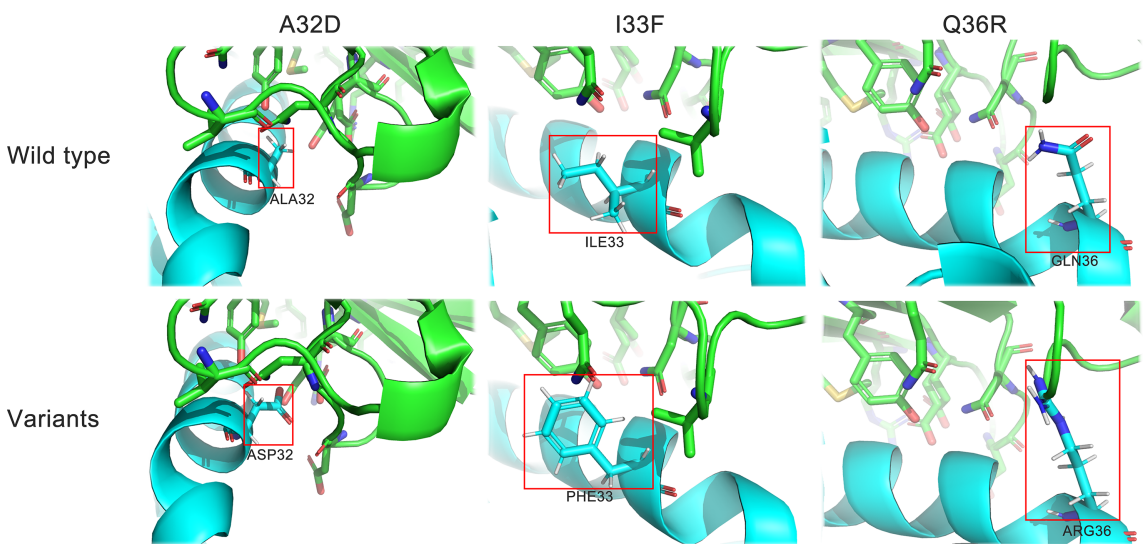


FIGURE 6 | Structure details of the variants. Cyan, U1 snRNPA; green, PD-L1. Stick representations in the red box show the mutated residues.

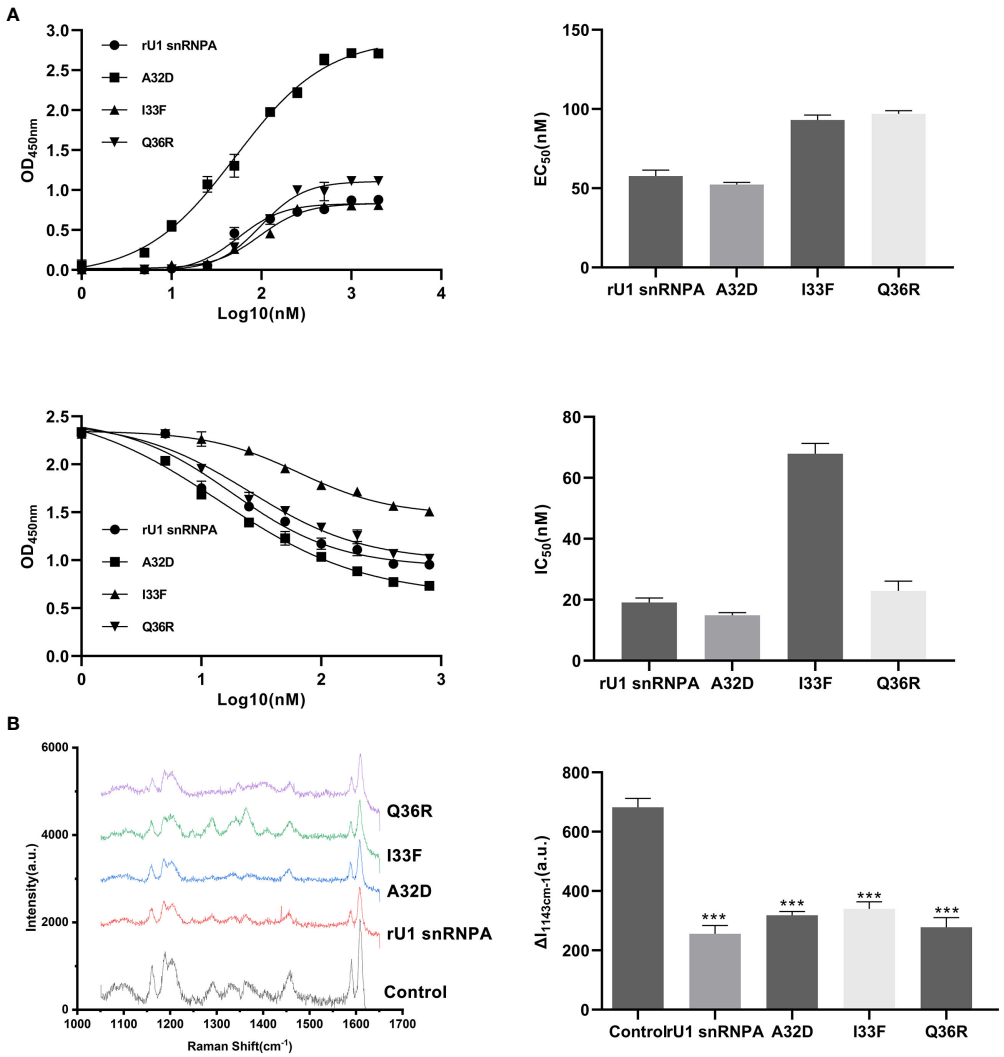


FIGURE 7 | Results of binding ability changes of variants. **(A)** Binding curves and inhibition curves of the variants. Charts of EC50 and IC50. Curve fitting was performed by using GraphPad Prism 8.0. The experiment was performed in double for each sample group. Data are shown as mean ± SD. **(B)** Comparison of the SERS spectra of the variants (both 1.5 μM). The characteristic peaks of 4-ABP are marked in gray. λex = 632.8 nm, t = 5 s, and accumulation times = 1. Data are shown as mean ± SD. ***P < 0.001.

residues on the interface of the U1 snRNPA/PD-L1 complex was not only to optimize and design the binder but also was conducive to analyze the key residues for interaction theoretically.

Increased Efficient Therapeutic Variant A32D

The effect of the variants on bio-activity was evaluated by T cell reactivation function and *in vitro* anti-melanoma efficacy. The experiment methods of the variants were the same as the wild types. In the A32D group of T cell, the reactivation assays showed that the levels of IFN-γ and TNF-α were higher than the wild-type groups, and others were higher than the control group but lower than the wild-type groups, although some groups did not have significant statistical differences (**Figure 8A**). From the results of *in vitro* anti-melanoma assay and killing activity assay, the variants still had efficacy to kill cells which expressed PD-L1 relying on CD4+T cells, and the efficacy was changed compared to the wild types (**Figure 8B**). Generally, *in vitro*, the A32D group had better anti-melanoma efficacy than the other groups, and other variants had similar or lower anti-melanoma efficacy than the wild types. The results of the variants in PD-L1-negative HEK293T cells co-cultured with CD4+ T cells or PD-L1-positive cells not co-cultured with CD4+ T cells did not significantly change the IFN-γ and TNF-α secretion (**Supplementary Figure S8A**) or killing activity (**Supplementary Figure S8B**). These results indicated that the variants of key residues led to the change of T cell reactivation function and *in vitro* anti-melanoma efficacy or killing activity to cells which expressed PD-L1. However, because only one residue had been mutated in each variant, they had not completely lost or most improved their binding ability to PD-L1, so their bio-activities were just partially changed.

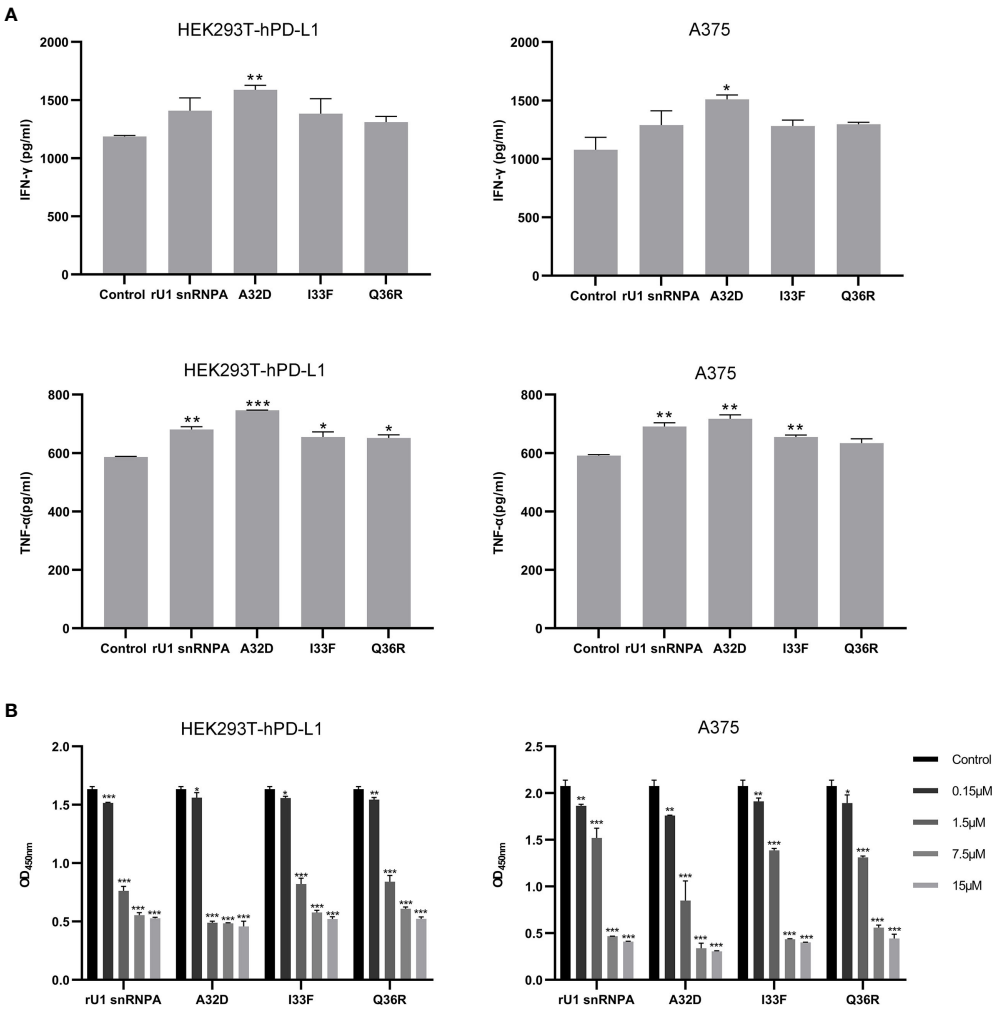


FIGURE 8 | Effect of variants on T cell reactivation function and anti-melanoma efficacy. **(A)** Effect of variants on IFN-γ and TNF-α secretion from CD4+ T cells co-cultured with HEK293T-hPD-L1 cells or A375 cells. **(B)** Anti-melanoma efficacy of variants mixed with CD4+ T cells in HEK293T-hPD-L1 cells or A375 cells. Data are shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

DISCUSSION

In recent years, except for chemical inhibitors, the development of non-IgG PD-L1 inhibitors seems to only focus on PD-1-based variants and peptides (6). So far, there were no other reports about the novel type of PD-L1 inhibitor from human-derived protein scaffolds library. The most important advantage of human-derived protein scaffolds is low or even no immunogenicity. The immunogenicity of therapeutic proteins is one of the serious problems hindering their development, especially when they are administered as multiple doses over prolonged periods (20). There are many factors that can influence the immunogenicity of therapeutic proteins, such as structural features (sequence variation and glycosylation), storage conditions (denaturation or aggregation caused by oxidation), and contaminants or impurities in the preparation (20). The clinical application of antibodies is also challenged by immunogenicity. The immunogenicity of antibody therapeutics can impact the safety and pharmacokinetic properties, which can impact the efficacy of the drugs (21). Therefore, understanding, controlling, and engineering around the potential immunogenicity is of great concern to the pharmaceutical industry (20). Although strategies, such as the humanization of antibodies, have reduced the immunogenicity to a certain extent, there is no solution that can better solve the problem of immunogenicity in the development of protein drugs (21). The human-derived protein scaffolds that we studied provided a new strategy to solve the problem of immunogenicity. Since the scaffolds were originally derived from humans, they have almost no immunogenicity in the human body, avoiding serious side effects in clinical treatment.

In addition, human-derived protein scaffolds are different from PD-L1 antibodies, which can avoid many disadvantages of the antibodies. First, the manufacturing cost of human-derived protein scaffolds like rU1 snRNPA can be lower than that of antibodies which had low yield from mammalian expression systems. Second,

the molecular weight of rU1 snRNPA is lower than antibodies, which may lead to better penetration into solid tumors. Compared with PD-1-based variants or peptides, human-derived protein scaffolds like rU1 snRNPA have a more ideal half-life with restriction of molecular weight and are more conducive to becoming an injectable immune-therapy drug. Ideal half-life also makes human-derived protein scaffolds like rU1 snRNPA better to control in terms of appropriate administration dosage to reduce adverse events. Compared with chemical inhibitors, human-derived protein scaffolds can better balance the ideal therapeutic effect and have low cytotoxicity. However, not all human-derived protein scaffolds can be developed into drugs—for example, enzymes and their analogues which interact with some small molecule substrates in the body and play extremely important functions are not suitable for development as therapeutic agents. In the same way, some scaffolds, such as interleukins, which act outside the cell cannot be developed into drugs as well; they are likely to interfere with the normal functions of the human body and cause a series of side effects. The U1 snRNPA selected in this study is located in the nucleus, which acts inside the cell, so it will not interfere with the normal function of the human body when it acts as a therapeutic agent outside the cell. In addition to the type of scaffolds, the molecular weight is also very important. It is necessary to fully consider the filtration of the glomerulus and the reabsorption of the renal tubules (22). In the subsequent development of a novel type of PD-L1 inhibitors from human-derived protein scaffolds library, these factors need to be paid attention to in order to obtain more ideal candidate inhibitors.

In anti-cancer assays, whether in melanoma A375 cells or breast cancer MDA-MB-231 cells or HEK293 cells expressing PD-L1, rU1 snRNPA has almost no anti-cancer activity without tumor-suppressed T cells. These results indicated that the rU1 snRNPA does not work by its own certain anti-tumor activity. Combined with the results of T cell reactivation assays, it can be determined that rU1 snRNPA exerts anti-tumor activity by reactivating tumor-suppressed T cells. Anti-cancer drugs also need to be specific and can target tumor cells but cannot or rarely kill normal cells to avoid serious side effects. In HEK293 cells without PD-L1 expression, rU1 snRNPA, as an inhibitor of PD-L1, had almost no reactivation of tumor-suppressed T cells or killing activity, which can preliminarily prove that rU1 snRNPA had specificity of targeting PD-L1 to exert anti-cancer activity. In the process of new drug development, many candidates have obtained ideal results in *in vitro* assays, but not in *in vivo* assays. Compared with antibodies, the human-derived protein scaffolds that we selected do not have IgG fragments and do not cause antibody-dependent cell-mediated cytotoxicity, so they cannot mediate killer cells to directly kill tumor cells (23). In order to investigate whether rU1 snRNPA can also obtain the ideal anti-cancer activity *in vivo*, we designed a melanoma–human immune system immunodeficiency mouse model to further evaluate the *in vivo* anti-melanoma efficacy of rU1 snRNPA. This mouse model simulated, as much as possible, the anti-tumor activity which rU1 snRNPA may exert on A375 cells in the human immune system. Fortunately, rU1 snRNPA had also achieved ideal results in an *in vivo* anti-melanoma assay, which means that rU1 snRNPA has potential for follow-up research.

CONCLUSION

The systematic screening of therapeutic proteins against the drug-target protein is a substantial challenge to *in silico* methods. In this study, the application of a human-derived protein scaffolds library and rigid molecular docking is a highly efficient and easily available tool for designing novel therapeutic protein drugs. Two major biological criteria for the screening of candidates are as follows: (a) do not interact with proteins in body liquids and cell surface receptors, such as cytokines, *etc.*, and (b) do not interact with small molecules related to life activities, such as enzymes. Our work suggested that the variant A32D of rU1 snRNPA, which we screened and improved, is a potential PD-L1 inhibitor.

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 animal study was reviewed and approved by the Institutional Animal Ethics Committee and Animal Care Guidelines of Jilin University.

AUTHOR CONTRIBUTIONS

CM and CL conceived the whole project. CL and FS managed the research group. CM and SQ calculated the *in silico* screening. CM and LS conducted the expression and purification. CM measured the bio-activity *in vitro* and SERS detection. ZL and SQ measured the anti-tumor efficacy *in vivo*. All authors contributed to the article and approved the submitted version.

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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.2021.781046/full#supplementary-material

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REVIEW
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Oncolytic Viruses and Cancer, Do You Know the Main Mechanism?

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The global rate of cancer has increased in recent years, and cancer is still a threat to human health. Recent developments in cancer treatment have yielded the understanding that viruses have a high potential in cancer treatment. Using oncolytic viruses (OVs) is a promising approach in the treatment of malignant tumors. OVs can achieve their targeted treatment effects through selective cell death and induction of specific antitumor immunity. Targeting tumors and the mechanism for killing cancer cells are among the critical roles of OVs. Therefore, evaluating OVs and understanding their precise mechanisms of action can be beneficial in cancer therapy. This review study aimed to evaluate OVs and the mechanisms of their effects on cancer cells.

Keywords: oncolytic virus, cancer immunotherapy, cancer vaccine, targeted treatment, immune checkpoint

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BACKGROUND

Millions of individuals are affected by cancer annually. Cancer is considered the leading cause of death and the most important barrier to the increase in life expectancy in the twenty-first century. In 2018, 18.1 million new cancer cases (17.0 million cancer cases excluding non-melanoma skin cancers) were reported. The mortality due to cancer in 2018 was 9.6 million (9.5 million, excluding non-melanoma skin cancers) (1). Significant developments in cancer treatment started in 1900. The achievements of this progress include the development of diagnostic, surgery, chemotherapy, hormone therapy, gene therapy, and cell therapy methods. Regardless of these advancements, human is still incapable of combating cancer, as none of the identified treatment methods could be used in all stages of cancer (2). Many of cancer patients experience a relapse of disease progression regardless of the primary response to treatment.

Furthermore, complete resection of the tumor is difficult or impossible in many cases (3). Immunotherapy has evolved as a practical treatment choice against malignant diseases during the past decades. Studies in oncolytic virotherapy (OVT) developed in the early twentieth century as an observational science for the cases of spontaneous regression of tumors were reported due to infection with specific viruses (4).

Oncolytic viruses (OVs) include a group of viruses that selectively affect and kill malignant cells, leaving the surrounding healthy cells unaffected. OVs have direct cytotoxic effects on cancer cells and augment host immune reactions and result in the destruction of the remaining tumoral tissue and establish a sustained immunity (5). Indeed, OVs function in four ways against tumor cells, including oncolysis, antitumor immunity, transgene expression, and vascular collapse (6). Regarding the fact

that cancer cells are developed to avoid detection and destruction by the host immune system and also to resist apoptosis, which are the critical responses of normal cells in limiting viral infections, OVs can kill cancer cells through a spectrum of actions ranging from direct cytotoxicity to induction of immune-mediated cytotoxicity. OVs can also indirectly destroy cancer cells by destroying tumor vasculature and mediating antitumor responses (7). Furthermore, in order to augment the therapeutic characteristics, modifications in OVs by genetic engineering such as insertions and deletions in the genome have been employed in many investigations; thus, additional antitumor molecules can be delivered to cancer cells and effectively bypass the widespread resistance of single-target anticancer drugs (8)

It should be noted that the use of OVs in cancer therapy was limited due to the pathogenicity and toxicity of these viruses in human cases. Recent advancements in genetic engineering have optimized the function of OVs through genetic modifications and therefore have become the issue of interest in OVT (9). Each virus tends to a specific tissue, and this tendency determines which host cells are affected by the virus and what type of disease will be generated. For instance, rabies, hepatitis B, human immunodeficiency virus (HIV), and influenza viruses affect neurons, hepatocytes, T lymphocytes, and respiratory tract epithelium, respectively. Several naturally occurring viruses have a preferential but not exclusive tendency towards cancer cells. This issue is more attributed to tumor cell biology compared to the biology of the virus.

OVs are generally categorized into two groups. One group is preferentially replicated in cancer cells and is not pathogenic for normal cells due to the increased sensitivity to the innate immune system's antiviral signaling or dependence on the oncogenic signaling pathways. Autonomous parvovirus, myxoma virus (MYXV; poxvirus), Newcastle disease virus (NDV; paramyxovirus), reovirus, and Seneca valley virus (SVV; picornavirus) are categorized in this group. The second group of OVs includes viruses that are either genetically modified for purposes including vaccine vectors such as mumps virus (MV; paramyxovirus), poliovirus (PV; picornavirus), and vaccinia virus (VV; poxvirus), or genetically engineered through mutation/deletion of genes required for replication in normal cells, including adenovirus (Ad), Herpes simplex virus (HSV), VV, and vesicular stomatitis virus (VSV; rhabdovirus) (10).

Furthermore, the mutation in cancer cells, drug adaptation, resistance, and cell immortality were effective in the initiation and speed of viral dissemination. Today, researchers are trying to discover and identify a new generation of OVs to save more patients' lives from cancer. Evaluation of OVs and identification of the exact mechanism of action of these viruses can be helpful in this way (11). This review study aimed to evaluate OVs and their mechanism of action against cancer cells.

METHODOLOGY

The key terms in the literature search included oncolytic virus, cancer, immunotherapy, innate immunity, adaptive immunity, virotherapy, viral therapy, oncolytic, and virus were searched in international databases, namely, Web of Science, PubMed, and Scopus from 2004 to 2021. The inclusion criterion was the evaluation of viruses using standard *in vivo* and *in vitro* laboratory methods. Exclusion criteria were lack of access to full text articles and incomplete description or assessment of diseases other than cancers.

RESULTS

The primary search yielded 1,450 articles. Finally, 47 articles were included in the review after eliminating irrelevant and duplicate studies. The characteristics of the 47 included articles are presented in **Table 1**, performed from 2004 to 2021. The OV families assessed in the studies included Ad, MV, PV, NDV, SFV, HSV, VV Reovirus, and bovine herpesvirus (BHV). The most commonly assessed virus was adenovirus (Ad) (n = 15), followed by the herpesvirus (HSV) (n = 12) and measles virus (MV) (n = 7). The least assessed viruses were BHV, SFV, and Reovirus (n = 1).

According to **Table 1**, OVs may employ multifunction against tumor cells; however, the most antitumor actions of OVs were related to cytolysis activity and inducing antitumor immunity (n = 26) in which adenovirus (n = 11) and HSV (n = 9) were the most responsible OVs in their categories, respectively. However, the last action was associated with vascular collapse. The collective data in **Table 2** exhibited a summary of clinical trials of OVs implicated in malignancies highlighting the most considerable focus on engineered VV by TK^{del} $GMCSF^{exp}$ (JX-594) on solid tumors supported by Jennerex Biotherapeutics Company. The majority of studies under clinical trials involve a transgene virus encoding an immune-stimulatory or proapoptic gene to boost the oncolytic features of the virus. As **Table 2** reveals, granulocyte–macrophage colony-stimulating factor (GM-CSF) and pro-drug-converting enzymes are the most popular transgenes, although many OVs encoding novel therapeutic cargos are in clinical development. Streby et al., in phase I clinical trial, examined the effects of HSV1716 on relapsed/refractory solid tumors. Despite the fact that none of the patients exhibited objective responses, virus replication and inflammatory reactions were seen in patients (58). In another clinical trial, Desjardins et al. reported a higher survival rate in grade IV malignant glioma patients who received recombinant nonpathogenic polio–rhinovirus chimera (59). In a phase I clinical trial, Rocio Garcia-Carbonero et al. discovered that enadenotucirev IV infusion was associated with high local CD8+ cell infiltration in 80% of tumor samples evaluated, indicating a possible enadenotucirev-driven immune response (60). TG4023, a modified vaccinia Ankara viral vector carrying the FCU1 suicide gene, was used in a phase I trial to convert the non-cytotoxic prodrug flucytosine (5-FC) into 5-fluorouracil (5-FU) in the intratumor. Finally, 16 patients with liver tumors were successfully injected; the MTD was not achieved, and a high therapeutic index was demonstrated (61). Dispenzieri et al. examined MV-NIS effects in patients with relapsed, refractory myeloma and reported satisfactory primary results (62).

TABLE 1 | The collective studies on OVs.

Virus	Cancer	Model	Effects	Mechanism	References
Adenovirus	Head and neck squamous cell carcinoma	Murine	Ad-derived IL-12p70 prevents the destruction of HER2.CAR-expressing T cells at the tumor site.	Enhanced antitumor effects of HER2 CAR T cells by CAd12_PDL1 Controlling of primary tumor growth and metastasis.	Shaw et al., 2017 (12)
	Renal cell carcinoma	Murine	HRE-Ki67-Decorin suppressed tumor growth and induced decorin expression in the extracellular matrix (ECM) assembly.	An effective anticancer treatment strategy may be chimeric HRE-Ki67 promoter-regulated Ad carrying decorin.	Zhang et al., 2020 (13)
	Lung cancer stem cell (LCSC)	Murine	Tumor necrosis factor (ZD55-TRAIL) increased cytotoxicity and induced A549 sphere cells apoptosis through a mitochondrial pathway	Treatment of lung cancer is possible by targeting LCSCs with armed oncolytic adenovirus genes.	Yang et al., 2015 (14)
	Leukemia	Murine	Induction of autophagic cell death Enhanced cell killing in primary leukemic blasts	Significant autophagic cell death	Tong et al., 2013 (15)
	Breast cancer	Murine	Tumor killing due to Sox2 and oct4 expression and Hoechst 33342 exclusion CD44+CD24−/low cells	A positive effect against advanced orthotopic was that CD44+CD24−/low-derived tumors were observed.	Eriksson et al., 2007 (16)
	Breast cancer	Murine	Delta24 can replicate and help the E1-deleted adenovector replicate in cancer cells	Spontaneous liver metastasis with Delta 24 virus therapy alone was less reduced than in combination with TRAIL gene therapy.	Guo et al., 2006 (17)
	Liver cancer stem-like cells	Murine	Significant apoptosis Inhibition angiogenesis in xenograft tumor tissues Inhibition of the propagation of cells occurred due to GD55	GD55 had a higher effect in suppressing tumor growth than oncolytic adenovirus ZD55.	Zhang et al., 2016 (18)
	B16F10	Murine	Infiltration of effector CD4+ and CD8+ T cells Increasing secretion of TNF-α and IFN-γ	Activation the immune system Creating a proinflammatory environment	Wei et al., 2020 (19)
	αvβ6-positive tumor cell lines of pancreatic and breast cancer	Murine	Cells expressing high levels of αvβ6 (BxPc, PANC0403, Suit2) were killed more efficiently by oncolytic $Ad5_{NULL}$-A20 than by oncolytic Ad5	$Ad5_{NULL}$-A20-based virotherapies efficiently target αvβ6-integrin-positive tumors	Davies et al., 2021 (20)
	Advanced metastatic tumors	Murine	Increase in CD8+ T cells Reduction of IFN-γ secretion	Specific immunity against tumor	Cerullo et al., 2010 (21)
	Breast cancer	Murine	Inflammation and neutrophil infiltration due to oncolytic adenovirus-GM-CSF.	Ad5/3-D24-GMCSF, combined with low-dose CP showed efficacy and antitumor activity	Bramante et al., 2016 (22)
	Solid tumors	Murine	CD8 cytotoxicity viruses efficiently lysed tumors	Significantly prolonged survival	Gürlevik et al., 2010 (23)
	Metastatic ductal breast cancer	Murine	Each virus featured 5/3 chimerism of a promoter controlling the expression of E1A and fiber, which was also deleted in the Rb binding domain for additional tumor selectivity	These viruses completely eradicated CD44 + low CD24−/cells *in vitro* Significant antitumor activity in CD44+ CD24−/low-derived tumors *in vivo*	Bauerschmitz et al., 2008 (24)
	Metastatic melanoma	*In vitro*	Activation and an increased costimulatory capacity of monocyte-derived antigen-presenting cells	A valuable immunotherapeutic agent for melanoma is ORCA-010	González et al., 2020 (25)
	Gastric cancer MKN45 and MKN7 cells	Murine	Cell death in stem cells such as CD133 resident cancer by stimulating cell-cycle-related proteins	Killing cancer cells	Yano et al., 2013 (26)
Herpesvirus	Bearing M3-9-M tumors	Murine	Increasing the incidence of CD4+ and CD8+ T cells and no correlation with the CD4+CD25+Foxp3+ regulatory T-cell populations in the tumor	An efficient therapy strategy for soft tissue sarcoma in childhood	Chen et al., 2017 (27)
	Breast cancer	Murine	Regulation of CD8+ T cell activation markers in the tumor microenvironment Inhibition of tumor angiogenesis	Tumor regression Anticancer immune response	Ghouse et al., 2020 (28)
	Colon carcinoma	Murine	Decreased inhibitory immune cells Increased positive immune cells in the spleen.	Generate tumor-specific immunity Elimination of primary tumors Developing immune memory to inhibit tumor recurrence and metastasis.	Zhang et al., 2020 (29)
	Ovarian carcinoma	Murine	DC maturation and tumor infiltration of INF-γ+ CTL	The antitumor immune responses are facilitated	Benencia et al. 2008 (30)
	Tumor	Murine	T-cell responses against primary or metastatic tumors	Antitumor immune response Prevention of tumor growth	Li et al., 2007 (31)
	STING low-metastatic melanoma	Murine	Release of DAMP factors Release of IL-1β and inflammatory cytokines Induction of host antitumor immunity	Induction of immunogenic cell death (ICD) Recruitment of viral and tumor-antigen-specific CD8+ T cells	Bommareddy et al., 2019 (32)

(Continued)

TABLE 1 | Continued

Virus	Cancer	Model	Effects	Mechanism	References
				STING expression as a predictive biomarker of T-Vec Response	
	Osteosarcoma cells	Murine	Antitumor efficacy *in vivo* Inducing antitumor immunity	The *in vitro* cytolytic properties of OVs are poor prognostic indicators of effective cancer virotherapy and *in vivo* antitumor activity	Sobol et al., 2011 (33)
	HCT8 human colon cancer cells	Murine	Cytotoxicity, viral replication, and Akt1 expression	Therapy of TIC-induced tumors with NV1066 slowed tumor growth and yielded tumor regression	Warner et al., 2016 (34)
	Glioblastoma-derived cancer stem-like cells (GBM-SC)	Murine	Infection with HSV G47Delta killed GBM-SCs and inhibited their self-renewal and the inability of viable cells to form secondary tumor spheres	Significant anti-tumor effect against xenografts in mice and effective killing of CSCs *in vitro*	Wakimoto et al., 2009 (35)
	Solid tumors	Human	The induction of adaptive antitumor immune responses	All patients were seropositive. No local recurrence was observed in patients and disease-specific survival was 82.4%	Harrington et al., 2010 (36)
	Breast, head and neck, and gastrointestinal cancers, and malignant melanoma	Human	Induction of adaptive anti-tumor immune responses	Biopsies contained residual tumor was observed in 19 patients after treatment that 14 of them showed tumor necrosis (extensive, or apoptosis)	Hu et al., 2006 (37)
	Metastatic melanoma	Human	ICP47 deletion increases US11 expression and enhances virus growth and replication in tumor cells	Overall survival at 12 and 24 months were 58% and 52%, respectively.	Senzer et al., 2009 (38)
Measles virus	Solid tumor	Murine	GOS/MV-Edm significantly increases viral replication in tumor mass	Increased survival in passive antiserum immunized tumor-bearing mice	Xia et al., 2019 (39)
	Orthotopic glioma tumor spheres and primary colon cancer	Murine	Overexpression of the CD133 target receptor or increased kinetics of proliferation through tumor cells	CD133-targeted measles viruses selectively removed CD133þ cells from tumor tissue	Bach et al., 2013 (40)
	Mesothelioma	Murine	Infiltration of CD68+ cells innate immune cells.	Oncolytic MVs is versatile and potent agents for the treatment of human mesothelioma.	Li et al., 2010 (41)
	Multiple myeloma	Murine	Induction of adaptive anti-tumor immune responses	Virus-infected T cells may induce systemic measles virus therapy in the presence of ABS antivirus.	Ong et al., 2007 (42)
	Breast cancer	*In vitro*	Inducing apoptosis	Induction of cell death leads to infection of breast cancer cells with rMV-BNiP	Lal and Rajala et al., 2019 (43)
	Breast cancer	*In vitro*	Increased percentage of apoptotic cells in infected MCF-7 cells	Significant apoptosis in breast cancer cell lines.	Abdullah et al., 2020 (44)
	T-cell lymphomas (CTCLs)	Human	An increase in the IFN-γ/CD4 and IFN-γ/CD8 mRNA ratio and a reduced CD4/CD8 ratio	MV can affect CTCL treatment.	Heinzerling et al., 2005 (45)
Newcastle disease virus	Lung cancer	Murine	Caspase-dependent apoptosis associated with increased caspase-3 processing and ADP-ribose polymerase cleavage.	A potential strategy for targeting lung CSCs	Hu et al., 2015 (46)
	B16 melanoma	Murine	Treatment with systemic CTLA-4 blockade was due to long-term survival and tumor rejection	Distant tumors are prone to systemic therapy with immunomodulatory antibodies using localized therapy with oncolytic NDV	Zamarin et al., 2014 (47)
	Lung cancer	Murine	DAMP release Autophagy induction	Inhibited tumor growth Trigger ICD	Ye et al., 2018 (48)
	GBM	Murine	GBM susceptibility to NDV is dependent on the loss of the type I IFN	Trigger the activation of immune cells against the tumor and show oncolytic effect	García-Romero et al., 2020 (49)
Vaccinia virus	Melanoma	Murine	PD-L1 inhibition Neoantigen presentation	Tumor neoantigen-specific T-cell responses	Wang et al., 2020 (50)
	Solid tumors	Murine	Activated the inflammatory immune status	Complete tumor regression long-term tumor-specific immune memory	Nakao et al., 2020 (51)
	Solid cancer	Murine	Replication was activated by EGFR/Ras pathway signaling, cellular TK levels, and cancer cell resistance to IFNs	Selectively cell lysis and stimulation of antitumoral immunity	Parato et al., 2012 (52)

(Continued)

TABLE 1 | Continued

Virus	Cancer	Model	Effects	Mechanism	References
M1 virus	Melanoma	Murine	$CD8^+$ T-cell-dependent therapeutic effects long-term antitumor immune memory Upregulating the expression of PD-L1	Immunogenic tumor cell death Restores the ability of dendritic cells to prime antitumor T cells	Yang Liu et al., 2020 (11)
	Bladder tumor	Murine	Inhibition of CCDC6 improve viral replication and then induced endoplasmic reticulum stress to facilitate M1 virus oncolytic effects.	CCDC6 inhibition resulted in better antitumor activity	Liu et al., 2021 (53)
Poxvirus	MC-38 colon adenocarcinoma tumors	Murine	Elicited TILs with lower quantities of exhausted PD-1^{hi}Tim-3^+ $CD8^+$ T cells and regulatory T cells	Tumor regression and improved survival	Mathilde et al., 2020 (54)
Poliovirus	Breast cancer	Murine	Primary oncolytic viral receptors are highly expressed in tumor cells and transmitted among cells.	Oncolytic PV recombinants may affect tumor cells by viral receptor CD155	Ochiai et al., 2004 (55)
Reovirus	Solid tumor	Murine	Induction of Golgi fragmentation and accumulation of oncogenic Ras in the Golgi body	Initiating apoptotic signaling events required for virus release and spread.	Garant et al., 2016 (56)
Adenovirus (Ad), Semliki Forest virus (SFV) and Vaccinia virus (VV)	Osteosarcoma	Murine	Activates immunogenic apoptosis Triggering phagocytosis and maturation of DCs Th1-cytokine release by DCs and antigen-specific T-cell activation.	Induction of T-cell-mediated antitumor immune responses. Increased cell death processes	Jing Ma et al., 2020 (57)

PD-L1, programmed death-ligand 1; Ad, adenovirus; MV, measles virus; GBM, glioblastoma; NDV, Newcastle disease virus; VV, Vaccina virus; Th, T helper; ICD, immunogenic cell death; EGFR, epidermal growth factor receptor; TK, thymidine kinase; IFN-I, type-I interferon; HSV, herpes simplex viruses; TIL, tumor infiltration lymphocyte; DC, dendritic cells; BHV, bovine herpesvirus; DAMP, damage-associated molecular pattern; Trail, TNF-related apoptosis-inducing ligand; GD-55, GOLPH2-regulated oncolytic adenovirus; GOS, graphene oxide arms PV, polio virus; LAPV, Israeli acute paralysis virus; CP, cisplatin; GM-CSF, granulocyte–macrophage colony-stimulating factor.

Cohn et al., in phase II clinical trial, evaluated the effects of oncolytic reovirus (Reolysin®) plus weekly paclitaxel in women with recurrent or persistent ovarian, tubal, or primary peritoneal cancer. The results did not show any improvement in the patient status (63), although Mahalingam et al. showed that REOLYSIN®, plus carboplatin and paclitaxel, is an effective treatment in advanced malignant melanoma (64). Packiam et al. showed that CG0070 (GM-CSF expressing adenovirus) has a 47% CR rate at 6 months for all patients and 50% for patients with carcinoma-*in situ* (65).

Geletneky et al. evaluated H-1 parvovirus (H-1PV) effects in recurrent glioblastoma patients and reported microglia/macrophage activation and cytotoxic T-cell infiltration in the infected tumors, proposing initiation of the immunogenic response (66).

Andtbacka et al., in a phase III study, evaluated Talimogene laherparepvec (T-VEC) in stage IIIc and stage IV malignant melanoma. T-VEC was the first approved OVs against melanoma in a phase III clinical trial. This virus compared with GM-CSF showed a higher durable response rate and overall survival (67). In another newest phase III study, Talimogene laherparepvec was approved by the Food and Drug Administration (FDA) in the USA, European Union, and Australia (68).

DISCUSSION

As a challenge in cancer therapy approaches (1), the exclusive features of oncolytic viruses have attracted plenty of researchers in recent years. OVs have the dramatic capability to selectively infect tumor cells leading to direct or indirect cancer cell death without harming normal cells (7). This study focused on some mechanisms employed by OVs against tumor cells, which are exactly various from virus to virus (**Figure 1**).

According to most studies, OVs can target cancer cells and benefit from tumor conditions in favor of replication in infected cells, eventually leading to oncolysis. Indeed, tumor cells tend to resist apoptosis and translational suppression, which are both compatible with the growth of several viruses (7). One of the main actions of OVs is to take advantage of immune-evading properties of cancer cells to escape from recognition and destruction by the immune system. Antiviral processes in normal cells are associated with the interferon pathway in which the secretion of type I interferon (IFN) cytokine can trigger an antiviral response and induce ISGs to block viral replication (69). This subsequently leads to cell apoptosis, as it is known that the IFN-I signaling regulates the expression of proapoptotic genes such as tumor necrosis factor alpha (TNF-α), FAS ligand, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (70).

Regarding the IFN-I signaling is defective in most tumor cells, it makes tumor cells susceptible to being infected by some OVs including NDV, VSV, MYXV, and raccoon pox virus (71–73). García-Romero et al. showed that NDV was able to replicate in glioblastoma (GBM) cancer stem cells (CSCs) due to type I IFN gene loss occurring in more than 50% of patients. Infection of GBM with NDV represents oncolytic and immunostimulatory properties through the production of type I IFN in non-tumor cells such as tumor infiltrated macrophages and DC or other cells present at the tumor microenvironment (49). NDV therapy also declines CSCs self-renewing capacity to improve their differentiation ability and facilitate cancer therapy (49, 74). OVs can also benefit from the abnormal expression of the proto-oncogene RAS which generally occurs in normal cells but actives in tumor cells (75). OV infection outcomes can be

TABLE 2 | The summary of clinical trials for oncolytic viruses.

Phase	Virus	Tumor	Interventions	Trial code	Country	Company
Phase I	JX-594	Refractory solid tumors	Intratumoral injection	NCT01169584	USA	Jennerex Biotherapeutics
	JX-594	Refractory solid tumors	Intravenous infusion	NCT00625456	Canada	Jennerex Biotherapeutics
	HSV-1, TBI-1401 (HF10)	Solid tumor with superficial lesions	Intratumoral administration	NCT02428036	Japan	Takara Bio Inc.
	Recombinant measles virus	Ovarian cancer Primary peritoneal cavity cancer	Intraperitoneal administration	NCT00408590	USA	Mayo Clinic
	GM-CSF-Adenovirus CGTG-102	Malignant solid tumor	In combination with low dose cyclophosphamide	NCT01598129	Finland	Targovax Oy
	Adenovirus VCN-01	Solid tumor	Intravenous administration with or without gemcitabine	NCT02045602	Spain	VCN Biosciences, S.L.
	REOLYSIN®	KRAS mutant metastatic colorectal Cancer	Intravenous administration with Irinotecan/ Fluorouracil/Leucovorin and Bevacizumab	NCT01274624	USA	Oncolytics Biotech
	Adenovirus VCN-01	Pancreatic cancer	Intratumoral injections with intravenous Gemcitabine and Abraxane®	NCT02045589	Spain	VCN Biosciences, S.L.
	JX-594	Hepatic carcinoma	Transdermal injection	NCT00629759	Korea	Jennerex Biotherapeutics
	Attenuated Vaccinia Virus, GL-ONC1	Solid organ cancers	Intravenous administration	NCT00794131	United Kingdom	Genelux Corporation
	Coxsackievirus Type A21	Melanoma	Intratumoural injection	NCT00438009	Australia	Viralytics
	REOLYSIN®	Pancreatic adenocarcinoma	Pembrolizumab (KEYTRUDA®)	NCT02620423	USA	Oncolytics Biotech
	Vaccinia Virus (GL-ONC1)	Head and neck carcinoma	With concurrent Cisplatin and radiotherapy	NCT01584284	USA	Genelux Corporation
Phase II	TBI-1401(HF10)	Melanoma	In combination with Ipilimumab	NCT03153085	Japan	Takara Bio Inc.
	HF10	Malignant melanoma	With Ipilimumab	NCT02272855	USA	Takara Bio Inc.
	OncoVEX^GM-CSF	Melanoma	Intratumoral injection	NCT00289016	United Kingdom	–
	Edmonston strain of Measles Virus Expressing NIS	Refractory multiple myeloma	Systemic Administration with cyclophosphamide	NCT02192775	USA	University of Arkansas
	Reovirus Serotype 3 REOLYSIN®	Non-small cell lung cancer	Intravenous administration with paclitaxel and carboplatin	NCT00861627	USA	Oncolytics Biotech
	JX-594	Hepatocellular carcinoma	Intratumoral injection	NCT00554372	USA	Jennerex Biotherapeutics
	CG0070	Non-muscle invasive bladder carcinoma	–	NCT02365818	USA	CG Oncology, Inc.
	Wild-type Reovirus REOLYSIN®	Bone and soft tissue sarcomas	Intravenous injection	NCT00503295	USA	Oncolytics Biotech
Phase I/II	Vaccinia Virus JX-594	Melanoma	Intratumoral injection	NCT00429312	USA	Jennerex Biotherapeutics
	Parvovirus H-1	Glioblastoma multiforme	Intratumoral/Intracerebral injection	NCT01301430	Germany	Oryx GmbH & Co. KG
	HSV1716	Malignant pleural mesothelioma	Intrapleural injection	NCT01721018	United Kingdom	Virttu Biologics Limited
	Ad-MAGEA3	Metastatic non-small cell lung cancer	With pembrolizumab	NCT02879760	Canada	Turnstone Biologics, Corp.
	REOLYSIN®	Recurrent malignant gliomas	Intralesional administration	NCT00528684	USA	Oncolytics Biotech
	JX 594	Colorectal carcinoma	Multiple intravenous with Irinotecan	NCT01394939	USA	Jennerex Biotherapeutics
	Vaccinia Virus GL-ONC1	Peritoneal Carcinomatosis	Intraperitoneal administration	NCT01443260	Germany	Genelux GmbH

affected by up-regulation of RAS in tumoral cells and further down-regulation of interferon-inducible genes due to activation of RAS/MEK signaling pathway that reduces viral response in tumoral cells (76). On the contrary with this attempt, Garant et al. demonstrated that reovirus could translocate and accumulate RAS into Golgi apparatus to increase apoptotic signaling events required for virus release (56). This highlighted that the outcomes of OVT are exclusively associated with the characteristics and type of OVs.

High expression of some viral receptors by cancer cells permits higher viral uptake in cancer cells than in normal ones. Some receptors such as CAR (77), laminin (78), CD155 (79), and CD46 (80) are overexpressed in various cancer cells which result in increased uptake of Ad (81), Sindbis virus (82),

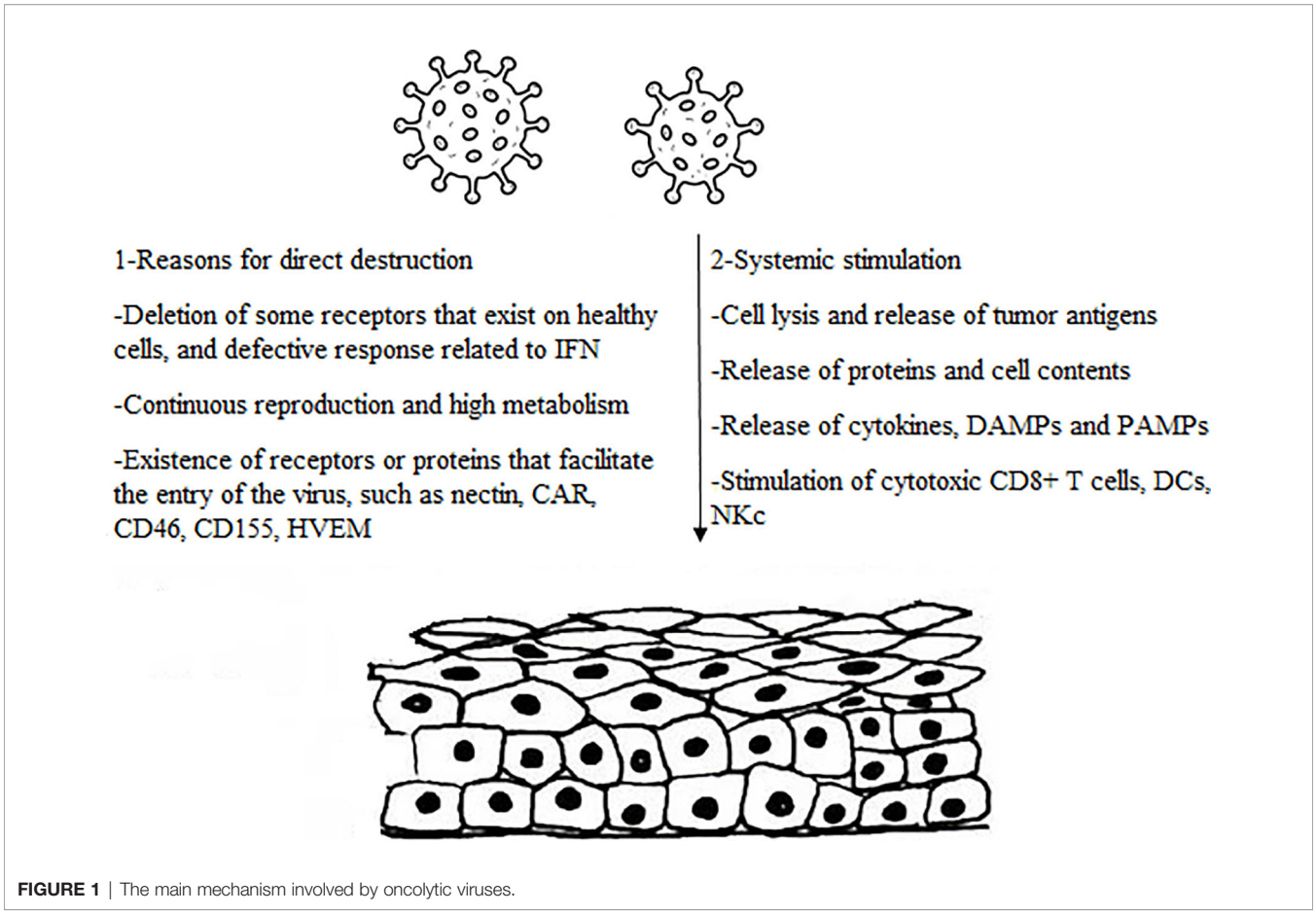

FIGURE 1 | The main mechanism involved by oncolytic viruses.

PV (83), and MV (84) respectively. Interestingly, some viral proteins are poisonous for neoplastic cells and can directly kill cells before viral replication. This was evidenced by the E3 death protein and E4orf4 proteins encoded by Ads and are toxic for cells that end in cytolysis at the time of virus exposure (3). However, deletion in specific viral genes can be another mechanism for the action of the OVs. These genes are necessary for the longevity of viruses in normal cells but not essential for viral activity in cancer cells. Thymidine kinase (TK) is an indispensable enzyme for nucleic acid metabolism encoded in infection with wild type vaccinia virus and enables the replicating of the virus in normal cells. Lister strain virus with TK gene deletion as a type of VV has shown a beneficial antitumor potency and cancer-selective replication *in vivo* since tumoral cells have a high TK content, which enables the virus to replicate in cancer cells regardless of the deletion in viral TK gene (85). In parallel with this study, Parato et al. analyzed the mechanism of cancer-selectivity by an engineered vaccina virus with TK deletion and epidermal growth factor (EGFR) and lac-Z transgenes observing the replication in tumor cells was related to activation of EGFR/RAS signaling, high cellular TK level and tumor cell resistance to IFN-I (52). These results displayed noticeably the beneficial implication of OVs with inherent and engineered mechanistic properties in cancer therapy approaches.

Oncolytic viruses may interfere with normal physiological process of tumor cells to induce the secretion of pro-inflammatory mediators or even lead to the exposure of tumor-associated antigens (TAA), pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) following apoptosis or oncolysis. These responses can also result in a change in tumor status from immune desert to inflamed status and further recruit a collection of immune cells such as cytotoxic T lymphocytes, dendritic cells, natural killer cells and phagocytic cells to induce immune cell death along with antiviral responses (86, 87).

Remarkably, most viruses continue their infection by expressing genes responsible for escaping the immune system and disseminating in host cells (88). Mutation in these genes can probably improve immune induction and thus increase the anti-tumoral responses regardless these mutations may reduce virus replication further (10). Thus, oncolytic viruses are often engineered to express various genes aided in the overall anti-tumor efficacy of the virus. Transgenes mostly include ranging from immune-stimulatory (IL-2, IL-4, IL-12 and GM-CSF) to pro-apoptotic (tumor necrosis factor alpha, p53 and TRAIL genes inserted into oncolytic viruses (87, 89–94). Interestingly, bystander effects of OVs through local release of cytokines can potentially cause immune response against nearby tumor cells even without direct antigen expression (95).

Furthermore, OVs can destroy tumor vasculature and impede sufficient intratumoral blood reserve, which is essential for tumor progression and metastasis (96). Breitbach et al. demonstrated that intravenous injection of JX-594, an engineered vaccine virus with TK deletion and overexpression of human granulocyte-monocyte colony-stimulating factor (hGM-CSF), led to replication of the virus in endothelial cells of the nearby tumor and disrupted tumor blood flow, which ultimately ended in intensive tumor necrosis within 5 days. Consistently, patients with advanced hepatocellular carcinoma, hypervascular and $VEGF^{high}$ tumor type, treated by JX-594 in phase II clinical trials confirmed the efficiency of the JX-594 OV in tumor vasculature disruption without toxicity to normal blood vessels in which inhibition of angiogenesis can passively result in tumor regression (97). This evidence may open promising technologies toward cancer therapy in a way tumor cells are targeted selectively and bypass the side effects of conventional approaches.

Recently, conditionally replication-competent adenoviruses (CRCAs) have been introduced as a successful method for cancer therapy. Sarkar et al. showed that Ad.PEG-E1A-mda-7, a cancer terminator virus (CTV), selectively replicated in cancer cells, inhibits their growth and induces apoptosis (98).

Qian et al. showed that ZD55 expressing melanoma differentiation-associated gene-7/interleukin-24 (ZD55-IL-24) affects B-lymphoblastic leukemia/lymphoma through upregulation of RNA-dependent protein kinase R, enhance phosphorylation of p38 mitogen-activated protein kinase, and induce of endoplasmic reticulum (ER) stress (99).

Azab et al. showed that Ad.5/3-CTV potently suppressed *in vivo* tumor growth in mouse (100).

Bhoopathi showed that Ad.5/3-CTV induces apoptosis through apoptosis-inducing factor (AIF) translocation into the nucleus, independent of the caspase-3/caspase-9 pathway (101).

In an interesting study, Bhoopathi et al. introduced a novel tripartite CTV "theranostic" adenovirus (TCTV) that targets virus replication, cytokine production, and imaging capabilities uniquely in cancer cells. This TCTV permits targeted treatment of tumors while monitoring tumor regression, with the potential to simultaneously detect metastasis due to the cancer-selective activity of reporter gene expression (102).

Greco et al. showed that ultrasound (US) contrast agents guided MB/Ad.*mda*-7 complexes to DU-145 cells successfully and eradicated not only targeted DU-145/Bcl-xL-therapy-resistant tumors but also nontargeted distant tumors (103).

T-VEC, adenovirus, and vaccinia virus are the most popular OVs in clinical trials. Approving T-VEC by FDA for the first time could pave the way for other OVs in the clinic. Oncolytic viruses have a broad therapeutic method; hence, their clinical development requires a multidisciplinary view. It is necessary to understand viral generation and viability in infected cells. To improve clinical trials, important factors such as viral entrance, replication, dissemination, oncolysis, and immune activation should be controlled. These factors can vary between tumor types and OVs. It is also critical to understand the immune composition of diverse cancers and the immunological repercussions of viro-immunotherapy.

CONCLUSION AND FUTURE DIRECTION

Cancer is among the most important causes of mortality worldwide, and many chemotherapies and radiotherapy approaches do not have a specific effect on cancer cells and are sometimes accompanied by side effects. Today, a biological war has evolved against cancer by genetically modifying natural pathogens to activate them against neoplastic cells. OVT is a promising therapeutic option in cancer therapy. The mechanisms of action of OVs differ entirely from the mechanism of action of chemotherapy, radiotherapy, surgery, and embolization. They can result in success in the treatment of cancers that are resistant to other therapeutic modalities. Better understanding and acquiring comprehensive information regarding OV therapy and the biology of cancer is an essential step in assessing and controlling cancer programs.

AUTHOR CONTRIBUTIONS

Conceptualization, WK and HE. Methodology, MF and RD. Validation, BJ. Data curation, MB. Writing—original draft preparation, HE and WK. Writing—review and editing, all. All authors have read and agreed to the published version of the manuscript.

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REVIEW
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Engineering Macrophages *via* Nanotechnology and Genetic Manipulation for Cancer Therapy

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Macrophages play critical roles in tumor progression. In the tumor microenvironment, macrophages display highly diverse phenotypes and may perform antitumorigenic or protumorigenic functions in a context-dependent manner. Recent studies have shown that macrophages can be engineered to transport drug nanoparticles (NPs) to tumor sites in a targeted manner, thereby exerting significant anticancer effects. In addition, macrophages engineered to express chimeric antigen receptors (CARs) were shown to actively migrate to tumor sites and eliminate tumor cells through phagocytosis. Importantly, after reaching tumor sites, these engineered macrophages can significantly change the otherwise immune-suppressive tumor microenvironment and thereby enhance T cell-mediated anticancer immune responses. In this review, we first introduce the multifaceted activities of macrophages and the principles of nanotechnology in cancer therapy and then elaborate on macrophage engineering *via* nanotechnology or genetic approaches and discuss the effects, mechanisms, and limitations of such engineered macrophages, with a focus on using live macrophages as carriers to actively deliver NP drugs to tumor sites. Several new directions in macrophage engineering are reviewed, such as transporting NP drugs through macrophage cell membranes or extracellular vesicles, reprogramming tumor-associated macrophages (TAMs) by nanotechnology, and engineering macrophages with CARs. Finally, we discuss the possibility of combining engineered macrophages and other treatments to improve outcomes in cancer therapy.

Keywords: macrophages, bioengineering, nanotechnology, cancer immunotherapy, chimeric antigen receptors

INTRODUCTION

Macrophages are a class of immune cells with highly diverse phenotypes and functions. Some macrophages residing in tissues are known as tissue-resident macrophages (TREMs), such as Kupffer cells in the liver and pulmonary macrophages in the lungs. TREMs have a long lifespan, participate in local immune responses, and are essential components to maintain internal

homeostasis (1–3). Peripheral monocytes can also be recruited to inflammatory tissues, where they differentiate into macrophages (4). In a typical inflammatory response caused by microorganisms, pathogen-derived molecules known as pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) in bacterial wall, can be detected by macrophages through a group of receptors called pattern recognition receptors (PRRs), which triggers the activation of macrophages (5–7). Activated macrophages can effectively eliminate pathogens by their potent phagocytic activity (5–7). They also recruit immune cells from blood and activate T cell response through antigen processing and presentation, thus playing a key role in both innate and acquired immunity (8–10).

Tumors are often accompanied by a certain degree of inflammatory response (11, 12). Macrophages in tumor tissues are collectively referred to as tumor-associated macrophages (TAMs). Tumor cells frequently overexpress some cytokines, such as macrophage colony-stimulating factor 1 (CSF-1) and monocyte chemoattractant protein-1, (MCP-1), which recruit a large number of macrophages into tumor sites (13). In addition, tumor blood vessels have an irregular structure and abnormal function; they are dilated, leaky, and inefficient at delivering oxygen, which causes hypoxia in tumor tissues (14). Hypoxia in turn induces the expression of vascular endothelial growth factor (VEGF), a key mediator of tumor angiogenesis, but is also a potent macrophage-recruiting cytokine (15). Therefore, macrophages are often the most abundant type of tumor-infiltrating immune cells (16–18). However, the activity of macrophages in tumors is often suppressed; they cannot kill tumor cells efficiently through phagocytosis and overexpress immunosuppressive cytokines, including IL-10 and TGF-β, thereby establishing an unfavorable tumor immune microenvironment (16–18). TAMs also promote tumor cell survival and metastasis and induce drug resistance by secreting growth factors or by direct cell-cell contact with tumor cells (19, 20). Therefore, in many cases, TAMs are protumorigenic, and identifying effective methods to modify TAMs to improve anticancer therapy is of great interest (16–18).

The application of nanotechnology in cancer therapy holds great promise (21, 22). Nanoparticles (NPs) are synthetic structures with a nanoscale dimension and can be generally divided into two categories: organic NPs (i.e., liposomes, polymer micelles) and inorganic NPs (i.e., gold, silver, iron oxide) (23). NPs have been used to deliver a variety of anticancer agents, such as traditional chemotherapeutic drugs (23), targeted drugs (24), and genetic materials [i.e., messenger RNA (25), small interfering RNA (26), and the CRISPR/Cas9 genetic editing system (27)]. Due to their distinctive physicochemical properties, NPs can enhance the delivery of anticancer agents to tumors by both passive and active mechanisms (21, 28). As mentioned above, tumor blood vessels have increased permeability, which allows NPs to pass through the leaky endothelium; meanwhile, due to defective lymphatic drainage, the extraverted NPs can accumulate in the tumor interstitium, leading to an increased local drug concentration, a process known as the enhanced permeability and retention (EPR) (29). However, in many cases, the passive mechanism and EPR are not sufficient (29), and by active targeting strategies, such as ligand-mediated systems (30), stimulus-responsive systems (31), and biological system (32), the efficiency of NP targeted delivery can be improved. For example, most tumors have an increased rate of glycolysis, leading to an acidic environment due to the accumulation of lactic acid. Based on this feature, various pH-responsive systems have been developed (33, 34), which effectively dissociate NPs and decrease their size in low-pH areas (inside the tumors), thereby enhancing their ability to deeply penetrate into tumors (35). Moreover, the NP surface can be modified by ligand molecules that can recognize specific receptors on the tumor cell surface, thus increasing the affinity between tumor cells and NPs, which is critical for effective internalization of NPs by tumor cells (36, 37).

Among various active strategies, biological NP delivery systems are attracting considerable interest (32). NPs can be loaded in cell membranes (CMs), extracellular vesicles (EVs), or even live cells for targeted delivery. Regarding live-cell NP carriers, research mainly focuses on immune cells (38), especially macrophages, as they are superior in their ability to migrate toward tumors. Many studies have demonstrated that NP-loaded macrophages (NPL-Ms) can directionally migrate to tumors and transport the payload to tumor cells, leading to a pronounced antitumor effect (39, 40). Moreover, after reaching tumors, these engineered macrophages can exert additional effects by stimulating anticancer immune responses (24, 41). In this review, we first introduce the origin, differentiation, and function of macrophages as well as the application of nanotechnology in anticancer therapy. Then, we elaborate on the activities, mechanisms, and limitations of the engineered macrophages. Finally, we discuss several new strategies in macrophage engineering and discuss their potential as novel anticancer therapeutics.

MACROPHAGES FUNDAMENTALLY IMPACT THE DEVELOPMENT OF CANCER

Macrophages are key players in inflammation and participate in the crosstalk between inflammation and cancer development (**Figure 1**). In a typical inflammatory response, macrophages can perform three basic functions: 1) pathogen clearance, i.e., eliminating pathogens through phagocytosis or secreting anti-infective substances (5–7); 2) immune activation, i.e., activating humoral and cellular immune responses by presenting antigens to T cells and modifying the immune microenvironment by releasing a variety of inflammatory factors (8–10); and 3) tissue repair, i.e., releasing factors in the late stage of inflammation that promote angiogenesis, coordinating the functions of a variety of interstitial cells, and mediating the repair of local tissue structure (42, 43). Macrophages can sense environmental stimuli and differentiate into functionally polarized subgroups (44–46), which is usually described as M1 or M2 differentiation, terms that were first used to describe the two functionally opposite statuses of macrophages that are induced *in vitro* (47, 48). Lipopolysaccharide (LPS) and interferon-gamma (IFN-γ) can promote the differentiation of

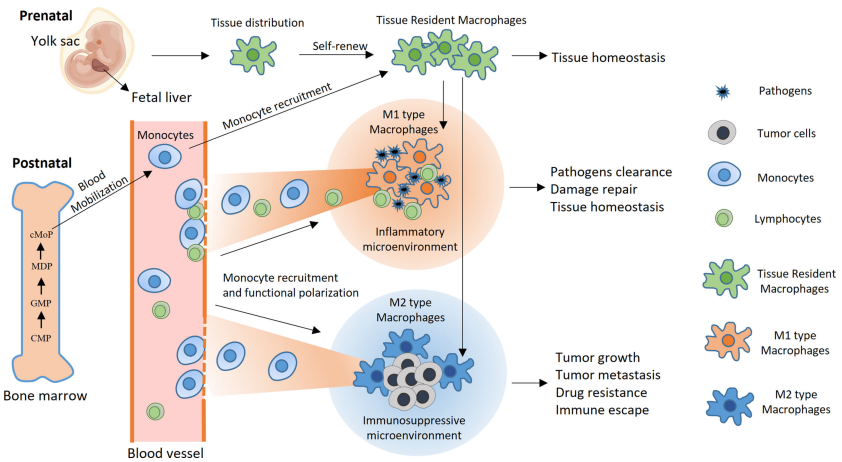

FIGURE 1 | Development, differentiation, and function of macrophages. Under physiological conditions, macrophages are highly versatile and widely present in almost all tissues and organs. Some macrophages that reside in tissues are called TREMs. TREMs originate mainly from yolk sac macrophage progenitors and fetal liver macrophages during embryonic development. After birth, TREMs maintain their number partially through self-renewal and sometimes through the recruitment of monocyte-derived macrophages. Pluripotent hematopoietic stem cells in bone marrow develop into monocytes through multiple stages, including common myeloid progenitors (CMPs), granulocyte-macrophage progenitors (GMPs), macrophage and dendritic cell precursors (MDPs), and common monocyte progenitors (cMoPs). In typical inflammation caused by pathogen infection, monocytes are mobilized from the bone marrow into the blood circulation and subsequently recruited into inflammatory sites, where they differentiate into M1 macrophages and efficiently phagocytose the pathogen. Inflammation also recruits lymphocytes and initiates antigen-specific immune responses with the help of macrophages and dendritic cells, ultimately resulting in pathogen clearance. At the late stage of inflammation, macrophages differentiate toward the M2 type and participate in the tissue repair process, leading to the restoration of internal homeostasis. In contrast, monocytes and TREMs preferentially differentiate toward M2 polarization after they enter the tumor microenvironment, wherein they promote tumor growth and metastasis, mediate resistance to cancer treatments and inhibit antitumor immune responses.

macrophages toward M1 polarization, characterized by high production of nitric oxide (NO), reactive oxygen species (ROS), and a series of proinflammatory cytokines, such as interleukin (IL)-1β and IL-12. M1 macrophages activate T helper type 1 (Th1)-type immune responses and have strong phagocytic and antigen-presenting activities. Hence, they are considered proinflammatory and tumor suppressive (49, 50). In contrast, IL-10, transforming growth factor-beta (TGF-β), and some other immunosuppressive factors, such as IL-4 and IL-13, can induce M2 macrophage differentiation. M2 macrophages participate in the Th2-type immune response, inhibit CD8+ T cell activities, and promote angiogenesis and tissue repair and therefore are believed to be anti-inflammatory and tumor-promoting factors (51, 52). However, recent studies have suggested that the extreme M1/M2 differentiation pattern induced *in vitro* cannot reflect the complex situation *in vivo*. For example, macrophages in the tumor microenvironment often exhibit some characteristics of both M1 and M2 macrophages (44, 53, 54). Although the dichotomy of M1/M2 macrophages is an oversimplification, it is still a meaningful way to describe the functionally poised status of macrophages in certain situations.

There is a close relationship between cancer and inflammation. Tumor growth is often accompanied by a certain degree of inflammation, and the underlying mechanisms are complex (11, 12). For example, chronic viral infection induces constant inflammation and contributes to the development of some types of cancer (55, 56). In addition, tumor blood vessels are often distributed abnormally and have a broken structure, and they cannot meet the oxygen and nutrition requirements of fast-growing tumor cells, resulting in hypoxia and nutrition deficiency within some tumor areas. Consequently, some tumor cells undergo apoptosis or necrosis and release proinflammatory substances, such as adenosine triphosphate (ATP) and high mobility group box 1 (HMGB1), inducing persistent low-grade inflammation and recruiting various immune cells into tumors (57, 58). Macrophages in tumor tissues are collectively referred to as tumor-associated macrophages (TAMs) and are often more numerous than other infiltrated immune cells (16–18). This in itself suggests that macrophages may have a tumor-promoting effect. Numerous studies have demonstrated that tumor cells often express high levels of chemokines, such as GM-CSF, M-CSF, and CXCL12, recruiting many monocytes from the circulation into local tumor sites (15). After entering tumors, monocytes differentiate into mature macrophages, followed by functional polarization toward M2-type TAMs, which is dictated by factors from the immunosuppressive tumor microenvironment. TAMs secrete factors such as CCL22, CXCL1, and PDGF, which bind to corresponding receptors on tumor cells, thereby promoting tumor growth and metastasis, as well as resistance to various cancer treatments (19, 20, 59–61).

In addition, TAMs contribute to the establishment of a deeper immunosuppressive tumor microenvironment by secreting soluble factors and cell-cell contact with other immune cells (54). For example, CCL20 secreted by TAMs recruits regulatory T cells that inhibit the response of effector T cells (62). Moreover, TAMs express low levels of major histocompatibility complex

(MHC)-II and costimulatory molecules on the cell surface, which greatly diminishes their ability to stimulate T cells (63). Although TAMs maintain the ability to phagocytose tumor cells to some extent, tumor cells often express high levels of CD47 molecules that bind to signal regulatory protein α (SIRPα) on the surface of TAMs, sending the "don't eat me" signal and inhibiting the phagocytic activity of TAMs (64). Although many studies have supported the notion that macrophages have tumor-promoting effects, some evidence suggests that macrophages play important antitumorigenic roles in some types of cancers, such as colorectal cancer and early-stage lung cancer (65, 66). More importantly, the functions of macrophages are highly plastic, and their anticancer activities can be reactivated by various means, including macrophage engineering *via* nanotechnology and genetic manipulation, which this review will focus on.

NANOTECHNOLOGY IN CANCER THERAPY

Recently, the application of nanotechnology in cancer therapy has attracted increasing attention (21, 22). NPs travel through the bloodstream to tumor sites, enter the interstitial fluid through the vascular wall *via* passive diffusion, and finally are taken up by tumor cells. However, tumor blood vessels have an abnormal structure, resulting in an uneven distribution of NPs, which often accumulate at the edge of blood vessels, resulting in limited anticancer activity of NPs (29, 67). Active targeting strategies, mainly the use of ligand-mediated systems, stimulus-response systems, and cell-mediated systems, are currently under intensive investigation for their potential to solve the above problem by targeted delivery of NPs to tumor tissues and enhanced tissue distribution and penetration.

The first category of active strategies is the use of ligand-mediated systems. In this strategy, ligands or antibody molecules that recognize biomarkers on tumor cells are present on the shell of NPs, thereby enhancing the interaction between NPs and tumor cells and promoting the transport of NPs to tumor tissues. Targeting biomarkers can be tumor-specific antigens or overexpressed oncoproteins, such as prostate-specific membrane antigen (PSMA) for prostate cancer (68, 69), epidermal growth factor receptor (EGFR) for lung cancer cells (36, 70), and human epidermal growth factor receptor 2 (HER2) for gastric cancer or breast cancer cells (37, 71). However, the outcomes of this strategy to date are often unsatisfactory *in vivo* due to various reasons, such as the high heterogeneity of tumor tissues and the fast clearance of NPs in circulation (72, 73).

The second category is stimulus-response systems. These systems use specific stimulus signals to promote the directional delivery of NPs to tumors and to boost the anticancer activities of NP-carried drugs (31, 74). The signals can be tumor intrinsic, such as an increased glutamine level (75), a decreased pH value (76), and hypoxia (77), or tumor extrinsic, such as a light source (78), a heat source (79), a magnetic field (80), or ultrasound (81). Among them, light-responsive systems may be the most well-studied systems because they can be readily controlled in a spatiotemporal manner, resulting in directional transport, improved tumor penetration and distribution, and controlled release of NP-carried drugs. For more information, please refer to the relevant reviews (82, 83).

The third method involves carrier cells or cell components. As mentioned earlier, the development of many cancers is accompanied by a certain degree of inflammation and immune cell infiltration. Immune cells can sense tumor-derived chemokines and actively move to tumor sites (84, 85). Interestingly, although hypoxia prevents the infiltration of T cells, it stimulates tumor cells to release a large number of macrophage-recruiting factors, such as CCL2, CSF-1, and VEGF, resulting in pronounced enrichment of macrophages in hypoxic tumor regions (15, 86). A series of studies have demonstrated that macrophages can be exploited as cell carriers to actively transport NPs into tumor sites (30, 87), and the following section will introduce the preparation, function, mechanisms, and limitations of NPL-Ms in cancer therapy.

ENGINEERING MACROPHAGES FOR NP DELIVERY IN CANCER THERAPY

NP Loading in Macrophages

There are two main sources of macrophages for NP loading. One source is primary macrophages, such as bone marrow-derived macrophages, alveolar macrophages, and peritoneal macrophages. The second source is cell lines, including the mouse macrophage cell lines RAW264.7 and J774A.1 and the human peripheral blood monocyte cell line THP-1 (24, 41, 88–91). NPL-Ms can carry a variety of NPs, including liposomes (92, 93), magnetic NPs (94, 95), polymeric NPs (96, 97), gold (AU) NPs (98–101), and others (102, 103). Because macrophages naturally phagocytose NPs (104, 105), NPL-Ms can be prepared by a simple coincubation method. Li et al. prepared RAW264.7 macrophages loaded with paclitaxel (PTX)-containing NPs. Intravenous injection of NPL-Ms significantly inhibited the growth of a breast cancer model (39). Ibarra et al. prepared mouse bone marrow-derived monocytes and THP-1 cells loaded with polymer NPs, and they showed that NP loading had no significant effect on the viability and function of macrophages, nor did it affect the differentiation of THP-1 cells into macrophages upon stimulation with phorbol 12-myristate 13-acetate (PMA). Moreover, these cells had a stronger NP loading ability after LPS stimulation (96). Electroporation can also be used to prepare NPL-Ms and might be a superior approach for loading easily degradable substances such as nucleic acids or enzyme precursors (106, 107).

NPL-Ms can be exploited for cancer therapy with *in situ* strategies. Because monocytes/macrophages efficiently phagocytose apoptotic bodies, Zheng et al. intravenously injected light-sensitive gold NPs encapsulated by apoptotic bodies, which were quickly engulfed by macrophages, thus generating NPL-Ms *in vivo*. These NPL-Ms effectively migrated to tumor sites and inhibited tumor growth and metastasis in a mouse tumor model (108). Circulating monocytes/macrophages

efficiently phagocytose damaged red blood cells (RBCs) *via* the complement-mediated opsonization effect. Based on that, Feng et al. designed a cell relay strategy that allowed monocytes in circulation to preferentially take up NPs. They first prepared NPs coated with artificially damaged RBCs that were used as primary carriers to deliver NPs to macrophages, generating NPL-Ms *in vivo*, which delivered NPs to tumors in a targeted manner, leading ultimately to enhanced anticancer activity in a rat tumor model (109).

In some cases, internalized nanomaterials may negatively affect macrophage function, or the encapsulated drugs in NPs are prematurely dissociated, which may reduce the efficacy of drug delivery or cause systemic toxicity (30). A plausible alternative is the so-called piggybacking method, i.e., binding NPs on cell surface, which has been tested with various cell types, including macrophages (38). Through various techniques that can be largely classified into two categories, noncovalent and covalent, NPs can be attached on cell surfaces without being internalized by the macrophage carrier, and transported to tumor sites (110–116). **Table 1** briefly describes the categories, principles, and mechanisms of major NP delivery methods with live macrophages, and readers are directed to more detailed reviews on this subject (112, 123, 134). **Table 1** also includes the methods of loading NPs in macrophage-derived cell membranes or extracellular vesicles, which will be discussed in the following section.

NPL-M Tumor Site Migration

In a study by Li et al., RAW264.7 macrophages loaded with fluorescent NPs were injected intravenously into normal nude mice, and these NPL-Ms were quickly distributed into the liver and intestine 1-2 h after injection; however, they were almost undetectable after 24 h, indicating fast clearance of the NPL-Ms. In contrast, in nude mice bearing subcutaneous xenograft tumors, the NPL-Ms infiltrated into tumor tissues shortly after injection and resided there for more than 48 h. These findings indicated that NPL-Ms directly migrated toward tumors and had a relatively long half-life in the tumor microenvironment (39). Hypoxia often occurs in tumors and drives the migration of monocytes/macrophages toward tumor sites. This feature renders macrophages a unique type of cell carrier to deliver NPs to hypoxic tumor areas. Choi et al. demonstrated that NPL-Ms carrying gold NPs could migrate toward hypoxic tumor spheres *in vitro* (98). An et al. loaded macrophages with anionic gold nanorods (AuNRs) for hypoxia-triggered photoacoustic (PA) imaging and photothermal therapy (PTT). The results indicated that NPL-Ms directionally migrated to hypoxic tumor sites and provoked significant antitumor effects (135).

Traditional cancer treatments, such as radiotherapy and chemotherapy, also affect the migration of macrophages to tumors. Evans et al. prepared NPL-Ms loaded with hypoxia-activated prodrug NPs and demonstrated that NPL-Ms accumulated in the hypoxic regions of mouse breast tumors. Moreover, the accumulation and anticancer activities of NPL-Ms were more significant when combined with chemotherapy (136). Miller et al. found that radiotherapy increased the intratumoral concentration of NPs in a mouse breast cancer model, which is related to the radiotherapy-induced increase in TAM infiltration. They found that a large number of TAMs accumulated around microvessels after radiotherapy, altered vascular permeability, and elicited dynamic bursts of NP extravasation. Depleting macrophages greatly diminished the effect of radiotherapy on the enrichment of NPs in tumor tissues (122). *In vivo* PET imaging can be performed using macrophages loaded with NPs containing (64)Cu. Based on that, Kim et al. demonstrated that chemotherapy or radiotherapy significantly increased the number of TAMs, thereby increasing the intratumoral NP concentration in mouse tumors (137).

Inducing M1 polarization may enhance the tumor homing activity of macrophages. Peng et al. found that M1 macrophages loaded with DOX-NPs effectively crossed the blood brain barrier (BBB) and exerted a strong inhibitory effect on a mouse glioma model (118). Li et al. prepared macrophages loaded with magnetic

TABLE 1 | NP loading in macrophage-based drug delivery.

Strategies	Categories	Method Descriptions and Mechanisms	REFs
Cell Encapsulation	*In vitro*	• Coincubation: cells uptake NPs through phagocytosis or other endocytosis mechanisms. • Electroporation: electroporation generates small pores on cell membrane for NPs to entry into cells.	(39, 40, 117–119)
	In vivo	• Functionalized NPs, NPs tethered on damaged red blood cell (RBC) membranes, or NPs cloaked in apoptotic bodies are engulfed by macrophages to form NP-loaded macrophages *in vivo*.	(108, 109, 120–122)
Surface Binding	Covalent coupling	• Modified NPs are coupled to functional groups (i.e., thiol, amine) on cells through various mechanisms, such as maleimide-thiol conjugation and disulfide bond formation. • - Complicated procedure, high binding strength, possibly impaired cell integrity	(114, 123, 124)
	Noncovalent binding	• Nonspecific adsorption: NPs are attached to outer cell membranes *via* hydrophobic or electrostatic binding. • Ligation-mediated binding: NPs modified with ligands or antibodies bind corresponding molecules on the cell surface. • - Simple procedure, low binding strength, high cell integrity	(110, 111, 113, 115, 116)
Membrane Coating	–	The procedure may involve the following steps: • Cell culture: such as tumor cells, RBCs, and immune cells; • Isolating the cell membrane by hypotonic treatment; • Coating NPs with the cell membrane by various methods, such as coincubation, extrusion, and sonication. • - NPs can be camouflaged in homogenous membranes from one cell type or heterogeneous fused membranes from two different cell types.	(95, 125–128)
EV Loading	–	• Extracellular vesicles (EVs) include exosomes and microvesicles derived from various cell types. • - The procedure is similar to that of membrane coating but is usually more sophisticated due to the complicated EV isolation procedure. EV-loaded NPs may have an increased ability to pass biological barriers due to their smaller size.	(129–133)

NPs. These NPL-Ms exhibited M1 polarization and had significantly enhanced tumor homing and anticancer activities in a mouse breast cancer model. In addition, NPL-Ms improved the tumor immune microenvironment, inhibited local M2 macrophages, and enhanced the antitumor immune response (138).

NPL-M Drug Release

There are relatively few studies on how NPL-Ms release NPs after reaching tumor tissues. In the piggybacking method (38), membrane-binding NPs are delivered to tumors with the help of macrophages in a targeted manner, and the subsequent release of the drug depends mainly on the design of the NP itself. In terms of NPL-Ms, regardless of whether they are formed *in vitro* or *in situ*, the mechanism of drug release and how the process is controlled remain elusive. Li et al. loaded macrophages with fluorescence-labeled PTX-NPs and then cocultured the macrophages with tumor cells *in vitro*. After 4 h, a fluorescent signal was detected in tumor cells that gradually increased and peaked at 12 h, during which time the signal in macrophages gradually decreased, indicating that the NPs were transferred from macrophages to tumor cells (39). Cells mainly ingest foreign substances through endocytosis, and ultimately, the ingested substances are either degraded or released from cells [please refer to the detailed reviews (139–141)]. Macrophages mainly engulf NPs through phagocytosis and pinocytosis. NPs are not rapidly degraded during intracellular trafficking in macrophages, so the potential adverse effects of the free drug are diminished. In addition, macrophages slowly release ingested NPs, which reduces the consumption of NPs before the macrophages reach tumors. For example, by comparing macrophages loaded with free PTX or PTX-NPs, Li et al. found that 26% of PTX-NPs vs. greater than 50% of free PTX were released before the macrophages reached the tumors (39).

NPL-Ms can transfer NPs or free drugs to tumor cells through other means. For example, tumor cells can interact with and exchange information with other cells through the microtubule network (142, 143). Guo et al. found that M1 macrophages loaded with DOX (DOX-M1) entered mouse tumors and exported DOX to tumor cells through tunneling nanotubes, leading to pronounced tumor cell killing (144). In another study, LPS was anchored to the cell membrane of macrophages loaded with DOX. These macrophages migrated to mouse tumors and rapidly killed tumor cells by transferring DOX to tumor cells through a microtubule network. In addition, cell membrane-anchored LPS induced the differentiation of local TAMs to M1 macrophages and promoted the antitumor immune response (145).

The process of NP release by macrophages is affected by many factors, including the physicochemical properties of NPs, the functional status of macrophages, and the tumor microenvironment. For example, Oh et al. reported that gold NPs with a high-aspect ratio exit macrophages more rapidly but tend to remain in tumor cells longer than those with a low aspect ratio (146). Ikehara et al. found that a mild temperature increase promoted the release of NPs by macrophages (147). In addition, macrophages showed higher drug release efficiency for polymeric or negatively charged copolymer NPs than for liposomal NPs or positively charged copolymer NPs (121, 148, 149). Interestingly, Soma et al. found that IFN-γ stimulation significantly promoted the release of NP-DOX by macrophages (150). During inflammation, activated macrophages release a large amount of cytokines and bioactive substances; therefore, activating macrophages may promote the release of NPs.

Limitations and Challenges

The concept of using macrophages as drug carriers is not new and has been studied for many years. However, it has not been applied in clinical practice. **Table 2** summarizes some recent preclinical studies using live macrophages for NP drug delivery. In the future, in-depth studies are needed to achieve a better understanding of the complex interaction among NPs, macrophages, and tumor cells. An ideal cell-mediated NP delivery system would have the following five characteristics: 1) an abundant source of cells into which NPs can be loaded efficiently; 2) no significant impairment of cellular function after NP loading; 3) directional migration toward tumors; 4) efficient release of NPs at tumor sites; and 5) effective uptake of the released NPs by tumor cells. Natural evolution has endowed macrophages with powerful phagocytic, migratory and secretory functions. With the advantages provided by nanotechnology, macrophages can be developed as prominent NP drug carriers. However, there are still many limitations and challenges. First, the sources of autologous macrophages are limited. It is currently impossible to obtain a large number of macrophages through *in vitro* expansion of autologous monocytes derived from patients, while the use of allogeneic macrophages carries a risk of rejection or graft-versus-host reaction. Second, loading NPs into macrophages or anchoring NPs on the surface of macrophages has complex effects on cell function, which remain not fully understood. Third, the local immunosuppressive microenvironment of tumors is closely related to tumor progression; however, there is currently much that is unknown regarding how NPL-Ms regulate the tumor immune microenvironment as well as T cell immune responses. Finally, although the pathways of NP internalization by tumor cells has been extensively studied, our knowledge about the cellular uptake of NPs with various properties by macrophages remains very limited (30, 153). How NP loading affects the function of macrophages in terms of phagocytosis, migration, and immune stimulation must be comprehensively evaluated in future studies. Moreover, although previous studies have shed some light on the possible pathways governing the intracellular trafficking of NPs in macrophages and their release at tumor sites (153, 154), which is depicted in **Figure 2**, precise mechanisms remain largely elusive and await more detailed investigations.

EMERGING CONCEPTS AND NOVEL STRATEGIES IN MACROPHAGE ENGINEERING

In recent years, new strategies have emerged in the field of macrophage engineering. For example, macrophage membranes and macrophage extracellular vesicles (MEVs) have been successfully utilized for NP loading; these approaches not only retain some characteristics of macrophages but also greatly expand

TABLE 2 | Macrophage-mediated NP drug delivery in some cancer studies.

NPs	Agents	Macrophage Information	NP Modification	Mechanisms and Features	Cancer Models	REFs
zSOC NPs; NLCs	PTX; DOX	• Raw 264.7 cells	–	• Targeted NP drug delivery	Breast cancer, SUB	(39)
rGO NPs	DOX	• Raw 264.7 cells	PEG-BPEI (PB) coating	• Enhanced NP loading by PB • NIR-triggered DOX release • Combined PTT and CT effects	Prostate cancer, SUB	(102)
NGs; PPy NPs	DOX	• Raw 264.7 cells	Hyaluronic acid (HA) coating	• Enhanced NP loading by HA • NIR-triggered DOX release • Combined PTT and CT effects	Breast cancer, SUB	(119)
AuNSs	–	• Raw 264.7 cells	Surface anionic charging	• Enhanced NP loading • PA imaging and PPT effects	Breast cancer, SUB	(40)
SNPs	DOX	• Raw 264.7 cells • M1 polarization upon NP loading	–	• Effective NP uptake, tumor site homing, and slow drug release • Drug release in exosomes	Glioblastoma, SUB	(151)
LNPs	Sorafenib	• Raw 264.7 cells • M1 polarization by LPS treatment	–	• Enhanced NP tumor site homing • Enhanced targeted drug therapy • Enhanced immune responses	liver cancer, SUB	(24)
AuNSs	–	• Raw 264.7 cells • LPS-treated or -untreated(M1 or M0 type macrophages)	–	• Enhanced NP loading, tumor site homing, and PTT effect by M1 macrophage polarization	Head and neck cancer, SUB, Xenograft	(117)
PLGA NPs	DOX	• Bone marrow-derived macrophage • M1 polarization by LPS and IFN-γ treatment	–	• Effective NP uptake, tumor site homing, and slow drug release • Crossing the BBB to brain tumors	Glioblastoma, orthotopic	(118)
ZnPc NPs	Oxaliplatin prodrug	• Bone marrow-derived macrophages • M1 polarization upon NP loading	–	• Drug release in low-pH sites • Combined PDT and CT effects • Enhanced immune responses	Breast cancer, SUB; Lung metastasis	(41)
Liposomes	DOX	• Primary peritoneal macrophages	–	• Targeted NP drug delivery	Lung cancer, SUB, Xenograft	(93)
PSMA NPs	Mertansine	• Bone marrow-derived Ly6c^{high} inflammatory monocytes	Legumain-sensitive peptide coating	• On-demand drug release by macrophages at l ung metastasis	Lung metastasis of breast cancer	(88)
CPNs	–	• Bone marrow-derived monocytes • Human monocytes THP-1 cells	–	• Crossing the BBB to brain tumors • PDT effects	Glioblastoma, orthotopic	(96)
Liposomes	–	• Human peripheral blood monocytes • Human peritoneal macrophages	Oligomannose coating	• Effective NP loading • Accumulation of the NPL-Ms in peritoneal micrometastatic sites	Gastric cancer metastatic model	(152)
SWNTs	–	• Circulating Ly-6C^{high} monocytes • Cell encapsulation *in vivo*	RGD peptide coating	• NP ligand functionalization • NPL-Ms generation *in vivo* in a selective macrophage subtype	Glioblastoma, SUB	(120)
PLGA NPs	Vincristine	• Circulating monocytes • Cell encapsulation *in vivo*	Binding on damaged RBC membranes	• Enhanced NP drug delivery by a cell relay strategy	Breast cancer, SUB in Rat	(109)
AuNRs	–	• Raw 264.7 cells (*in vitro* encapsulation) • Circulating Ly-6C^{high} monocytes (*in vivo* encapsulation)	CpG coating; Cloaking in apoptotic bodies	• Immune stimulation by CpG • PTT effects	Breast cancer, SUB	(108)

AuNRs, gold nanorods; AuNS, gold nanoshells; BBB, blood–brain barrier; CPNs, conjugated polymer nanoparticles; CT, chemotherapy effects; DOX, doxorubicin; LNPs, lipid nanoparticles; NGs, nanogels; NLCs, nanostructured lipid carriers; OMLs, oligomannose-coated liposomes; PA, photoacoustic; PDT, photodynamic therapy; PLGA, polylactic-co-glycolic acid; PSMA, poly (styrene-co-maleic anhydride); PTT, photothermal therapy; PTX, paclitaxel; rGO, reduced graphene oxide; SNPs, silica-based nanoparticles; SOC, N-Succinyl-N'-octyl chitosan; SUB, subcutaneous tumor model; SWNTs, single-walled carbon nanotubes; ZnPc, photosensitizer zinc phthalocyanine.

the compatibility and loading capacity of NPs (**Figure 3**). Another research hotspot involves targeting macrophages with NPs, thereby enhancing the phagocytic function of macrophages and promoting the differentiation of macrophages toward the M1 type. In addition, the success of CAR-T technology has inspired studies of macrophage engineering with CARs for cancer immunotherapy.

Macrophage Membrane-Coated NPs (MMC-NPs) and Macrophage Extracellular Vesicle-Coated NPs (MEVC-NPs)

In preparing MMC-NPs, the structure of the macrophage cell membrane is disrupted by physical or ultrasonic methods, and then, the cellular contents are removed. After coincubation with NPs, the cell membrane spontaneously closes to form MMC-NPs (155). MMC-NPs have several important advantages. First, the use of the cell membrane eliminates the potential adverse effects of NP loading on the function of macrophages. In addition, it does not cause immune rejection if the autologous cell membrane is used and thus significantly prolongs the half-life of NPs in circulation. Moreover, many macrophage membrane proteins are retained on the surface of MMC-NPs, which may facilitate tumor homing (156). Xuan et al. prepared a macrophage membrane-coated gold nanoshell (AuNS). These MMC-NPs accumulated in tumor sites through the interaction between macrophage membrane

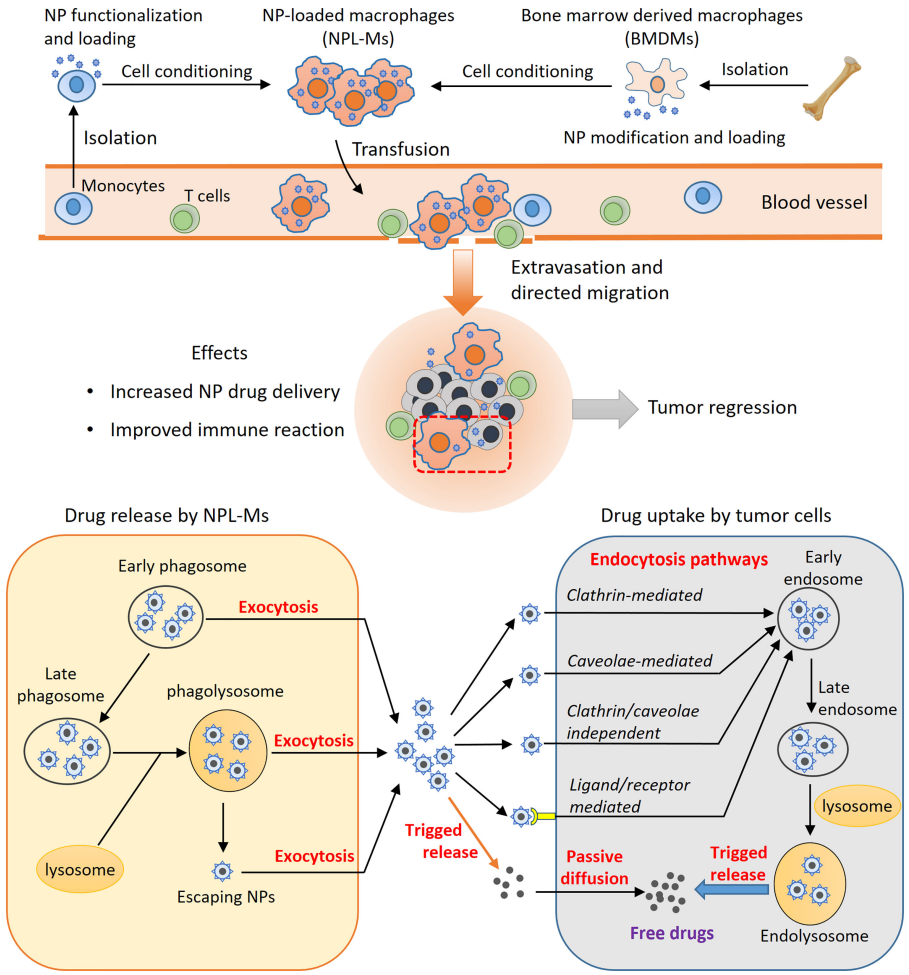

FIGURE 2 | The principles of macrophage-based NP drug delivery. Live macrophage carriers are mainly from peripheral monocytes, bone marrow-derived macrophages, or macrophage cell lines. M1-type macrophage differentiation can be induced, and NPs can be functionalized. After administration, the NPL-Ms migrate to tumors, enhancing drug delivery and anticancer immune responses. The efficiency of this strategy depends on controlled drug release by NPL-Ms and effective drug uptake by neighboring tumor cells. Through exocytosis, NPs recycled from early phagosomes or matured phagolysosomes or NPs that escape from phagosomes can be released through the exocytosis mechanism. Tumor cells uptake NPs through various endocytosis pathways, such as the clathrin-mediated, caveolae-mediated, and clathrin/caveolae-independent pathways. NPs functionalized by surface ligands can be recognized by corresponding receptors on tumor cells and effectively internalized by endocytosis. Consequently, the internalized NPs are sorted into early endosomes, late endosomes, and eventually endolysosomes where NPs can be triggered to release free drugs. Free drugs released from NPs in the intracellular space can enter into tumor cells by passive diffusion.

molecules and adhesion molecules on the vascular endothelial cells of tumor tissue, leading to significant antitumor effects in a mouse breast cancer model. Compared with NPs coated with erythrocyte membranes, MMC-NPs were more effectively enriched in tumor tissues. In addition, due to the membrane fusion effects, the uptake of MMC-NPs by tumor cells was significantly improved compared to that of free NPs (127). Zhang et al. prepared MMC-NPs loaded with pH-sensitive PTX-NPs. Upon reaching the tumor tissue, these MMC-NPs released PTX-NPs in response to the weakly acidic environment in the tumor stroma; after internalization by the tumor cells, the PTX was quickly dissociated from the PTX-NPs in the highly acidic environment of lysosomes inside the tumor cells and exerted significant anticancer effects in a mouse breast cancer model (125).

Extracellular vesicles (EVs) are cell-derived and membrane-coating particles carrying cell-specific DNA, RNA, and proteins. They are usually divided into three categories based on their size and origin: exosomes (30-150 nm), microvesicles (MVs, 50 nm-1 μm), and apoptotic bodies (50 nm-5 μm) (157). EVs can be efficiently internalized by other cells, mediating the exchange of biological substances between cells and playing important roles in tumor progression (158–160). The potential application of macrophage-derived exosomes and MVs in cancer therapy has attracted great attention recently because of their excellent biocompatibility and high NP-loading capacities (161, 162). Kim et al. found that free PTX coated with M1 macrophage-derived exosomes (PTX-M1-exos) had strong anticancer effects in a mouse model of pulmonary tumor metastases (133). They

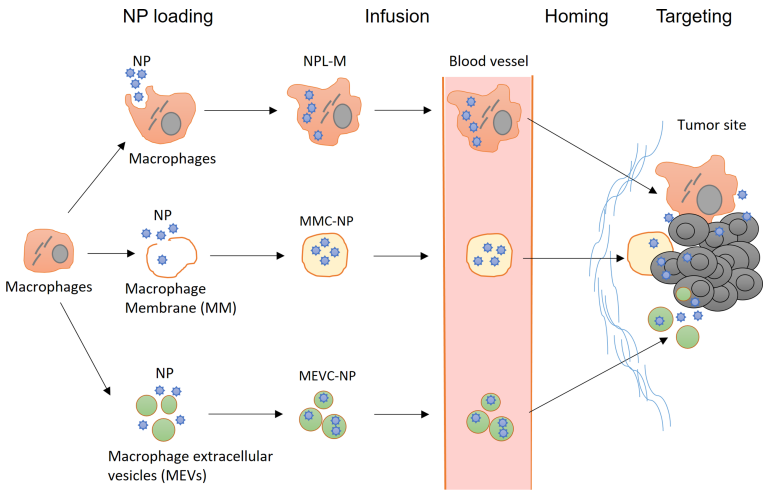

FIGURE 3 | Application of nanotechnology in the engineering of macrophages. (Top) After infusion, NPL-Ms actively migrate to tumor tissue and release NPs locally, resulting in enhanced antitumor effects. (Middle) Macrophage membrane-coated NPs (MMC-NPs) have a prolonged half-life in circulation and a strong affinity at the tumor site for vascular endothelial cells that facilitate their tumor site homing and accumulation. (Bottom) Macrophage-derived extracellular vesicle-coated NPs (MEVC-NPs) can infiltrate tumor sites, where they are taken up by tumor cells, inducing significant cell death.

demonstrated that PTX-M1-exos were more effectively internalized by tumor cells than NPs-PTX, as indicated by the nearly complete colocalization of PTX-M1-exos with cancer cells 4 h after intranasal administration (133).

The communication between tumor cells and macrophages *via* exosomes is believed to play an important role in tumor development (163, 164). Interestingly, tumor cells efficiently take up EVs derived from macrophages (129, 131, 133, 165), although the underlying mechanism is not very clear. It was reported that the acidic tumor microenvironment may promote membrane fusion between exosomes and tumor cells (166). In addition, macrophage-derived exosomes may carry certain cell membrane proteins capable of specifically binding to tumor cells, thus promoting membrane fusion and exosome internalization (167, 168). Moreover, after entering tumor cells, exosomes may alter intracellular transport pathways to prevent their rapid release from tumor cells (169), thus allowing more drugs to enter the cytoplasm and nucleus and exert a more significant therapeutic effect (132).

In addition to improving drug delivery, macrophage-derived EVs also regulate antitumor immune responses. For example, Choo et al. found that exosome-mimetic nanovesicles (M1NVs) derived from M1 macrophages were enriched in tumor tissue after intravenous infusion, which induced the differentiation of TAMs from M2 to M1 macrophages and thus enhanced the effect of anti-PD-1 immunotherapy in tumor-bearing mice (170). Wei et al. found that macrophage-derived microparticles could be preferentially taken up by TAMs in tumor tissues, thereby exerting immunomodulatory effects in tumor-bearing mice (171). Cheng et al. reported that after subcutaneous injection, M1 macrophage-derived exosomes could be taken up by both macrophages and dendritic cells in lymph nodes, where they secreted large amounts of Th1-type cytokines and enhanced antitumor immune responses in a melanoma mouse model (172). In summary, using macrophage membranes or macrophage-derived EVs as carriers can improve drug loading and partially solve the shortage of cell sources. These novel drug carriers can not only target tumor sites but also activate antitumor immune responses and therefore hold great promise in cancer therapy (173–175).

Targeting TAMs *via* Nanotechnology for Improved Anticancer Activity

As described earlier, reprogramming TAMs from the M2 to M1 differentiation status may be an effective cancer treatment strategy (176, 177). To this end, nanotechnology is very useful. A variety of NP designs were reported to be capable of targeting TAMs specifically and inducing M1 differentiation, leading to potent anticancer activities in preclinical models. For example, given that mannose specifically binds to the CD206 receptor on the surface of M2 macrophages, Zhao et al. prepared mannose-encapsulated NPs containing polyinosinic-polycytidylic acid (poly IC) that are capable of inducing M1 differentiation. NPs are preferentially taken up by M2 macrophages and induce M1 polarization, thereby leading to pronounced antitumor effects (178). Qiang et al. prepared M2-targeting NPs (M2NPs) by coating the NPs with an M2 macrophage-binding peptide and loaded them with small interfering RNA (siRNA) targeting colony-stimulating factor-1 receptor (CSF-1R), which plays a critical role in M2 differentiation. M2NPs effectively targeted M2-type TAMs and induced M1 differentiation, thereby inhibiting the growth of tumors in tumor-bearing mice (179).

In addition, multifunctional NPs can be generated for better treatment outcomes. Zhang et al. constructed NPs containing mesoporous Prussian blue (MPB) with a surface modified by low-molecular-weight hyaluronic acid. After tail vein injection,

the NPs selectively accumulated in M2 TAMs in tumors, leading to reprogramming from M2 to M1 macrophages. In addition, the NPs generated oxygen through the catalytic decomposition of endogenous hydrogen peroxide (H_2O_2) and thus corrected hypoxia in the tumor microenvironment, acting as *in situ* O2 generators (180). Han et al. loaded NPs with CpG oligodeoxynucleotides (CpG-ODN), baicalin, which has immunomodulatory functions, and the human melanoma antigen $Hgp100_{25–33}$. The NPs were further coated with an RBC membrane carrying galactose that facilitated the targeted delivery of the NPs to TAM by binding galactose-type lectin (Mgl) on the TAM cell surface (181). The results demonstrated that these multifunctional NPs promoted M1 differentiation and enhanced the antigen-specific immune response, thereby exerting a significant antitumor effect in melanoma tumor-bearing mice (181).

CD47 on the tumor cell surface binds to SIRPα on the surface of macrophages, which activates the Src homology region 2 (SH2) domain phosphatases SHP1 and SHP2 and thereby transmits a "don't eat me" signal to macrophages. Ramesh et al. prepared NPs containing two types of inhibitors: a CSF1-R inhibitor capable of promoting M1 reprogramming and an SHP2 inhibitor that blocks CD47-SIRPα signal transduction and thus enhances phagocytosis. In addition, they coated NPs with anti-CD206 to improve the efficacy of M2-type TAM targeting. The results demonstrated that these multifunctional NPs exerted a significant antitumor effect, mainly through modifying TAMs in breast cancer and melanoma mouse models (182). In addition, the CRISPR/Cas9 gene editing system can also be delivered to macrophages using NPs. Lee et al. used gold NPs to carry the Cas9 protein and sgRNAs targeting the *PTEN* gene. These NPs were mainly phagocytosed by macrophages residing in the liver and spleen after tail vein injection, leading to a gene-editing efficiency of greater than 8% in macrophages (183). Nanotechnology can also be used to transport mRNA or siRNA to a specific cell population in a targeted manner (184, 185). For example, NPs carrying *PTEN* mRNA were effectively delivered to $PTEN^{null}$ cancer cells, and restoration of PTEN expression induced immunogenic death of cancer cells and thus induced potent antitumor immune responses in melanoma tumor-bearing mice (186). In summary, by combining nanotechnology and a variety of approaches, TAMs can be modified in a targeted manner, and their anticancer activities can be promoted.

Equipping Macrophages With CARs *via* Genetic Manipulation

The concept of CARs was first tested in T cells, and the application of CAR-T cells in the treatment of blood cancers was successful (187, 188). As shown in **Figure 4**, T cell CARs are mainly composed of an extracellular domain of a single-chain variable fragment (Scfv) that specifically recognizes target molecules, a transmembrane (TM) domain, and an intracellular domain responsible for signal transduction. This design confers T cell tumor cell-specific cytotoxicity in an MHC-independent manner. However, to date, CAR-T therapy has have a limited effect in solid tumors (187, 189), and researchers have begun to ask whether CAR-modified macrophages (CAR-Ms) could be useful in cancer therapy. It is known that the "eat me" signal molecules on tumor cells, such as lipid phosphatidylserine (PS), are recognized by corresponding scavenger receptors on macrophages, resulting in the activation of phagocytosis (190,

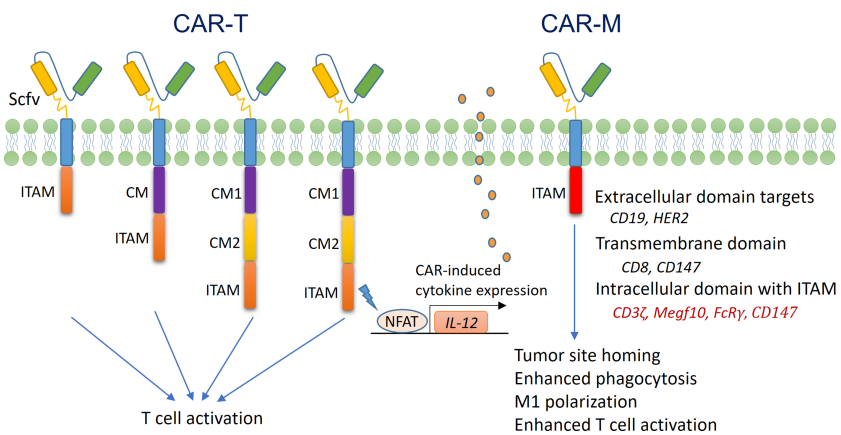

FIGURE 4 | Structure and function of CAR-T cells and CAR-Ms. (Left) The structure of first-generation T cell CARs mainly includes an ScFV extracellular domain that recognizes tumor antigens, a TM domain, and an intracellular domain that contains ITAM and is responsible for signal transduction (usually derived from the intracellular domain of CD3ζ). The structure of second-generation T cell CARs includes an additional intracellular signal transduction domain from costimulatory molecules (CMs), such as CD28 and 4-1BB. The structure of third-generation T cell CARs includes two or more CM domains, which further enhance T cell activation. The structure of fourth-generation CARs includes a nuclear factor of activated T cells (NFAT)-responsive gene expression cassette, which drives the expression of an immunoregulatory gene, such as IL-12. Once CAR-T cells are activated, NFAT translocates to the nucleus and activates the expression of IL-12, thereby promoting anticancer activity. (Right) Currently, the structure of macrophage CARs is based on that of first-generation T cell CARs. The intracellular domain of CD3ζ, FcRγ or Megf10 is used for signal transduction. In addition, CAR-Ms are preferentially fixed at the M1 differentiation status, with enhanced phagocytic and antigen presenting activities.

191). In addition, Fcγ receptors (FcγRs) on macrophages mediate antibody-dependent cellular phagocytosis (ADCP) by binding to the Fc segment of the IgG antibody (190, 191). The basic structures of these abovementioned phagocytic receptors all include an extracellular domain, a TM domain, and an intracellular domain, similar to those of CAR molecules. Ligation of the extracellular domains of these receptors induces phosphorylation of tyrosine in the immunoreceptor tyrosine-based activation motif (ITAM) of the intracellular domain of these receptors, leading to cytoskeletal and membrane remodeling events that promote the ingestion of tumor cells by macrophages (192).

A series of recent studies have demonstrated that the antitumor activity of macrophages can be enhanced by modifying phagocytic receptors with CAR technology (193–196). Morrissey et al. prepared mouse CAR-Ms by lentiviral transduction. The extracellular domain of the CAR recognized CD19, and the TM domain was derived from CD8 (194). They found that the intracellular domains from either Megf10 or FcRγ molecules were able to mediate the specific phagocytosis of CD19-expressing Raji B cells by the CAR-Ms. Interestingly, replacement of the intracellular domain with that of CD3ζ (which contained three ITAMs and had high homology with FcRγ) achieved a similar effect (194). Klichinsky et al. prepared CAR-Ms with human peripheral blood monocytes. The CAR molecules had an extracellular domain that recognized human epidermal growth factor receptor 2 (HER2) and an intracellular signal domain from CD3ζ (193). The CAR-Ms were able to specifically recognize and phagocytose $HER2^+$ tumor cells, and a single-dose infusion of the CAR-Ms significantly inhibited the growth of $HER2^+$ xenograft tumors. Importantly, after infusion, the CAR-Ms accumulated in liver and tumor tissues and survived *in vivo* for at least 2 months (193). In the preparation of CAR-Ms, delivering CAR genes into macrophages is technically challenging. The authors demonstrated that a replication-incompetent chimeric adenoviral vector (Ad5f435) not only efficiently transferred the CAR genes into macrophages but also induced M1 differentiation. Such CAR-Ms activated CD4+ Th1 cells and, more importantly, CD8+ cytotoxic T cells through cross-presentation, thereby promoting a strong antitumor effect (193). Zhang et al. prepared CAR-Ms to target the extracellular matrix rather than tumor cells, with the aim of enhancing immune infiltration into solid tumors (195). The TM and intracellular domains of the CAR molecules were all derived from CD147, which drives the expression of matrix metalloproteinases (MMPs) in macrophages. The CAR-Ms were detected in tumor tissues 24 h after tail vein injection, and their numbers peaked at 3 d, during which time the collagen content in the tumor stroma was significantly decreased due to the increased activity of MMPs. Further analysis revealed that the anticancer effect of the CAR-Ms in tumor-bearing mice was associated with increased $CD3^+$ T cell infiltration (195).

CAR-M technology holds great potential for the treatment of solid tumors. However, at present, this field is still in its infancy, and there are many challenges. For example, most solid tumors lack suitable tumor-specific antigens for CAR design. In addition, the impact of different TM domains and intracellular domains on the function of CAR-Ms remains unclear. In the clinical application of CAR-T cells, cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) are the two most serious side effects, both of which may be related to excessive inflammatory cytokines derived from CAR-T cells (197). A recent study utilized the intracellular domain of the MERTK kinase to develop CAR-Ms. These CAR-Ms effectively eliminated SARS-CoV-2 virus *in vitro* by enhanced phagocytosis without upregulation of proinflammatory cytokine expression (198). Such results indicate that it is possible to optimize the design of CAR-Ms to reduce their potential side effects. In the context of cancer therapy, inducing M1 differentiation may be preferred, as it can improve the phagocytic activity of CAR-Ms; however, such manipulation may have unpredictable side effects and needs to be carefully evaluated using preclinical models.

CONCLUSION AND PERSPECTIVE

Macrophages are extremely versatile and possess a variety of antitumor properties. They can kill tumor cells directly by phagocytosis or indirectly by activating other immune cells. However, in the tumor microenvironment, their antitumor activities are often inhibited (192). With the rapid development of nanotechnology and transgenic technology, engineering macrophages has become an important research direction in cancer therapy (199). Numerous studies have demonstrated that engineered macrophages can actively migrate to tumor tissues and kill tumor cells effectively. However, they can also migrate to normal tissues and organs after infusion. Considering the relatively long lifespan of these cells, their migration, distribution, and potential toxicity to normal tissues needs to be closely monitored *in vivo*, and novel techniques such as macrophage imaging might be useful in this regard (193, 200). It is of great significance to investigate how to better control the migration of engineered macrophages to reduce their accumulation in normal tissues. Studies have shown that chemotherapy, radiotherapy, and immunotherapy (such as STING agonist treatment) can all stimulate inflammation to a certain extent, thereby transforming cold tumors into hot tumors (201–203). Such transformations could improve the directional migration of engineered macrophages to tumor sites, thus enhancing their therapeutic effects while reducing potential off-target or on-target toxicities.

Notably, when NPs or macrophage membrane-coated NPs are used to deliver genetic materials into macrophages, including DNA, mRNA, noncoding RNA, and the CRISPR system, the efficacy of genetic modification seems to be greatly improved (25, 204–206). However, at present, our understanding of the interactions between these gene carriers and macrophages, in terms of phagocytosis, transport, and release, is very limited, and further investigation is needed. In addition, after engineered macrophages enter tumors, their activities may be antagonized

by local TAMs that are usually immunosuppressive; therefore, conducting in-depth studies is important to determine whether the pre-existing TAMs will significantly impact the function of engineered macrophages, or vice versa. In this regard, methods for local TAM depletion can be used in sequential combination with engineered macrophages (207, 208), i.e., disruption of the immunosuppressive microenvironment dominated by depleting TAMs followed by activation of antitumor immune responses by supplying engineered macrophages.

Reprogramming macrophages from M2 to M1 polarization can be achieved through various means, such as by using IL-12, CD40 agonists, or CSF-1R inhibitors (209–211). In addition, "don't eat me" molecules, such as CD47 and MHC-I, on tumor cells inhibit the phagocytic function of macrophages by binding SIRPα or LILRB1, respectively, on macrophages (64, 212). Therefore, interference with these "don't eat me" molecules may further enhance phagocytosis by engineered macrophages. These methods could further promote the anticancer activities of engineered macrophages. Finally, if needed, methods of TAM depletion *in vivo* can serve as a safeguard to remove engineered macrophages that have serious side effects.

AUTHOR CONTRIBUTIONS

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Grabbing the Bull by Both Horns: Bovine Ultralong CDR-H3 Paratopes Enable Engineering of 'Almost Natural' Common Light Chain Bispecific Antibodies Suitable For Effector Cell Redirection

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A subset of antibodies found in cattle comprises ultralong CDR-H3 regions of up to 70 amino acids. Interestingly, this type of immunoglobulin usually pairs with the single germline VL gene, V30 that is typically very conserved in sequence. In this work, we have engineered ultralong CDR-H3 common light chain bispecific antibodies targeting Epidermal Growth Factor Receptor (EGFR) on tumor cells as well as Natural Cytotoxicity Receptor NKp30 on Natural Killer (NK) cells. Antigen-specific common light chain antibodies were isolated by yeast surface display by means of pairing CDR-H3 diversities following immunization with a single V30 light chain. After selection, EGFR-targeting paratopes as well as NKp30-specific binders were combined into common light chain bispecific antibodies by exploiting the strand-exchange engineered domain (SEED) technology for heavy chain heterodimerization. Biochemical characterization of resulting bispecifics revealed highly specific binding to the respective antigens as well as simultaneous binding to both targets. Most importantly, engineered cattle-derived bispecific common light chain molecules elicited potent NK cell redirection and consequently tumor cell lysis of EGFR-overexpressing cells as well as robust release of proinflammatory cytokine interferon-γ. Taken together, this data is giving clear evidence that bovine bispecific ultralong CDR-H3 common light chain antibodies are versatile for biotechnological applications.

Keywords: bovine ultralong CDR-H3 antibodies, bispecific antibodies, effector cell redirection, NK cell engagers, common light chain, antibody engineering, yeast surface display

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INTRODUCTION

The human body is continuously exposed to potentially life-threatening opponents such as bacteria, viruses or cancerous cells. In order to assert oneself, antibodies (Abs) play a fundamental role in host defense by recognizing foreign antigen in an adaptive fashion. The high specificity for a given antigen in conjunction with humoral and cellular effector functions mediated by the Fc-part of IgG isotypes renders this class of Abs as very promising molecules for therapy (1, 2). This is exemplified by the fact that as of 2021 around 100 therapeutic antibody derivatives have been granted marketing approval by the FDA (3). However, one obvious obstacle of monoclonal antibodies for therapeutic purposes results from their monospecific nature since diseases are typically multifaceted e.g. with respect to their origin or disease mediators (4, 5). Consequently, tremendous efforts were made within the last decades to engineer antibodies for bi- and multispecificity (6), culminating in the approval of four bispecific entities until now (7, 8) - including Catumaxomab that has been withdrawn in 2017 (9). Moreover, a steady incline in investigational bispecifics that are entering clinical development on a yearly basis can be observed (9, 10). Most of the molecules that are currently investigated in clinical trials are so called asymmetric formats (9). This type of bispecific antibody (bsAb) resembles the IgG-like architecture of conventional monoclonal antibodies as closely as possible. Here, each Fab arm targets a different antigen in a monovalent manner. Consequently, two different heavy chains as well as two separate light chains need to be expressed and even more importantly, assembled precisely to represent a functional bsAb. To facilitate heavy chain heterodimerization as well as specific heavy and light chain pairing, several different technologies have been developed (11). In this respect, the issue of accurate heavy and light chain assembly can be obviated by engineering common light chain bsAbs i.e. bispecifics where both Fabs share the identical light chain (12–14). Besides, an unprecedented multitude of different bsAb formats has been engineered (15), including bi- and multispecifics derived from camelids (16–18) or sharks (19–21).

A fraction of about 10% of the immunoglobulin repertoire found in cattle produces antibodies with exceptionally long CDR-H3 regions of up to 70 amino acids (22). Typically, the vast majority of clones harboring ultralong paratopes adopts a characteristic structure that can be divided into a stalk region composed of an ascending as well as a descending β-strand and a disulfide-rich globular architecture referred to as knob (**Figure 1**) (23). Usually, one distinct V gene segment, IGHV1-7 is utilized for the construction of bovine ultralong CDR-H3 antibodies as well as one particular germline D segment, IGHD8-2, encoding for the stalk-knob structure. IGHD8-2 is diversified in a process involving cytidine deaminase with a strong bias towards the introduction of cysteine residues causing extraordinary structural diversity through the formation of different disulfide bond patterns predominantly in the knob region (22, 24). Consequently, it is the knob region that plays a pivotal role for antigen binding, whereas the stalk as well as the VH scaffold seem to have a stabilizing function (22, 23). Intriguingly, ultralong CDR-H3 heavy chains typically pair with a single VL gene, VL30 that generally is relatively sequence conserved (25). In this respect, several of the published crystal structures of ultralong CDR-H3 antibodies share a CDR-identical light chain (24, 26, 27). Hence, these molecules comprise an almost natural source of common light chain antibodies.

In this work, we have engineered EGFR and NKp30 targeting cattle-derived bispecific common light chain antibodies that can be utilized to efficiently redirect NK cells in order to kill EGFR-overexpressing tumor cells. EGFR is a receptor tyrosine kinase overexpressed in an array of different tumors (28–30). We have recently described the generation of a platform process for isolating ultralong CDR-H3 antibodies targeting EGFR by combining cattle immunization with yeast surface display (31). To this end, bovine ultralong CDR-H3 regions were PCR-amplified and grafted onto a fixed IGHV1-7 scaffold. Subsequently, this CDR-H3-only diversity was combined with a single VL30 light chain enabling the facile isolation of EGFR-specific antibodies. In this work, we have isolated multiple NKp30-specific ultralong CDR-H3 antibodies by exploiting the same platform process involving the identical VL30 light chain. NKp30 is an activating NK cell receptor that can be addressed in a bispecific fashion to efficiently trigger NK-cell mediated target cell lysis (32–34). Following isolation of NKp30-specific paratopes by yeast surface display (35, 36), NKp30-addressing clones as well as EGFR targeting variants both sharing the identical light chain were combined into common light chain bispecifics by employing the strand-exchange engineered domain (SEED) technology for heavy chain heterodimerization (**Figure 1**) (37). The vast majority of resulting common light chain bsAbs displayed favorable biophysical properties as well as simultaneous binding to both antigens in the nanomolar range. Most importantly, generated IgG-like bsAbs facilitated significant NK cell-mediated lysis of EGFR-overexpressing A431 tumor cells as well as a robust release of proinflammatory cytokine interferon-γ (IFN-γ). Taken together, our data demonstrates that cattle-derived bispecific common light chain ultralong CDR-H3 antibodies can be readily engineered that seem to be versatile for biomedical applications such as effector cell redirection.

MATERIAL AND METHODS

Immunization

As previously described, three cattle (*Bos taurus*) with approximately one year of age were immunized using recombinant human NKp30 extracellular domain comprising a *C*-terminal hexahistidine tag (ECD; produced in-house) in a cocktail approach with recombinant *C*-terminal his-tagged EGFR ECD (produced in-house) at preclinics GmbH, Germany (31). Animal care and invasive procedures were in accordance with local animal welfare protection laws and regulation (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Dezernat 33 – Tierschutzdienst. Number: 33.19-42502-05-17A210). Six immunizations were performed in total over the period of 84 days (d0, d28, d42, d56, d70, d84). To this end, 200 μg of NKp30 (in a volume of 2 ml) were mixed with 2 ml Fama adjuvant (GERBU Biotechnik) and injected subcutaneously at multiple sites. After immunization (day 88) 250 ml

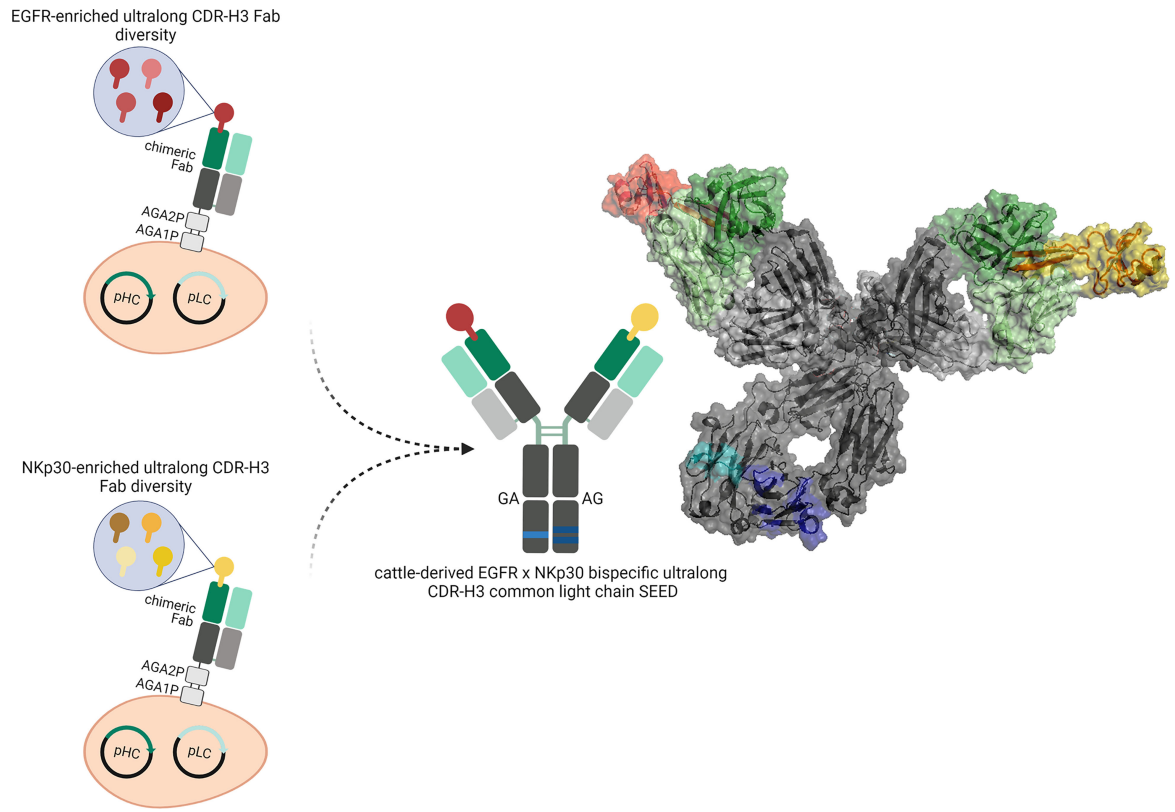

FIGURE 1 | Overview about the generation of cattle-derived ultralong CDR-H3 common light chain bispecific antibodies. After immunization of cattle and library generation antigen-specific paratopes are enriched against both targets (shown in red color and yellow color). To this end, ultralong CDR-H3 regions encoding for stalk/knob architectures are specifically amplified and grafted onto a fixed chimeric Fab scaffold utilizing a single light chain. After selection, common light chain paratopes are reformatted into an IgG-like bispecific format exploiting a heavy chain heterodimerization technique (e.g. the SEED technology). Schemes generated using biorender (www.biorender.com). Model constructed with PYMOL v0.99 based on pdb entries 5dk3 and 5ilt. Individual paratopes based on stalk/knob structures are colored in red and yellow, respectively. Fixed VH region based on IGHV1-7 shown in dark green, utilized VL30 exploited as common light chain shown in light green. Constant regions of the heavy chains colored in dark grey, CLλ shown in light grey. Use of heavy chain heterodimerization technology resulting in two distinct heavy chains indicated by the use of dark blue and light blue segments.

of blood per specimen was collected followed by RNA extraction and cDNA synthesis.

Yeast Surface Display Library Generation

The library construction process involving strains, reagents as well as plasmids has been described in detail elsewhere (31). In brief, *S. cerevisiae* strain EBY100 *MATa (URA3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL (pIU211:URA3)*) was utilized for the generation of the heavy chain diversity, whereas BJ5464 cells (*MATα URA3-52 trp1 leu2Δ1his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*) harboring the light chain plasmid (pLC) encoding for a specific VLλ30 (**Supplementary Figure S1**) was exploited. Primer sets for specific ultralong CDR-H3 amplification are given in **Supplementary Table 1**. For PCR-based amplification 1 µl of cDNA pooled from all three specimen was used in a final volume of 50 µl as well as Q5 High-Fidelity 2x Master Mix (New England Biolabs; NEB). Conditions were as followed: 98°C for 3 min, 35 cycles of 30 s at 98°C and 50 s at 72°C, followed by 2 min at 72°C. PCR products were purified by Wizard® SV Gel and PCR Clean-up System (Promega). Gap repair cloning was employed for library construction according to Benatuil and co-workers (38). Therefore, 12 µg CDR-H3 PCR product as well as 3.5 µg *Not*I and *Eco*RI (both New England Biolabs) digested heavy chain destination plasmid (pHC) were used per electroporation reaction. The resulting library size was roughly estimated by dilution plating on SD-Trp agar plates. In order to accomplish Fab display, EBY100 cells comprising the heavy chain diversity as well as BJ5464 cells harboring the single light chain were combined by yeast mating (39, 40).

Selection of NKp30-Targeting Ultralong CDR-H3 Fabs

Library sorting was facilitated by growing diploid library cells overnight in SD-Trp-Leu medium at 30°C and 120 rpm agitation. Subsequently, library cells were transferred to SG-Trp-Leu medium supplemented with 10% (w/v) polyethylene glycol 8000 at an OD_{600} of 1.0 and incubated for 2 days at 20°C and 120 rpm. Afterwards, cells were washed twice with PBS (Sigma Aldrich) and incubated

with *C*-terminally hexahistidine tagged recombinant human NKp30 ECD (produced in-house or Abcam) at a concentration of 1 µM for 30 min on ice. Cells were washed thrice, followed by simultaneous detection of functional Fab display and antigen binding. To this end, cells were labeled with light chain specific goat F(ab')2 anti-human lambda R-phycoerythrin (R-PE) (SouthernBiotech, diluted 1:20) as well as Penta-His Alexa Fluor 647 Conjugate antibody (Qiagen, diluted 1:20) for sorting round one or SureLight® APC Anti-6X His tag® antibody (abcam, diluted 1:20) for sorting round two. Eventually, library cells were washed thrice with PBS and selected by fluorescence-activated cell sorting (FACS) on a BD FACSAria™ Fusion cell sorter (BD Biosciences). In the first round of selection, a total number of approx. 5 x 10^8 cells were sorted to ensure adequate coverage of the library. For the second round of library sorting about 5 x 10^7 cells were exploited.

SEEDbody Expression and Purification

Monovalent SEED antibody derivatives of NKp30-targeting cattle-derived ultralong CDR-H3 paratopes as well as bispecific common light chain SEEDbodies were designed in-house, synthesized and subcloned into pTT5 vector backbone by GeneArt (Thermo Fisher Scientific). Therefore, NKp30-specific VH regions were placed onto the AG chain of the SEEDbody encoding for human constant regions. EGFR-targeting VH domains were grafted onto the GA chain, also encoding for human constant regions. In both heavy chains we implemented amino acid mutations L234A, L235A, P329G to abolish Fc-mediated immune effector functions (41). The bovine VLλ30 region was fused to human CLλ. For monovalent (one-armed) SEEDbody expression of NKp30-addressing cattle derived entities, respective AG chain plasmids were co-transfected with the light chain plasmid as well as a paratope-less GA chain plasmid (i.e. the GA chain starting from the hinge region) in a 2:1:1 (AG:GA:LC) ratio. For bsAb expression, AG chain plasmids encoding for NKp30 paratopes were combined with GA chain plasmids encoding for EGFR-specific cattle-derived common light chain paratopes as well as with the light chain plasmid in a 2:1:1 (AG:GA:LC) ratio. In general, 25 ml Expi293™ cells were transfected with the respective expression vector mixtures according to the manufacturer's recommendations and protocols (Thermo Fisher Scientific). Supernatants were collected after five days and purified using MabSelect chromatography resin (GE healthcare). Subsequently, buffer was exchanged to PBS pH 6.8 *via* Pur-A-Lyzer™ Maxi 3500 Dialysis Kit (Sigma Aldrich/Merck KGaA) for 24 h at 4°C. Optionally, in case of low yields, a concentration step was executed using Amicon Ultra-4 Centrifugal Filters (MW cutoff 10 kDa, EMD Millipore). Protein concentrations were determined on the QIAexpert system (Qiagen). Analytical size exclusion chromatography was exploited to determine aggregation propensities using a TSKgel SuperSW3000 column (4.6 × 300 mm, Tosoh Bioscience LLC) in an Agilent HPLC system with a flow rate of 0.35 ml/min.

Biolayer Interferometry

All BLI measurements were performed on the Octet RED96 instrument (ForteBio, Pall Life Science) at 25°C and 1000 rpm, agitation. To assess binding as well as for kinetic measurements, cattle derived bsAbs were loaded onto anti-human Fc (AHC) sensors at a concentration of 5 µg/ml (in PBS) for 3 min followed by 60 s of sensor rinsing using kinetics buffer (KB; PBS + 0.1% (v/v) Tween-20 + 1% (w/v) BSA). Subsequently, association to the respective antigen was measured at varying concentrations (100 nM, 50 nM, 25 nM and 12.5 nM for EGFR and depending on the bsAb at 100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM and 3.125 nM for NKp30) for 300 s followed by dissociation in KB for 300 s. For analyzing simultaneous binding on the protein level, bsAbs were loaded onto AHC sensors at a concentration of 5 µg/ml (in PBS) for 3 min followed by 60 s of sensor rinsing in KB. Afterwards, a first association step was performed using 100 nM NKp30 (Abcam) for 200 s followed by a second association in EGFR at 100 nM for 200 s. To perform competition assays with B7-H6, bsAbs were loaded onto AHC sensors at a concentration of 5 µg/ml (in PBS) for 3 min followed by 60 s of sensor rinsing in KB. A first association was performed using NKp30 at 100 nM for 100 s followed either by 100 s in KB or 100 s in 1000 nM B7-H6 ECD. Data was fitted (1:1 binding model) and analyzed using ForteBio data analysis software 8.0 as well as Savitzky-Golay filtering.

Flow Cytometry

Cellular binding was assessed on a Sartorius iQue3 flow Q1 cytometer and the IntelliCyt ForeCyt software was used for analysis. For each experiment, 800-1800 cells per well were measured. To this end, 10^5 cells/well were seeded and incubated for 1 h on ice with bsAbs at 100 nM in PBS supplemented with 1% (w/v) BSA after two initial washing steps with PBS+1 % (w/v) BSA. Following antibody incubation, two additional washing steps with PBS+1% (w/v) BSA were performed with subsequent Alexa Fluor® 488 AffiniPure Fab Fragment Goat Anti-Human IgG (Fc specific) (Jackson ImmunoResearch) detection antibody staining (200 nM) at 4°C for another 30 min. After two washing steps with PBS+1% (w/v) BSA, 20 µg/ml propidium iodide (Invitrogen) was used to label dead cells in a total volume of 100 µl/well. Controls were included, e.g. anti-HEL IgG, cells without antibody incubation as well as cells labeled with the detection reagent only. For the detection of simultaneous binding, A431 cells were seeded and labeled equivalently with bsAbs. Following bsAb incubation at a concentration of 100 nM and two washing steps, his tagged NKp30 (ECD, Acro Biosystems) was added at 200 nM for 30 min. After two additional washing steps, cells were incubated with 400 nM of detection antibody (Penta His Alexa Fluor® 488 Conjugate (Qiagen)) for 30 min and 20 µg/ml propidium iodide (Invitrogen). Controls were included, e.g. anti-HEL IgG, cells without antibody incubation, cells labeled with the detection reagent only as well as cells treated with a bispecific and detection antibody, but not with NKp30.

Killing Assay

The killing assay has been described in detail elsewhere (18). In brief, PBMCs were isolated from blood of healthy donors by density gradient centrifugation. NK cells were enriched using the EasySep™ Human NK Cell Isolation Kit (Stemcell Technologies). After overnight incubation in complete medium using low dose recombinant human IL-2 (100 U/ml, R&D systems), cells were adjusted to 0.625 x 10^6 vc/ml. EGFR positive A431 cells or EGFR negative ExpiCHO™ cells were

stained with CellTracker™ Deep Red Dye (ThermoFisher). Target cells were seeded into 384-well clear bottom microtiter plates (Greiner Bio-One) at 2500 cells/well in 20 µl volume and incubated for 3 h. Afterwards, NK cells were added at different E:T ratios (i.e. 1:1, 5:1, 10:1 and 20:1). BsAbs were added at concentrations as indicated. An EGFR targeting Fc immune effector silenced antibody derivative was utilized as negative control. SYTOX™ Green Dead Cell Stain (Invitrogen, 0.03 µM) was dispensed to the assay followed by plate incubation and on-line measurement for 24 h in the Incucyte® system. Lysis was normalized to maximum lysis triggered by therapeutic antibody cetuximab or to target cells cultivated with 30 µM staurosporine (Merck Millipore). Overlay signals allowed for analysis of dead target cells only.

Cytokine Release Assay

The EasySep™ Human NK Cell Isolation Kit (Stemcell Technologies) was employed to isolate NK cells derived from PBMCs of healthy human donors. Cell were incubated overnight in complete medium supplemented with 100 U/ml recombinant human interleukin-2 (R&D Systems). Subsequently, 2.500 A431 cells were seeded in 384 well plates. After 3 h of incubation, NK cells were added at an E:T ration of 5:1 followed by the addition of cattle-derived bsAbs at a final concentration of 50 nM. An EGFR targeting Fc immune effector silenced antibody derivative was utilized as negative control. After 24 h incubation supernatants were collected and analyzed utilizing the human IFN-γ HTRF kit (Cisbio) by following the manufacturer's instructions. Plates were measured with PHERAstar FSX (BMG Labtech) and data were analyzed by MARS software (v.3.32, BMG) enabling a 4-parameter logistic (4PL $1/y^2$) model fitting of the standard curve.

RESULTS

Isolation of Chimeric NKp30-Targeting Ultralong CDR-H3 Fab Fragments

We have previously described the generation of a platform process for the isolation of ultralong CDR-H3 antibodies by combining cattle immunization and yeast surface display (31). The same strategy involving the same library was applied in this study for the isolation of NKp30-specific antibodies. In brief, as already described earlier (31) we specifically amplified ultralong CDR-H3 regions from cDNA obtained from the peripheral blood mononuclear cell (PBMC) repertoire of cattle that were immunized with recombinant human NKp30 ECD. Subsequently, a heavy chain library was constructed by grafting the amplified CDR-H3 diversity onto a fixed bovine IGHV1-7 scaffold fused to human domain CH1 and AGA2P by gap repair cloning into *S. cerevisiae* strain EBY100. The resulting library with approximately 5 x 10^7 unique clones was then combined by yeast mating with BJ5464 cells harboring a single light chain plasmid encoding for a bovine V30 paratope fused to a human CLλ region (39, 40). Afterwards, the resulting diploid yeast cell Fab library was screened by fluorescence activated cell sorting (FACS) to isolate ultralong CDR-H3 common light chain paratopes specific to NKp30. To this end, a two-dimensional labeling strategy was applied to simultaneously select for full-length Fab display in addition to NKp30 binding (**Figure 2A**). Using an antigen concentration of 1 µM for selection, we were able to enrich for a NKp30-targeting population within two rounds of FACS. Sequencing of 192 clones of the sorting output revealed the isolation of 17 unique CDR-H3 paratopes on the protein level with a length ranging from 56 to 66 residues and an even number of four to eight Cys residues within that region (**Figure 2B**). Since the isolated paratopes have to be functional in a strictly monovalent fashion when reformatted into common light chain bsAbs, we initially produced all 17 cattle-derived Abs in a one-armed SEED format (**Supplementary Figure 2**). For this, we exploited the SEED technology which relies on beta-strand exchanges of IgG and IgA CH3 constant domains, preferably resulting in heavy chain heterodimerization (37). The bovine x human chimeric ultralong CDR-H3 Fab fragments were genetically fused to the AG chain of the SEED molecule, while for generating monovalent versions the GA Fc chain was expressed without paratope. For all the molecules in this study we introduced amino acid exchanges L234A, L235A, P329G into both heavy chains (41) to abolish Fc-mediated immune effector functions. After expression and protein A purification we analyzed binding to recombinant human NKp30 ECD in a biolayer interferometry (BLI) experiment using an antigen concentration of 100 nM (data not shown). This revealed a total number of 13 NKp30-specific monovalent bovine x human ultralong CDR-H3 antibody derivatives (unfunctional: 63E04, 63C05, 63F02 and 63H12).

Generation, Biophysical, and Biochemical Characterization of Bispecific Bovine x Human Chimeric Ultralong CDR-H3 Common Light Chain Antibodies Targeting NKp30 and EGFR

All 13 remaining NKp30-specific ultralong CDR-H3 paratopes were subsequently combined with two EGFR-targeting ultralong CDR-H3 antibodies (60F06 and 60H05) we have previously generated (**Supplementary Figure 3**). Notably, both EGFR specific entities represent unique clonotypes, based on sequence similarity of CDR-H3, allowing for a more thorough characterization in terms of biophysical, biochemical as well as functional properties of generated ultralong common light chain bispecifics. For bsAb production, all 13 NKp30 targeting paratopes were expressed on the SEED AG chain, while EGFR-specific clones were genetically fused to the GA chain of the SEED. In addition to both Fc-effector silenced heavy chains, the same chimeric light chain based on V30 exploited for antibody discovery was utilized for common light chain bsAb expression (**Figure 1** and **Supplementary Figure 1**). Following production and single step purification by protein A, all 26 bispecifics were scrutinized in terms of expression yields as well as target monomer species by analytical size exclusion chromatography (SEC), as shown in **Table 1**. Except for two molecules, expression yields post protein A purification were in the double digit milligram per liter scale, which can be considered as acceptable for transient protein production, especially given the high complexity of this kind of bsAb. Of note, tranfections were

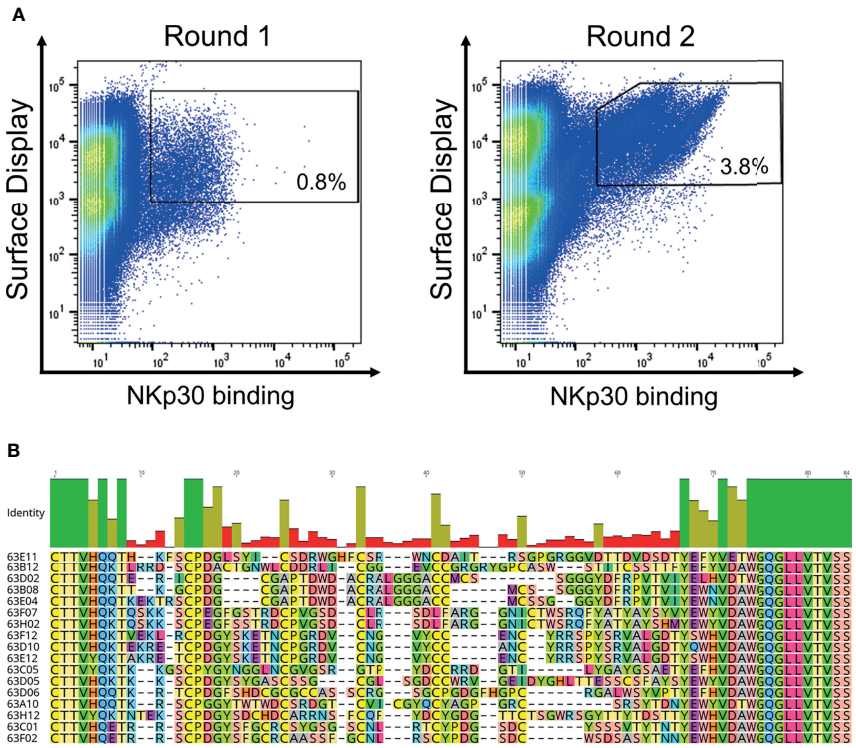

FIGURE 2 | Yeast surface display based selection of NKp30 targeting chimeric bovine x human Fab fragments by yeast surface display as well as sequence analysis after enrichment. **(A)** Within two sorting rounds a NKp30-binding population was enriched. A two-dimensional sorting strategy was applied to label for functional Fab assembly as well as for NKp30 binding. To this end, library cells were incubated with recombinant human his-tagged NKp30 at a concentration of 1 μM followed by staining using secondary detection reagents directed against the his-tag as well as against the constant region of the human lambda chain. **(B)** CDR-H3 alignment of sequence unique ultralong CDR-H3 paratopes obtained after library sorting. Sequence of IGHJ2-4 is also shown. Amino acids given in 1-letter code and in different colors. Alignment generated with Geneious Prime® v2021.1.1.

performed in a 2:1:1 ratio (i.e. AG plasmid: GA plasmid: light chain plasmid). By modifying plasmid ratios for transfection, expression yields might be further optimized. Interestingly, there was a clear trend for higher yields for all bispecific molecules based on EGFR-targeting paratope 60H05 in direct comparison to 60F06. This is highlighting the impact of individual paratopes on protein production. Aggregation properties were determined by analytical SEC and unveiled more than 90% target peak for 24 out of 26 common light chain bispecifics (**Table 1** and **Supplementary Figures 4, 5**). Only 63D06x60H05 with 88.8% target peak and 63H02x60H05 with 88.9% main peak were slightly below this threshold, indicating rather favorable biophysical properties of the herein engineered cattle-derived bsAbs.

Subsequently, we determined binding kinetics to both antigens, EGFR and NKp30, as exemplarily shown for bsAb 63D02x60F06 and 63H02x60F06 in **Figures 3A, B** (**Table 1**, **Figure 3** and **Supplementary Figures 6, 7**). In accordance with affinity measurements conducted previously (31), all bsAbs incorporating EGFR-directed paratope 60H05 specifically bound to recombinant human EGFR ECD in the lower double digit nanomolar range, whereas bsAbs harboring EGFR-specific ultralong CDR-H3 common light chain paratope 60F06 displayed affinities for EGFR in the single digit to lower double digit nanomolar range. Affinities for NKp30 ranged from picomolar to double digit nanomolar binding demonstrating a wide range of affinities of isolated NKp30-binding ultralong CDR-H3 common light chain paratopes. Only minor to moderate differences in kinetics were observed for identical NKp30 binding sites when reformatted with the two different EGFR-addressing paratopes. This is giving some evidence that main binding characteristics remain largely unaffected when individual common light chain ultralong CDR-H3 paratopes are incorporated into different bsAbs. Moreover, all cattle-derived chimeric bsAbs were capable of simultaneously binding to both, EGFR as well as NKp30 recombinant human ECDs (**Figure 3C** and **Supplementary Figures 6, 7**).

Bispecific Bovine x Human Chimeric Ultralong CDR-H3 Common Light Chain Antibodies Targeting NKp30 and EGFR Elicit Significant NK-Cell Mediated Lysis of EGFR-Overexpressing Tumor Cells as Well as Robust Proinflammatory Cytokine Release

We also set out to scrutinize whether generated cattle-derived chimeric ultralong common light chain bsAbs could trigger

TABLE 1 | Biophysical and biochemical characterization of cattle-derived ultralong CDR-H3 common light chain bispecific antibodies.

Bispecific molecule	Yield [mg/L]	SEC [%]	KD EGFR [M]	kon EGFR [1/Ms]	koff EGFR [1/s]	KD NKp30 [M]	kon NKp30 [1/Ms]	koff NKp30 [1/s]
63A10x60F06	14,6	97,9	7,4E-09	9,0E+04	6,6E-04	2,0E-08	6,0E+05	1,2E-02
63B08x60F06	11,3	96,3	2,0E-08	6,9E+04	1,4E-03	3,4E-09	1,3E+06	4,3E-03
63B12x60F06	14,5	98,6	1,5E-08	7,1E+04	1,1E-03	2,0E-09	1,4E+06	2,7E-03
63C01x60F06	14,9	96,9	1,4E-08	7,3E+04	1,0E-03	8,5E-08	8,3E+05	7,0E-02
63D02x60F06	14,7	98,5	1,4E-08	6,0E+04	8,4E-04	4,7E-09	1,5E+06	7,1E-03
63D05x60F06	19,2	96,8	6,5E-09	7,9E+04	5,1E-04	9,5E-10	1,9E+06	1,9E-03
63D06x60F06	7,9	94,4	1,0E-08	8,3E+04	8,3E-03	1,1E-08	3,6E+05	4,1E-03
63D10x60F06	9,2	97,8	1,7E-08	7,0E+04	1,2E-03	8,9E-08	4,8E+05	4,2E-02
63E11x60F06	46,1	97,3	1,3E-08	7,9E+04	1,0E-03	1,4E-09	1,9E+05	2,7E-04
63E12x60F06	26,4	100	1,0E-08	7,0E+04	7,3E-04	9,6E-08	6,7E+05	6,5E-02
63F07x60F06	40,0	99,6	9,0E-09	7,1E+04	6,3E-04	8,2E-10	1,3E+06	1,1E-03
63F12x60F06	29,0	99,2	1,0E-08	7,8E+04	7,9E-04	2,3E-08	8,0E+05	1,9E-02
63H02x60F06	10,1	94,5	1,1E-08	8,4E+04	9,5E-04	1,2E-09	1,2E+06	1,4E-03
63A10x60H05	27,0	93,8	2,0E-08	9,1E+04	1,9E-03	1,4E-08	3,0E+05	4,1E-03
63B08x60H05	16,2	93,7	2,3E-08	1,4E+05	3,2E-03	5,4E-09	1,1E+06	6,0E-03
63B12x60H05	16,4	94,4	2,2E-08	7,8E+04	1,7E-03	2,4E-09	1,2E+06	2,8E-03
63C01x60H05	34,4	93,8	1,9E-08	1,0E+05	2,0E-03	6,3E-08	8,2E+05	5,2E-02
63D02x60H05	15,3	95,8	1,9E-08	9,0E+04	1,7E-03	1,6E-09	1,6E+06	2,5E-03
63D05x60H05	30,1	93,8	2,1E-08	9,2E+04	1,9E-03	7,8E-10	1,5E+06	1,2E-03
63D06x60H05	15,1	88,8	1,8E-08	9,6E+04	1,7E-03	1,7E-08	2,7E+05	4,6E-03
63D10x60H05	37,7	92,0	1,9E-08	9,2E+04	1,8E-03	4,4E-08	5,5E+05	2,5E-02
63E11x60H05	63,9	91,9	2,0E-08	1,1E+05	2,1E-03	3,1E-09	2,3E+05	6,9E-04
63E12x60H05	47,2	96,2	1,5E-08	9,7E+04	1,4E-03	6,9E-08	9,0E+05	6,2E-02
63F07x60H05	61,6	99,5	2,2E-08	1,2E+05	2,6E-03	1,3E-09	1,3E+06	1,6E-03
63F12x60H05	44,4	98,0	1,1E-08	1,0E+05	1,1E-03	6,1E-08	4,9E+05	3,0E-02
63H02x60H05	19,1	88,9	2,8E-08	6,7E+04	1,9E-03	2,5E-09	1,1E+06	2,7E-03

efficient NK cell redirection, resulting in killing of EGFR-overexpressing tumor cells. To this end, we ranked all 26 engineered common light chain bispecifics in a killing assay exploiting EGFR-positive cell line A431 as well as peripheral blood mononuclear cell (PBMC)-isolated NK cells of three healthy donors (**Figure 4A**). All molecules were assessed for their killing capacities at a concentration of 50 nM. This revealed that besides one NKp30-targeting paratope that was unfunctional in combination with both EGFR-directed antigen binding sites (similar to an EGFR-targeting Fc-silenced negative control), 12 out of 13 NKp30-directed binders triggered tumor cell lysis in conjunction with both EGFR-paratopes to some extent (**Figure 4A**). Amongst those, seven NKp30-directed paratopes (63B08, 63B12, 63D02, 63D05, 63E11, 63F07 and 63H02) were robust in eliciting

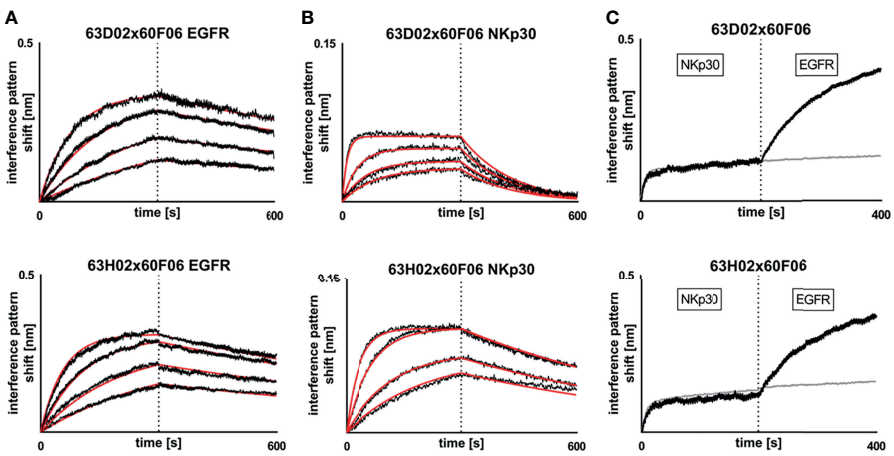

FIGURE 3 | Biochemical characterization of chimeric ultralong CDR-H3 common light chain bispecific antibodies *via* Biolayer interferometry. Kinetic measurements against recombinant human EGFR extracellular protein **(A)** or recombinant human NKp30 ECD **(B)**. Bispecific entities 63D02x60F06 (top) or 63H02x60F06 (bottom) were loaded onto sensor tips. After sensor rinsing, antigen binding was conducted at different concentrations (100 nM, 50 nM, 25 nM and 12.5 nM for EGFR and 50 nM (25 nM for 63H02), 12.5 nM, 6.25 nM and 3.125 nM for NKp30) for 300 s, followed by a dissociation step in kinetics buffer for 300 s. **(C)** Simultaneous binding of 63D02x60F06 (top) or 63H02x60F06 (bottom) bispecifics against NKp30 ECD and EGFR ECD. Bispecifics were loaded to the sensor tips. After sensor rinsing two consecutive association steps were performed at 100 nM (Nkp30) and 100 nM (EGFR) for 200 s each.

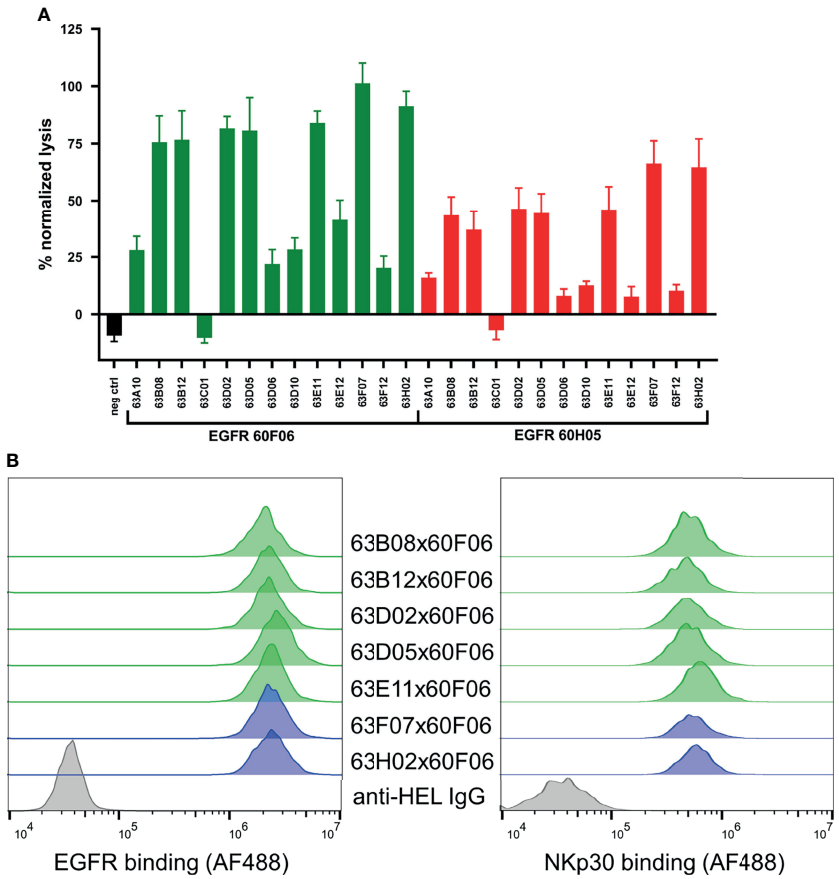

FIGURE 4 | Killing capacities of 26 generated cattle derived common light chain bispecifics **(A)** and cellular binding (**B**, left) as well as simultaneous binding to A431 and NKp30 ECD (**B**, right). Fluorescence-microscopy based killing assay using EGFR-positive A431 target cells and PBMC-purified NK effector cells at an E:T ratio of 5:1 **(A)** as well as cattle derived bsAbs at a concentration of 50 nM. A monospecific EGFR targeting Fc effector silenced negative control was included (black). Individual bsAbs based on EGFR targeting paratope 60F06 shown in green and entities based on EGFR-specific binder 60H05 given in red. Data was normalized to allow comparison of the independent experiments. Graphs show normalized means ± SEM of n = 3 different healthy donors. (**B**, left) Cellular binding of selected 60F06 based cattle-derived ultralong CDR-H3 common light chain bsAbs to EGFR expressing A431 cells at 100 nM. B7-H6 competing molecules shown in green, molecules targeting another epitope on NKp30 given in blue. An anti-HEL IgG control was included (grey). Cellular binding properties were detected *via* a fluorophore conjugated anti-human Fc antibody (**B**, right) Simultaneous binding properties of generated bispecifics. A431 cells were incubated with engineered common light chain bispecifics at 100 nM (green: B7-H6 competitors, blue: B7-H6 non competitors) followed by incubation with his-tagged NKp30 ECD at 200 nM. Simultaneous binding was detected *via* a fluorescence-labeled anti-his antibody.

killing of A431 cells. B7-H6, the cell bound ligand of NKp30, is upregulated on tumor cells and absent on most normal cells (42). In this respect, B7-H6 acts as 'danger signal' providing a positive input for NK cell activation *via* the NKp30 axis. To characterize epitope coverage more thoroughly, we investigated whether those seven NKp30 targeting cattle derived binders address a similar region as the natural ligand on NKp30 (**Supplementary Figure 8**). Within this set of bispecifics, five NKp30 paratopes competed with B7-H6 for binding to NKp30, while two NKp30 directed antigen binding sites did not show competition (63F07 and 63H02). Interestingly, when reformatted as bispecific together with EGFR-specific binder 60F06 killing capacities were more pronounced than for 60H05 SEEDbodies, clearly indicating dependencies of cytotoxic synapse formation on the tumor targeting antigen binding site of the bispecific molecule.

To get a more profound understanding on NK cell redirection, we focused on the seven NKp30-targeting cattle-derived paratopes that mediated robust killing in initial assays reformatted as bispecific common light chain SEED with 60F06. As expected, all seven cattle-derived common light chain bsAbs showed specific binding to EGFR-overexpressing tumor cell line A431 with similar mean fluorescence intensities (**Figure 4B**, left). Additionally, we set out to assess simultaneous binding on the cellular level. On PBMC-derived NK cells, NKp30 is only expressed at very low levels with approximately 1000 molecules per cell (34). Due to this, binding to NKp30 is hardly detectable *via* flow cytometry (33). To this end, we exploited an indirect binding assay to detect simultaneous binding. At first, EGFR-positive A431 cells were coated with cattle-derived ultralong CDR-H3 common light chain bsAbs. Subsequently, his-tagged

NKp30 ECD was added and simultaneous binding was monitored *via* application of a his-tag specific fluorophore-coupled detection antibody. This resulted in specific interactions of NKp30 with A431 cells for all seven bispecific molecules (**Figure 4B**, right). Hence, all bispecifics bound simultaneously to cell surface expressed EGFR and the soluble form of trigger receptor NKp30.

Afterwards, those molecules were assessed more meticulously in killing assays using PBMC-isolated NK cells of eight healthy donors in a dose-response curve ranging from 0.005 pM to 500 nM (**Figure 5A** and **Table 2**). All seven common light chain bsAbs triggered significant lysis of EGFR-overexpressing A431 cells in a dose-dependent manner with potencies (EC_{50}killing) in the picomolar range. In this regard, potencies ranged from 219 pM for 63B08x60F06 to 807 pM for 63H02x60F06 and also efficacies were similar among this selected set of cattle-derived ultralong CDR-H3 bispecifics. In this regard, potencies ranged from 219 pM for 63B08x60F06 to 807 pM for 63H02x60F06 and also efficacies were similar among this selected set of cattle-derived ultralong CDR-H3 bispecifics (**Figure 5B**). Additionally, for none of those molecules we observed significant NK cell mediated killing of EGFR-negative CHO cells (**Supplementary Figure 9**).

Furthermore, cytotoxic capacities of engineered ultralong CDR-H3 common light chain bsAbs were scrutinized at varying effector cell (i.e. NK cell) to target cell ratios (**Figure 5C**). In line with their natural ability to spontaneously lyse tumor cells (43, 44), basal killing activities, i.e. killing without bsAb redirection of NK cells were significantly amplified by increasing E:T ratios. For all assessed constructs, NK cells efficiently triggered lysis of EGFR-overexpressing cells even at low ratios. Efficacies in the presence of bsAbs were substantially higher compared to NK cell mediated tumor cell lysis alone throughout all different settings. In this respect, nearly half-maximal lysis was already observed at a 1:1 E:T ratio under saturating conditions. At a 5:1 E:T ratio maximal overall lysis was almost achieved and higher ratios only minorly affected efficacies. Most importantly, the different generated bispecifics did not behave appreciably different under these variable conditions. Next, we analyzed redirection capabilities of the different bsAbs on the individual donor level to get a glimpse on donor to donor variation (**Figure 5D**). At an E:T ratio of 5:1, all seven distinct cattle-derived ultralong CDR-H3 bispecifics behaved quite similar with only subtle differences in eliciting lysis of EGFR-overexpressing tumor cells by PBMC-isolated NK cells from

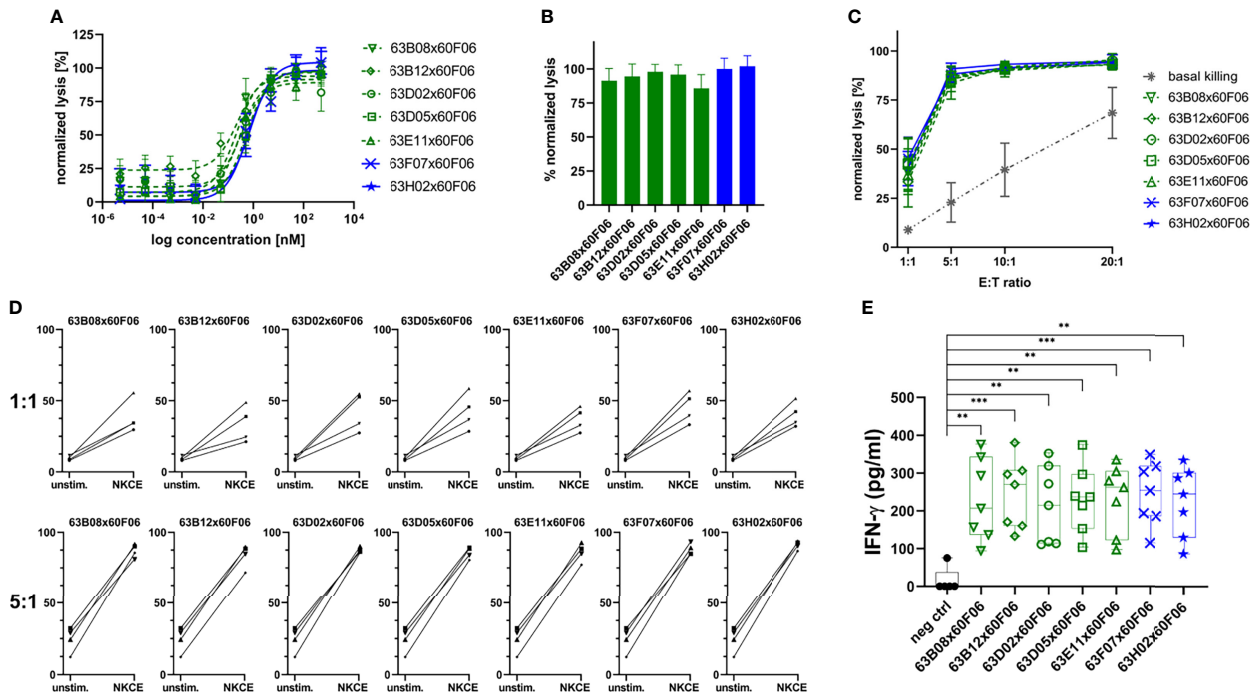

FIGURE 5 | Characterization of selected cattle-derived ultralong CDR-H3 common light chain bsAbs in terms of killing capacities and cytokine release. Fluorescence-microscopy based killing assay using EGFR-positive A431 target cells and PBMC-purified NK effector cells at an E:T ratio of 5:1. Analysis of dose-dependent **(A)** and maximum **(B)** target cell killing. B7H6 competitors shown in green, B7-H6 non competitors given in blue. Data was normalized to allow comparison of the independent experiments. Graphs show normalized means ± SEM of n = 8 different healthy donors. **(C)** Maximum killing capacities at different effector to target (E:T) ratios. Cattle derived common light chain bsAbs were applied at a concentration of 50 nM. Grey: basal killing activities of NK cells i.e. without addition of bsabs. Data was normalized to allow comparison of the independent experiments. Graphs show normalized means ± SEM of n = 3-4 different healthy donors. **(D)** Donor specific lysis capacities at an E:T ratio of 1:1 and 5:1. BsAbs were added at 50 nM (NKCE). Unstim: Basal killing of NK cells in the absence of bsAbs. Data was normalized to allow comparison of the independent experiments. Graphs show normalized means ± SEM of n = 4 different healthy donors. **(E)** NK cell-mediated IFN-γ using cytokine HTRF kits for quantification. Purified NK cells were co-cultured with A431 cells for 24 h at an E:T ratio of 5:1 prior to analysis. Graphs show box and whisker plots as superimposition with dot plots of 7 individual experiments. *** p≤0.001, ** p≤0.01. B7-H6 competing molecules shown in green, molecules targeting another epitope on NKp30 given in blue.

TABLE 2 | NK cell mediated target cell dependent cytotoxic properties and IFN-γ release triggered by selected cattle derived ultralong CDR-H3 common light chain bispecific antibodies.

Bispecific molecule	EC50 killing [pM]	Max killing [%]	Mean IFN-γ release [pg/mL]
63B08x60F06	219	91.4	229.3
63B12x60F06	225	94.5	245.1
63D02x60F06	325	98.0	214.4
63D05x60F06	540	95.9	231.0
63E11x60F06	273	85.8	232.0
63F07x60F06	598	100	245.6
63H02x60F06	807	102	226.1

individual healthy donors. Moreover, variations in donor to donor responses were barely neglectable. Intriguingly, when lowering the E:T ratio to 1:1, differences were quite more profound. While again the different bsAbs behaved overall rather similar, NK cells from individual donors triggered considerably different maximum killing levels under saturating conditions of applied antibodies. In this respect, efficacies varied between approximately 20% lysis to more than 50% killing. Essentially, all seven generated bispecifics were able to efficiently trigger lysis of A431 cells by redirecting NK cells from all individual donors at low E:T ratios.

Finally, we also looked at the targeted release of IFN-γ as an *in vitro* indicator of a potential targeted inflammation of tumors. To this end, A431 cells were incubated with NK cells either in the presence of cattle-derived common light chain bsAbs or in the presence of an EGFR-targeting Fc-silenced control molecule (**Figure 5E**). All ultralong CDR-H3 bispecifics robustly induced effector-type cytokine production of IFN-γ (**Table 2**). In contrast to this, only negligible levels were detected for the control molecule when added to co-cultured A431 and NK cells. Additionally, only a minor release of IFN-γ was detected when the bispecific molecules were added to EGFR-negative CHO cells that were co-cultured with PBMC-derived NK cells (**Supplementary Figure 10**).

DISCUSSION

Bispecific antibodies pave the way for completely novel modes of action and consequently emerged as promising molecules for therapeutic intervention (9, 15). To investigate whether cattle-derived ultralong CDR-H3 paratopes can be efficiently engineered into bispecific antibody formats, we have generated bovine ultralong CDR-H3 paratopes directed against NKp30 that share the same light chain with EGFR-specific ultralong CDR-H3 paratopes we have previously isolated and characterized (31). Fascinatingly, in cattle, ultralong CDR-H3 heavy chains typically pair with a single VL gene, VL30 that is relatively sequence conserved (25). As such, bovine ultralong antibodies can be almost considered as a natural source of common light chain paratopes. In general, it has been shown that this non-classical type of immunoglobulin is predisposed to address epitopes that might only be inefficiently targeted by conventional antibodies. Stanfield and colleagues, for instance, demonstrated that a broadly neutralizing anti-HIV ultralong CDR-H3 paratope hits an epitope on the gp120 CD4 binding site (25) that is typically recessed for conventional antibodies (45). Hence, it is tempting to speculate that ultralong CDR-H3 antibodies might enlarge the 'druggable' target space.

For the isolation of ultralong CDR-H3 entities we have specifically amplified this region from the PBMC-repertoire of immunized cattle and engrafted it onto a fixed chimeric Fab heavy chain that was paired with a single VL30 region we have previously used for the isolation of bovine ultralong EGFR-specific antibodies (31). By exploiting yeast surface display, NKp30-specific antibodies were readily obtained within two rounds of FACS sorting and eventually, after soluble antibody expression, 13 unique clones showed specific binding to this target. Subsequently, those were reformatted as bispecific common light chain antibodies with two different ultralong CDR-H3 paratopes directed against EGFR by employing the same light chain that has been used for YSD. Essentially, the vast majority of the generated 26 molecules showed 'early signs' of favorable biophysical properties as well as simultaneous binding to both antigens on the protein level, a prerequisite for effector cell recruitment. When the same NKp30 targeting cattle derived ultralong CDR-H3 common light chain paratopes were reformatted into bsAbs harboring different EGFR directed paratopes, we observed only minor to moderate differences in affinities. Albeit an impact of the EGFR based ultralong CDR-H3 paratope cannot be entirely excluded, this is indicating that main binding properties of these antigen binding sites remain mostly unaffected when produced as asymmetric common light chain bsAb. Positioning effects resulting in impaired affinities have been described for multiple different bispecific antibody platforms (18, 46–49). It will be interesting to investigate whether such effects can be observed for cattle-derived ultralong CDR-H3 common light chain binders, when reformatted into more complex formats.

In a first killing assay, 24 out of 26 bispecific common light chain derivatives mediated significant NK cell mediated lysis of EGFR-overexpressing A431 cells with one NKp30-targeting paratope remaining unfunctional when combined with both EGFR-directed paratopes. Maximum killing was more pronounced for NKp30-directed clones when reformatted with EGFR-binder 60F06. Interestingly, 60F06 seems to target the same subdomain on the ECD of EGFR as 60H05 but does not compete with it for binding to EGFR (31). Hence, it targets a different epitope. In general, differences in killing capacities of the generated molecules are not unexpected, given the multiple parameters that have a major impact on cytolytic synapse formation of effector cells (including synapse distance as well as the epitopes that are targeted on the tumor associated antigen as well as on the effector trigger molecule) (50). Most importantly, all seven bsAbs that were scrutinized more meticulously in terms of killing abilities elicited robust NK cell

mediated lysis of tumor cells in a targeted fashion with negligible killing of EGFR-negative cells. Potencies were in the picomolar range for all the molecules tested. As such, cytotoxic capacities were similar to those reported for NK cell engagers based on the natural ligand of NKp30 referred to as B7-H6 or affinity optimized versions thereof (32–34). Only at a low E:T ratio of 1:1 we observed differential killing on the single donor level i.e. donor to donor variations. Notwithstanding, significant killing was observed for all the donors tested in this particular setting. Furthermore, at higher E:T ratios all donors behaved quite similar in robustly triggering NK cell mediated killing of tumor cells. Variations in maximum lysis on the donor to donor level have been previously reported for NK cell engagers by Peipp and co-workers (34). For their HER2-specific bifunctional immunoligand harboring B7-H6, differences in efficacies were even observed at higher E:T ratios of 10:1. Finally, all cattle-derived common light chain bispecific compounds significantly triggered the release of IFN-γ in a strictly tumor cell targeted manner. Considering the multiple pleiotropic effects of IFN-γ such as inhibiting suppressive immune cell subsets (51, 52) and NK, NKT and T cell trafficking into tumors through the induction of chemokine production (53), this might be envisioned to result into a targeted inflammation of tumors when applied *in vivo*. In conclusion, our data suggest that cattle-derived ultralong CDR-H3 paratopes enable the facile generation of common light chain bispecifics suitable for effector cell redirection.

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 reviewed and approved by Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (LAVES), Dezernat 33 – Tierschutzdienst. Number: 33.19-42502-05-17A210.

AUTHOR CONTRIBUTIONS

SZ, HK, and LP conceived and designed the experiments. LP, PA, and DK performed experiments. LP, DK, PA, and SZ analyzed the data. SZ and LP wrote the manuscript. SK and BV gave scientific advice. All authors contributed to the article and approved the manuscript.

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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.2021.801368/full#supplementary-material

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CASE REPORT
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Tumor Flare Reaction in a Classic Hodgkin Lymphoma Patient Treated With Brentuximab Vedotin and Tislelizumab: A Case Report

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Background: Tumor flare reaction (TFR) is a clinical syndrome, which is mainly associated with painful and swollen lymph nodes or splenomegaly, slight fever, bone pain, and skin rash during treatment with immune-related drugs, causing difficulty in distinguishing TFR from disease progression. Brentuximab vedotin (BV) and programmed death 1 (PD-1) inhibitor are two ideal drugs used for the treatment of classic Hodgkin lymphoma, but few studies have reported their adverse effects in association with TFR. The efficacy and safety of monotherapy or combination therapy with these drugs needs to be further evaluated. It is essential to determine whether treated patients can develop TFR, thus enabling more accurate diagnosis and treatment.

Case presentation: A 26-year-old female patient, diagnosed with classic Hodgkin lymphoma, had received 2 + 3 cycles of ABVD chemotherapy (a combination of adriamycin, bleomycin, vinblastine, and dacarbazine) and 4 cycles of PD-1 inhibitor (tislelizumab) therapy but exhibited poor efficacy. Subsequently, she was given combination therapy of BV (100 mg) + tislelizumab (200 mg). However, a slight fever, painful and swollen axillary lymph nodes, multiple skin rashes with pruritus, joint pain, and fatigue with poor appetite appeared during the treatment. Ultrasound (US) scans revealed that multiple lymph nodes were significantly enlarged. After treatment with low-dose dexamethasone and cetirizine, the symptoms were alleviated. A biopsy of the left axillary lymph node revealed that lymphoid tissue exhibited proliferative changes, without tumor cell infiltration. These findings were consistent with the clinical and pathological manifestations of TFR.

Conclusion: Combination therapy with BV and PD-1 inhibitor was effective in the treatment of relapsed or refractory classic Hodgkin lymphoma. The results suggest that the combination therapy may cause TFR, and biopsy and also continuous imaging observation are important to determine the disease stage. This approach allows clinicians to decide whether to continue the current treatment plan, and alerts them to the occurrence of excessive activation of the immune system.

Keywords: brentuximab vedotin, tislelizumab, tumor flare reaction, classic Hodgkin lymphoma, immune related adverse event

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INTRODUCTION

Brentuximab vedotin (BV) is a biological agent with an immune function, which is composed of three components, namely, an anti-CD30 monoclonal antibody(cAC10), a potent antimicrotubule agent (monomethyl auristatin E, MMAE), and a dipeptide linker that can be cleaved by proteases in lysosomes (1, 2). In the human body, after binding to CD30 on the cell surface, BV is internalized and the linker is cleaved to release MMAE, exerting its cytotoxic effect. Then, MMAE can inhibit tubulin aggregation, disrupt the intracellular tubulin skeleton and arrest the cell cycle (G2/M phase) inducing the apoptosis of the target cells (3). The induction of apoptosis by the anti-tubulin action of MMAE, and activation of the innate immune system by an antitumor immune reaction that induces immunogenic cell death through endoplasmic reticulum stress are two major molecular mechanisms of BV (4). BV as monotherapy or in combination with chemotherapy has been reported to produce satisfactory outcomes in patients with relapsed or refractory Hodgkin lymphoma (5–8). Common BV-associated adverse effects include neutropenia, thrombocytopenia, peripheral sensory neuropathy, fatigue, rash, fever, constipation, nausea, poor appetite, and infection (1, 3, 4, 9–12).

Programmed death 1 (PD-1) inhibitor, a human or humanized IgG4 monoclonal antibody, is an immune checkpoint inhibitor that has shown promise for the treatment of relapsed or refractory Hodgkin lymphoma (8, 13). However, fatigue, poor appetite, rash, pruritus, diarrhea, nausea and infection are common adverse effects (14). Immune associated adverse effects have also been reported, namely, hypothyroidism, pneumonia, hepatitis, colitis and skin rash (15, 16), with tumor flare reaction (TFR) also recognized as one of complications (17, 18). Tislelizumab is a humanized IgG4 anti-PD-1 monoclonal antibody and its adverse effects are similar to those elicited by nivolumab or pembrolizumab (16).

Targeted therapy has been widely used for the treatment of numerous malignant tumors. CD30 and PD-1 are two ideal therapeutic targets for classic Hodgkin lymphoma, and a number of relevant clinical trials have been carried out (1, 2, 7, 8). The efficacy of combination therapy with the two drugs is still under evaluation, with reported common adverse effects at present being fatigue, nausea, rash, pruritus, vomiting, diarrhea, and infusion-related adverse reactions (4). That a PD-1 inhibitor can cause TFR has been previously reported (18), but there are few reports on TFR associated with BV (1, 19). One was reported in a phase II study of relapsed/refractory systemic anaplastic large-cell lymphoma with BV that 4 patients experienced painful enlargement of lymph nodes and erythema after the administration of BV, without pathological biopsy of the enlarged lymph nodes (1). Another study described 2 patients who experienced TFR to BV after Lenalidomide treatment (19). However, there are no specific reports of TFR caused by BV with PD-1 inhibitor treatment of hematological malignancies. The present study reports a case of TFR in a patient who received combined administration of BV and tislelizumab and explores the possible mechanism and diagnostic significance of TFR caused by immune-related adverse effects of drugs.

CASE PRESENTATION

A 26-year-old female patient was admitted to the First Affiliated Hospital of Zhejiang University School of Medicine (Hangzhou, China) in October 2019 due to persistent pain of the right side of the neck and left side of the axilla lymphadenopathy for more than 10 days. The lymph node on the right side of the neck was biopsied, and the pathology suggested nodular sclerosis classic Hodgkin lymphoma (**Figure 1A**), a subtype of classic Hodgkin lymphoma. Immunohistochemistry (IHC) results of CD30 (+) (**Figure 1B**), CD15 (+), PAX5 (+) (**Figure 1C**), Bcl-2 (+), MUM-1 (+), Bcl-6 (partial +), CD21 (FDC+), Ki-67 (+, 60%), PD-1 (small lymphocytes +, 20%), CD3, CD5, CD7, CD20, CD45, anaplastic lymphoma kinase (ALK), and EMA were all negative. There was no obvious abnormal lymphocyte group in bone marrow smears or after bone marrow immunophenotyping. A bone marrow biopsy revealed that the proliferation of hematopoietic tissue was active. The chromosomal analysis showed 46, XX (20). In October 2019, primary positron emission tomography-computed tomography (PET/CT) showed that there were multiple enlarged lymph nodes in the bilateral neck, bilateral inguen, and left axilla, and that the size of the left axillary lymph node was 3.1 × 2.2 cm (**Figure 2**). The patient was diagnosed as having nodular sclerosis classic Hodgkin lymphoma IIIA, and she subsequently received ABVD chemotherapy (a combination of doxorubicin, bleomycin, vinblastine, and dacarbazine) for 2 cycles. In December 2019, the secondary PET/CT showed that the size of the left axillary lymph node was significantly smaller and that glucose metabolism of FDG was reduced (**Figure 2**). The patient subsequently received 3 cycles of ABVD chemotherapy. In May 2020, a tertiary PET/CT examination showed that the left axillary lymph node was larger and that metabolism was increased (**Figure 2**), following local recurrence after treatment. A biopsy of the left axillary lymph node was undertaken, and the pathology indicated classic Hodgkin lymphoma, nodular sclerosis type (**Figure 1D**). The IHC results of testing of CD30 (+) (**Figure 1E**), CD15 (+), PAX-5 (+ weak) (**Figure 1F**), Bcl-2 (+), MUM1 (+), CD21 (FDc+), Ki-67 (+80%), Bcl-6, CD3, CD5, CD7, CD20, CD45, ALK, EMA, and EBER were all negative. Considering the disease progression of the patient, she received tislelizumab (200 mg Q3W) for 4 cycles. During that period, the left axillary lymph node of the patient was reduced in size. Next, the patient was scheduled to receive auto-SCT. She underwent the PET/CT in August, and we mobilized and collected autologous stem cells in September, without continuing tislelizumab treatment. In August 2020, the quaternary PET/CT revealed that multiple lymph nodes in the bilateral neck, the left clavicle and left axilla area were slightly enlarged and exhibited mildly increased metabolic activity (**Figure 2**) suggesting that there was still residual tumor activity after targeted therapy. Thus, combined therapy of BV (100 mg) + tislelizumab (200 mg) was administered to the patient in October, 2020. Before the treatment, the lymph nodes of the patient were not significantly changed as revealed by US and CT scans. Overall, the patient did not exhibit disease progression from August to the time she received combination therapy in

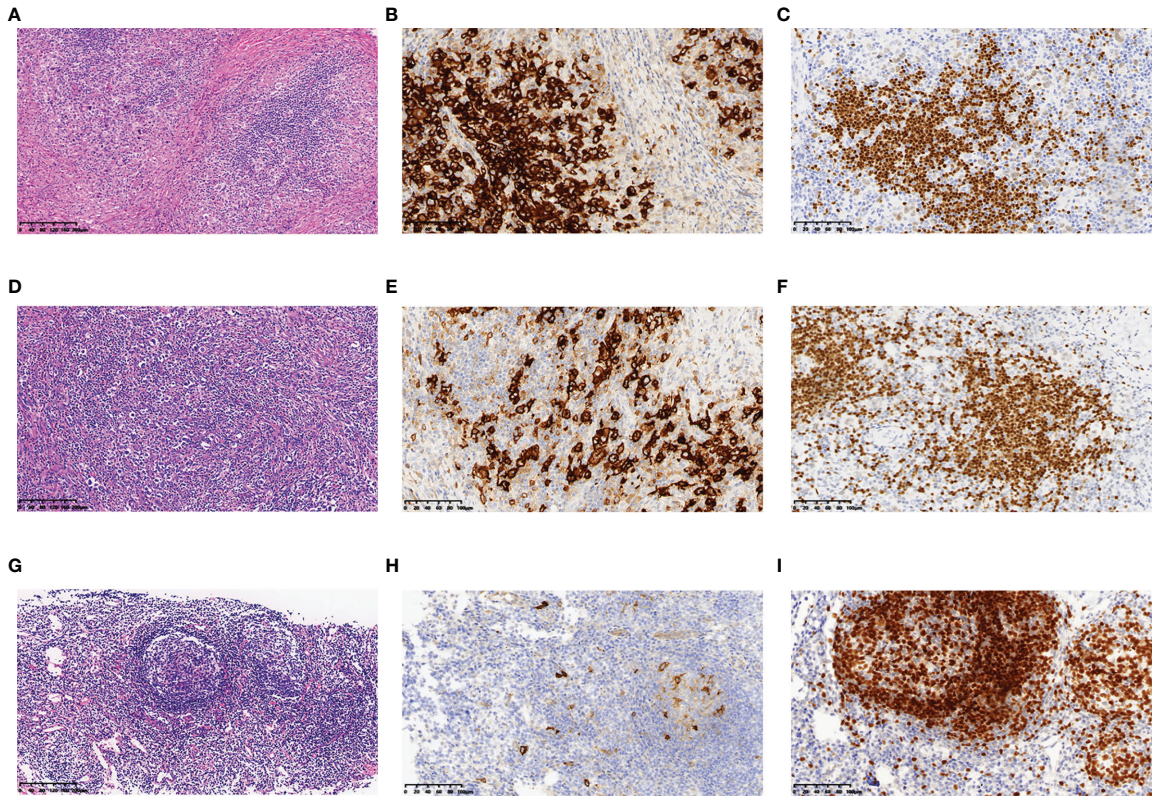

FIGURE 1 | Histopathology and immunohistochemistry of the lymph nodes of this patient. In October 2019, **(A)** HE staining revealed nodular sclerosis classic Hodgkin lymphoma (10×), IHC staining showed that lymphoma cells were positive for **(B)** CD30 (20×), **(C)** PAX-5 (20×). In May 2020, **(D)** HE staining revealed nodular sclerosis classic Hodgkin lymphoma (10×), IHC staining showed that lymphoma cells were positive for **(E)** CD30 (20×), **(F)** PAX-5 (20×). In December 2020, **(G)** HE staining revealed lymphoid tissue had a proliferative change (10×), IHC staining showed that lymphoma cells were negative for **(H)** CD30 (20×), and positive for **(I)** PAX-5 (20×).

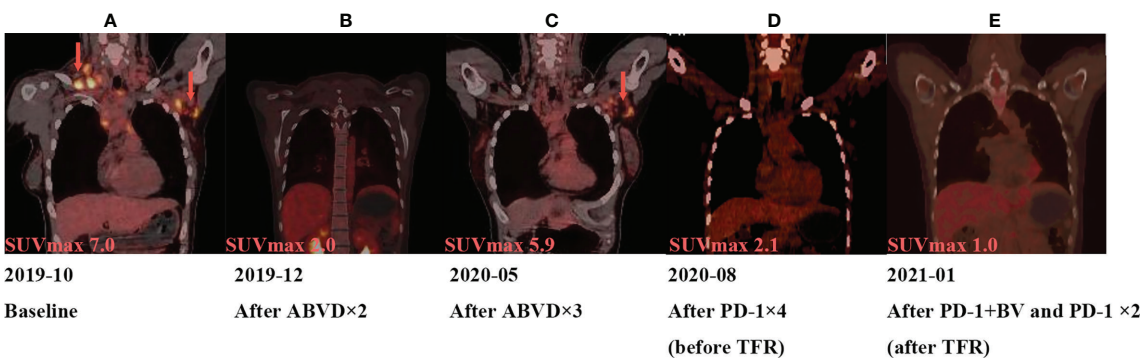

FIGURE 2 | Tumor assessment during treatment by PET. **(A)** In October 2019, the size of the left axillary lymph node was 3.1 × 2.2 cm, and maximum standard uptake value (SUVmax) from 18F-FDG is 7.0. **(B)** In December 2019, the size of the left axillary lymph node was 1.51 × 0.84 cm, SUVmax is 2.0. **(C)** In May 2020, the left axillary lymph node was1.69 × 1.69 cm, and SUVmax is 5.9. **(D)** In August 2020, the left axillary lymph node was 1.5 cm, and SUVmax is 2.1. **(E)** In January 2021, PET/CT showed the SUVmax is 2.1.

October. However, the patient had a slight fever, with the highest temperature being 37.6 °C after 2 days of this round of treatment, experienced pain in the axillary lymph nodes and had multiple skin rashes with pruritus all over the body after 1 week, accompanied by joint pain, fatigue and poor appetite for about 15 days. Before these reactions, the patient had no history of autoimmune or hyperimmune reactivity. Testing for the presence of inflammatory cytokines revealed that interleukin-4

(IL-4) concentrations decreased from 3.41 to 0.1 pg/ml, IL-2 and tumor necrosis factor-α (TNF-α) from 1.54 or 4.13 to 0.1 pg/ml, respectively. Interferon-γ (IFN-γ) concentrations increased from 2.95 to 15.43 pg/ml, IL-6 from 8.15 to 12.96 pg/ml, and IL-17A from 51.12 to 176.68 pg/ml (**Table 1**). The US and CT scans showed that multiple lymph nodes were notably enlarged all over the body compared to before treatment (**Figure 3**). Next, the patient was given dexamethasone (5 mg i.v. QD) for 4 days combined with cetirizine (10 mg p.o. QD) for 3 days. The body rash of the patient subsided and pruritus was alleviated. During December, 2020, US scans revealed that the sizes of the bilateral cervical, axillary and inguen lymph nodes were reduced. A biopsy of the left axillary lymph node showed that lymphoid tissues exhibited a proliferative change (**Figure 1G**). The results of IHC indicated that CD30 (−) (**Figure 1H**), CD3 (+), CD20 (+), Ki-67 (around 5%+), CD5 (+), CD10 (germinal center +), Bcl-2 (+), Bcl-6 (germinal center +), MUM1 (scattered W+), PAX-5 (+) (**Figure 1I**), CD21 (FDC+), VD23 (FDC+), cyclinD1c-Myc, ALK and EBER were all negative. During the wait for auto-SCT, the patient received a further 2 cycles of PD-1. In January 2021, the quinary PET/CT scans showed there was no obvious increase in glucose metabolism of FDG in the area of the lymph nodes, after the lesion had shrunk and tumor activity inhibited (**Figure 2**). Then the patient underwent auto-SCT. At present, she has been regularly followed-up and remains disease free for 10 months since transplant without the need for anti-tumor treatment. The patient never received radiation therapy. The clinical course of the patient is shown in **Figure 4**.

DISCUSSION

TFR, the pseudoprogression of hematological malignancies, is a clinical syndrome caused by drugs with actions on the immune system, and often occurs in patients who are diagnosed with chronic lymphocytic leukemia (20), mantle cell lymphoma (21), Hodgkin lymphoma (22) or Waldenström's macroglobulinemia (23, 24). TFR often manifests as painful and enlarged lymph nodes or splenomegaly in patients following treatment with immune-related drugs. It may be accompanied with symptoms of fever, bone pain, skin rash or lymphocytosis (25, 26), which are mainly mild and self-limiting and often appear during the initial phase of treatment. The course of the disease generally evolves over 7–14 days (27). In most cases, a patient with TFR can be given non-steroidal anti-inflammatory drugs to relieve the discomfort (25). But if the patient has severe symptoms or a history of immune checkpoint inhibitor related adverse effects, corticosteroid should be administered, and therapy stopped if necessary (21, 25). It may not be suitable to use multiple immune-related drugs at the same time, for example two immune checkpoint inhibitors.

In the present study, the patient neither had obvious discomfort after 4 treatments of intravenous tislelizumab infusion, nor significantly larger lymph nodes according to the results of PET/CT scans. After the first administration of BV combined with tislelizumab, low-grade fever, swollen and painful lymph nodes, multiple skin rashes with pruritic, joint pain, fatigue and poor appetite appeared. The symptoms of the patient improved after treatment with glucocorticoid and antihistamine therapy, which was consistent with the clinical manifestations of TFR. US and CT scans showed that the lymph nodes were significantly enlarged after combination therapy of BV and tislelizumab, and re-examination showed that multiple lymph nodes were significantly reduced with no anti-tumor treatment. These findings were in line with pseudoprogression determined retrospectively through imaging observations (28). The medical team considered the reaction to be TFR and decided to give the patient auto-SCT. As hospital beds were scarce in our center, the patient continued PD-1 treatment at home during the waiting time. Before retreatment of PD-1, US scans showed that there were still a number of swollen lymph nodes in the axilla and neck regions. In order to determine whether the patient was progressing or had TFR, we took a lymph node biopsy, which showed proliferative changes but no tumor cell infiltration was detected. In retrospect, it was indeed a pity we did not to take a lymph node biopsy during the onset of TFR.

The specific mechanism of the TFR remains to be elucidated, but may be related to the excessive activation of the immune system (25, 29) and the secretion of inflammatory cytokines (30). In the present study, the significant change of concentration of IL-17A might indicate the occurrence of inflammation, but other concentrations were still within the reference range. In addition, TFR is correlated with the activation and infiltration of NK cells and T cells in cancer foci and changes in the tumor microenvironment (26, 29). However, the biopsy of lymph node did not reveal infiltration of NK or cytotoxic T cells on the day 20 of treatment in a case that was TFR associated with lenalidomide in a follicular lymphoma patient (31). The pathophysiological changes of related lymph nodes or involved lesions after the occurrence of TFR have not been fully clarified, while the rapid appearance may indicate that the initial stage of TFR is mediated by non-antigen-specific effectors, and the later stage antigen-specific immune effects (29).

TFR is an important component of the antileukemic effect of lenalidomide. Chanan-Khan et al. pointed out that the intensity of TFR after the administration of lenalidomide in chronic

TABLE 1 | Changes of inflammatory cytokines.

Date	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)	IL-17A (pg/ml)	TNF-α (pg/ml)	IFN-γ (pg/ml)
2020-09-01 (Before TFR)	1.54	3.41	8.15	2.88	51.12	4.13	2.95
2020-10-30 (TFR)	0.1	0.1	12.96	2.74	176.68	0.1	15.43
2020-12-04 (After TFR)	1.77	3.8	4.23	2.82	59.61	4.18	6.69

IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.

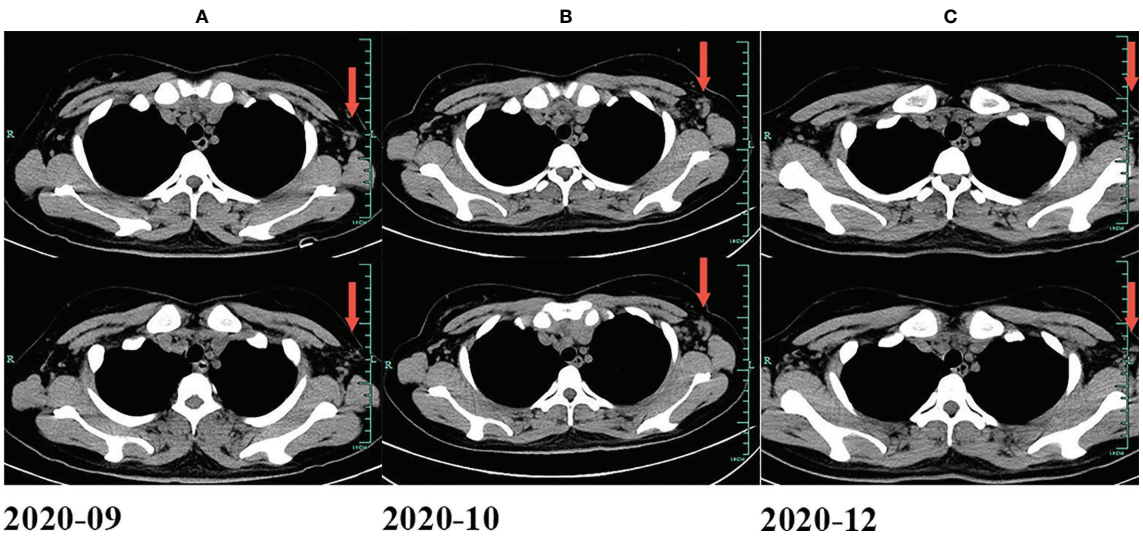

FIGURE 3 | Changes of lymph nodes evaluated by CT. **(A)** In September 2020. **(B)** In October 2020. **(C)** In December 2020.

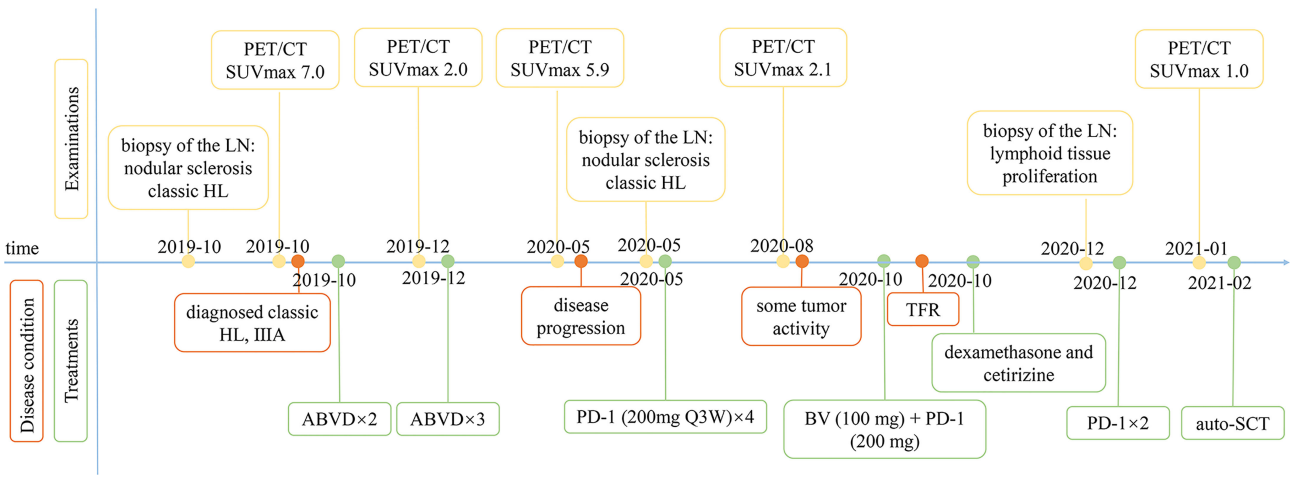

FIGURE 4 | Clinical course of the patient. US, ultrasound; LN, lymph node.

lymphocytic leukemia patients was related to the complete response rate (25). To the best of our clinical knowledge, it is difficult to distinguish TFR or pseudoprogression from disease progression by one measurement of tumor size or metabolism using immediate imaging examinations. Skoura et al. reported a case of false-positive 18F-FDG PET/CT after rituximab therapy, the patient was finally diagnosed with TFR. PET/CT examination of that patient revealed increased metabolic activity of enlarged lymph nodes after R-CHOP (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone) treatment and allogeneic transplantation, whereas biopsy of the lymph node revealed extensive reactive T cell infiltration, with no signs of lymphoma cells. Re-examination of PET/CT scans showed no obvious enlargement or increased metabolic activity of lymph nodes after 3 months (32). Isolated measurements of the sizes of lymph nodes by US, CT or other imaging methods to evaluate changes in the severity of lymphoma are imprecise, and even the use of single PET/CT for the assessment for the changes of disease does not provide an accurate diagnosis, leading to difficulty in distinguishing TFR from disease progression and whether to terminate the original effective treatment therapy.

BV-related immune complications are not common, but include progressive multifocal leukoencephalopathy and acute pancreatitis (33, 34). A case of a Hodgkin lymphoma patient treated with BV, who developed progressive multifocal leukoencephalopathy, indicated that BV can reduce the number of $CD30^+$ T cells, which may be related to immune surveillance, and also inhibition of the TNF signaling pathway (33). Additionally, Gandhi et al.

demonstrated that low CD30 expression was detected in the pancreas of a patient and two healthy controls by multispectral imaging, suggesting that BV targeted to the unexpected low-level $CD30^+$ pancreas may be the foundation of this adverse effect (34). A limited number of reports on TFR induced by BV exist, but the underlying mechanisms are still unclear. In a clinical study of BV combined with nivolumab for relapsed or refractory Hodgkin lymphoma patients, BV or nivolumab was administered on day 1 or day 8 in the treatment first cycle (4). For our patient, the two immune-related targeted drugs were simultaneously used during the first cycle, which may have triggered the TFR of the patient. In addition, CD30 is a member of the TNF receptor family and TNF-α can induce the nuclear factor-κB (NF-κB) pathway and regulate the local microenvironment to enhance intracellular killing (33). Therefore, BV-induced TFR may be related to the CD30 and TNF pathway, but further research is required to verify unequivocally this hypothesis.

In case of abnormal conditions, such as rapid enlargement of lymph nodes during the administration of immune-related therapeutic drugs or targeted therapies, clinicians should be additional aware of the occurrence of TFR that may be related to excessive activation of the immune system. In this clinical research, combination therapy using BV and tislelizumab may cause TFR. Thus, biopsy and continuous imaging observation are important to indicate the disease changes, enabling clinicians to determine the disease stage and real effects of the drugs of a patient, to facilitate the development of further accurate diagnostic and treatment schemes.

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 human participants were reviewed and approved by the Clinical Research Ethics Committee–IIT Ethics Review Group, The First Affiliated Hospital, College of Medicine, Zhejiang University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DH and WH carried out the clinical management of the patient. FY and WW reviewed and analyzed the data. CZ and YZ wrote the manuscript. JH and ZC supervised the entire study. All authors contributed to the article and approved the submitted version.

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REVIEW
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Semaphorins as Potential Immune Therapeutic Targets for Cancer

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Semaphorins are a large class of secreted or membrane-bound molecules. It has been reported that semaphorins play important roles in regulating several hallmarks of cancer, including angiogenesis, metastasis, and immune evasion. Semaphorins and their receptors are widely expressed on tumor cells and immune cells. However, the biological role of semaphorins in tumor immune microenvironment is intricate. The dysregulation of semaphorins influences the recruitment and infiltration of immune cells, leading to abnormal anti-tumor effect. Although the underlying mechanisms of semaphorins on regulating tumor-infiltrating immune cell activation and functions are not fully understood, semaphorins can notably be promising immunotherapy targets for cancer.

Keywords: semaphorins, cancer, immunotherapy, immune cell, therapeutic targets, tumor microenvironment

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INTRODUCTION

Relative to traditional cancer treatments, tumor immunotherapy has shifted the paradigm for the treatment of cancer (1). Particularly, the emergence of immune checkpoint inhibitors (ICIs, such as CTLA-4 and PD-1/PDL-1 inhibitor) (2) and adoptive cell therapy (chimeric antigen receptor T cells, CAR-T) (3) represents a turning point for tumor treatment. However, due to the existence of multiple immunosuppressive mechanisms in the tumor microenvironment (TME), tumor cells can get rid of the surveillance and immune killing effects of the immune system under various immune escape pathways.

Abbreviations: APC, antigen presentation cell; AML, acute myeloid leukemia; BTLA, B- and T-cell lymphocyte attenuator; CTLA-4, cytotoxic T lymphocyte-associated antigen-4; CTL, cytotoxic T lymphocytes; CIML, cytokine-induced memory-like; DCs, dendritic cells; GBM, glioblastoma; GC, germinal center; HNSCC, head and neck squamous cell carcinoma; HPV, human papillomavirus; ILT-4, immunoglobulin-like transcript 4; ICANS, immune effector cell-associated neurotoxicity syndrome; LAG-3, lymphocyte-activation gene 3; MDSCs, myeloid-derived suppressor cells; Nrps, Neuropilins; NK, natural killer cells; PD-1, programmed cell death-1; PD-L1, programmed cell death-ligand 1; PLGF, placental growth factor; PDPN, podoplanin; PDX, patient-derived xenograft; PanNET, pancreatic neuroendocrine cancer; SDF1, stromal cell-derived factor 1; TME, tumor immune microenvironment; TAMs, tumor-associated macrophages; Tregs, regulatory T cells; TILs, tumor-infiltrating lymphocytes; TIM-3, mucin domain-3 protein; TLS, tertiary lymphatic structure; VEGF, vascular endothelial growth factor.

Semaphorins, initially characterized as axon guidance factors, are membrane-bound or secreted proteins that participate in cell-to-cell communication and functions (4). Semaphorins play versatile roles in pathophysiological processes, including cancer, immune diseases, and bone diseases, which can be used as novel targets for drugs for preventing or treating various diseases (5–7). There are more than 20 kinds of semaphorins in vertebrates, which can be divided into class 3–7 categories. Class 3 semaphorins are secreted proteins, whereas the others are membrane-bound proteins, and membrane-bound Class 4 semaphorins can be shed into soluble forms by proteolytic cleavage under certain circumstances (6, 8, 9).

Semaphorins contain a common "sema domain", the domain for receptors binding. The main receptors of semaphorins are Neuropilins or Plexins families. The most membrane-bound semaphorins directly bind to conservative plexins that also contain a "sema domain". Plexins can be classified into four classes, A–D, and transfer signals mediated by small GTPases (10), whereas soluble class 3 semaphorins transmit signals requiring neuropilins (Nrps) as co-receptors (11). Nrps are divided into two isoform subtypes Nrp1 and Nrp2. Nrp1 is essential for immune response and identified as the co-receptor of VEGF to mediate angiogenesis (12), and Nrp2 exerts a significant role in VEGF-C/D/VEGFR-3-mediated tumor lymphangiogenesis and lymphatic metastasis (13). Moreover, there are a few semaphorins that require additional receptors to participate in biological activities. For instance, Sema4A can bind to TIM2 (14), Sema4B to CLCP1 (15), Sema4D to CD72 (16), and Sema7A to integrin β1 (17) (**Figure 1**).

Accumulating evidence indicate that semaphorins are dysregulated and play versatile and multifaceted regulatory roles in several hallmarks of cancer, including angiogenesis (18), metastasis (19), tumor immune escape, and tumor-associated inflammation (20–22). Semaphorins can contribute to tumor progression by modulating immune responses between tumor cell and tumor-infiltrating immune cells in TME. The immunological function of semaphorins is widespread, mainly due to membrane-bound semaphorins or their receptors widely distributed on the surface of immune cells and tumor cells. The so-called immune semaphorins can act as attractants to regulate the recruitment of macrophages, natural killer cells (NK), dendritic cells (DCs), and cytotoxic T lymphocytes (CTL) to

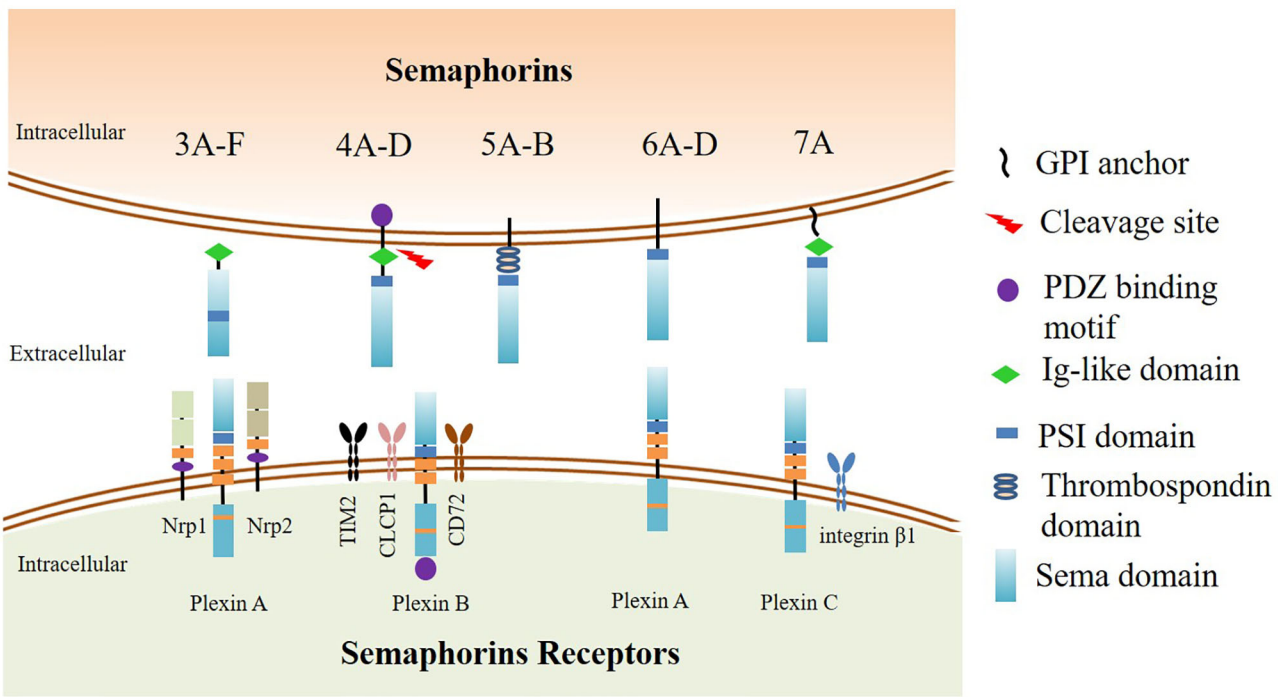

FIGURE 1 | The classification and structure of semaphorins and their receptors. The upper part: Class 3 semaphorins are secreted proteins. Class 4 to 6 semaphorins are membrane-bound proteins. Sema7A is the only GPI-linked protein in the semaphorin family. The N-terminus of the semaphorins is Sema domain. Adjacent to the downstream area of the Sema domain is the plexin-semaphorin-integrin (PSI) domain. Class 3, 4, and 7 semaphorins contain an immunoglobulin-like domain located downstream to the PSI domain. Class 4 semaphorins have a PDZ binding motif. The lower part: The receptors of semaphorins. The most membrane-bound semaphorins directly bind to conservative plexins, which are classified into four classes A–D. Plexin A proteins are mainly associated with class 5 and 6 semaphorins, whereas Plexin B proteins are mainly associated with class 4 and 5 semaphorins, and Plexin C proteins are bound with Sema7A. Secreted class 3 semaphorins transmit signals requiring neuropilins (Nrps) as coreceptors. Neuropilins are divided into two subtypes, Nrp1 and Nrp2. There are a few semaphorins that require additional interactors to participate in biological activities. Sema4A binds to TIM2, Sema4B binds to CLCP1, Sema4D binds to CD72, and Sema7A binds to integrin β1.

the TME (23). For instance, Sema3A, Sema4C, and Sema4D have been found to promote tumor progression by enrichment of tumor-associated macrophages in TME (24, 26). On the other hand, Sema3A, as a tumor suppressor, has been reported to restrict the proliferation of pro-tumoral macrophages and repress tumor growth (27). The role of Sema4A is also intricate in tumor immunity. Sema4A expression enhances B-cell infiltration, which contributes to favorable outcome for head and neck squamous cell carcinoma (28), and Sema4A expression on DCs activates CTL and exerts anti-tumor in Lewis lung cancer (29), whereas Sema4A maintains the stability and function of Tregs in melanoma (30). Due to the versatile and multifaceted regulatory roles of semaphorins in tumor-infiltrating immune cells, semaphorins with their receptors could mediate intricate cross-talking between tumor cells and the microenvironment. This review mainly illuminates the regulatory effects and potential mechanisms of representative semaphorins on tumor-infiltrating immune cells, as well as the potential application of semaphorins as therapeutic targets for tumor immunotherapy.

SEMAPHORINS AND TUMOR ASSOCIATED MACROPHAGES

Tumor-associated macrophages (TAMs, Mϕ) are main infiltrating cell groups in tumor stroma and closely associated with tumor angiogenesis, invasion, and metastasis. TAMs have two opposing phenotypes, anti-tumorigenic M1-Mϕs and pro-tumorigenic M2-Mϕs. M1-Mϕs function as inhibiting tumor progression by secreting pro-inflammatory cytokines (IFN-α/β/γ and IL-12) and chemokines (CXCL9 and CXCL10), which can attract CTL and NK cell to restrict tumor growth (31, 32). M2-Mϕs suppress tumor immunity and accelerate tumor progression by secreting immune suppressive factors, such as cytokines (TGF-β and IL-10) and chemokines (CCL2, CCL17, CCL22, and CCL24) (33, 34). TAMs can also secrete pro-angiogenic factor vascular endothelial growth factor (VEGF), placental growth factor (PLGF), and Sema4D to promote angiogenesis, and express podoplanin (PDPN, lymphatic marker) to promote lymphangiogenesis in paracrine and autocrine pathways, leading to tumor vascular and lymphatic metastasis (18, 35). Studies have shown that semaphorins play significant roles in the migration and polarization of TAMs.

Sema3A

Sema3A, a secreted protein, plays paradoxical roles in TME in different types of tumors. In breast cancer, Sema3A is a tumor suppressor, downregulated in tumor and negatively correlated with tumor stage. *In vivo*, Sema3A overexpression increases $CD11b^+F4/80^+$ Mϕs accumulation but not $CD11b^+Ly6C^+$ monocytic cells, and reduces $4T1\text{-}3A^+$ tumor growth in immune complete BALB/c mice. Sema3A regulates intratumoral M1-Mϕs ($CD11b^+Ly6G^-Ly6C^{low}MHCII^{high}$) and M2-Mϕs ($CD11b^+Ly6G^-Ly6C^{low}MHCII^{low}$) differentiation by binding to its receptor Nrp1, and increases M1-Mϕs proliferation but represses M2-Mϕs by enhancing CSF1-mediated phosphorylation of Akt and MAPK, inducing $CD8^+$ T cells and NK cells to repress tumor growth (27).

However, in Lewis lung cancer, Sema3A binding to Nrp1 and PlexinA1/PlexinA4 coreceptors promotes tumor growth by TAM infiltration and pro-tumorigenic function in hypoxic areas (24). Under the tumor hypoxia environment, Sema3A is upregulated, attracting TAMs from the vascularized and perfused area to the hypoxic area by binding to Nrp1/PlexinA1/PlexinA4/VEGFR1 (24). Interestingly, when the expression of Nrp1 on TAMs is downregulated, TAMs are stopped migrating from normoxic regions to hypoxic region by Sema3A/PlexinA1/PlexinA4-mediated stop signals. The redistribution of TAMs weakens their angiogenic and immunosuppressive ability and hinders orthotopic and spontaneous tumor growth (36, 37). In terms of glioblastoma (GBM), the expression of Sema3A is significantly higher in tumor tissues relative to adjacent normal tissues. Sema3A derived from GBM elicits TAMs (microglial cell) accumulation, and antibody blockage of Sema3A (anti-Sema3A, F11) exhibits notable tumor inhibitory effect through downregulating TAMs recruitment in patient-derived xenograft (PDX) models (38). In addition, upregulation of Sema3A boosted the phosphorylation of downstream PI3K and AKT by binding to Nrp1, and enhanced the enrichment of M2-Mϕs to promote resistance to androgen deprivation therapy in prostate cancer (39). Another study has demonstrated that blockage of Sema3A/Nrp1 could also enhance anti-tumor response by increasing M1-Mϕs and decreasing M2-Mϕs in colorectal carcinoma (40). These studies indicate that Sema3A, particularly binding to its receptor Nrp1 on TAMs, regulates the recruitment and differentiation of TAMs in TME. Targeting SEMA3A and Nrp1 has proved to be a novel approach for multiple malignances.

Sema4C

Sema4C, a transmembrane protein, is overexpressed in multiple types of malignant tumors, including breast cancer, esophageal cancer, gastric cancer, and rectal cancer (41, 42). In breast cancer, the functions of Sema4C in macrophage recruitment contribute to tumor malignant properties. Sema4C with plexin-B2 receptor promotes macrophage infiltration in TME, and promotes tumor growth and progression by activating the NF-κB pathway to induce CSF-1 production in breast cancer (25). Additionally, Gao Qinglei found that MDA-MB-231 with shSema4C attracted few macrophages relative to empty vector control cells in *in vitro* migration assays (25). Membrane-bound Sema4C could be cleaved by matrix metalloproteinases to produce soluble Sema4C. A multicenter retrospective study demonstrated that soluble Sema4C was a potential biomarker for breast cancer diagnosis (43). Thus, not only could membrane-bound Sema4C be a promising target to macrophage for immunotherapy, but soluble Sema4C could also be a diagnostic biomarker for breast cancer.

Sema4D (CD100)

Sema4D, also known as CD100, is a transmembrane molecule of 150 kDa of semaphorins IV subfamily, and upregulated in multiple tumor tissues, such as lung, colon, and breast cancer (44–46). Additionally, Sema4D is the first semaphorin member known to be widely expressed on immune cells (16). CD72, Plexin-B1, and Plexin-B2 are the receptors of Sema4D. CD72 is mainly expressed in immune cells and regulates immune response by combining with Sema4D, whereas Plexin-B1 and Plexin-B2 are widely expressed on endothelial cells in multiple tissues and can trigger MET tyrosine kinase signals to promote angiogenesis by interacting with Sema4D ligand (47, 48).

Zhou Yan-Bing's research found that Sema4D and CD68 (TAMs marker) expression were significantly higher in gastric tumor tissues than that in adjacent normal tissues and correlated with histological differentiation type, TNM stage, and lymphatic metastasis by clinicopathological features analysis of 290 gastric patients (26). *In vitro*, they further found that gastric carcinoma SGC-7901 cells showed great morphological changes after non-contact co-culture of M2-Mϕs: cubic tumor epithelial cell with blunt edge and high confluence shifted to narrow interstitial cell-like shape with long spindle and less confluence. TAMs enhanced the expression of Sema4D on SGC-7901 cells, and promoted invasion and metastasis abilities of SGC-7901 cells *in vitro*. It indicated that targeting Sema4D might be able to bring favorable prognosis for gastric patients. However, anti-Sema4D treatment with a specific antibody (Mab67, Vaccinex) shrank tumor bulk and improved survival rates in pancreatic neuroendocrine cancer (RIP1-Tag2) mice in a short period, but conversely promoted lymph node metastasis consistent with an increase in TAMs after anti-Sema4D treatment (49). To further identify the mechanism of TAMs promoting metastasis, the study of Oriol Casanovas found a significant increase in stromal cell-derived factor 1 (SDF1, CXCL12, a pro-invasive molecule) after anti-Sema4D treatment through a mouse cytokine array. In the presence of anti-Sema4D antibodies, macrophages secrete SDF1, which leads to stronger tumor cell migration by binding to CXCR4 receptor.

Sema7A

Sema7A, also known as CD108, the only GPI-linked protein in the semaphorin family, promotes neutrophil migration under hypoxia stimulation (50). Sema7A increases α1β1-integrin macrophages in viral myocarditis (51). Sema7A can recruit macrophages not only in viral infection, but also in TME. Elder and Tamburini found that Sema7A might be involved in macrophage-mediated lymphangiogenesis in breast cancer (52). Sema7A promotes macrophages podoplanin (PDPN) expression, migration, and adhesion of the lymphatic epithelial cell, resulting in breast cancer lymphatic metastasis. PDPN-expressing macrophages (PoEMs) can activate integrin β1 (Sema7A receptor) to bind to lymphatic endothelial cells expressing galectin 8 (GAL8) and cause lymphatic vessel remodeling, lymphangiogenesis (35). Lymphangiogenesis depends on PDPN-CLEC-2 (PDPN receptor) interaction and Sema7A-integrin β1 interaction. Therefore, CD68, Sema7A, and PDPN are associated with poor prognosis of breast cancer patients with lymphatic metastasis (52).

TAM infiltration, especially pro-tumorigenic M2-Mϕs, are related with poor prognosis of multiple cancer types. Sema4C, Sema4D, and Sema7A can be considered as promising biomarkers of TAM infiltration and can be used as prognostic indicators of cancer. Moreover, depletion of TAMs by Sema4D blockage to decrease M2-Mϕs recruitment and aggregation, to eliminate TAMs-associated angiogenesis and metastasis, is a potential strategy to cancer treatment. However, the strategy of TAM depletion may lead to a decline in ability of tumor antigen presentation. Therefore, reprogramming TAM polarization from M2-Mϕs to M1-Mϕs by altering Sema3A expression can be another effective approach to enhance anti-tumor effects.

SEMAPHORINS AND T LYMPHOCYTES

The presence of CAR-T targeting to tumor-infiltrating lymphocytes (TILs) has greatly improved clinical outcome in cancer, particularly for hematologic malignancies, but fail to effectively eliminate cancer cells. Due to insufficient expression of MHC-I or the presence of immunosuppressive signals, the anti-tumor effect of CTL is greatly compromised and displays dysfunctional states (53, 54). PD-1, CTLA-4, T-cell immunoglobulin, and mucin domain-3 protein (TIM-3), lymphocyte-activation gene 3 (LAG-3, CD223) (55), B- and T-cell lymphocyte attenuator (BTLA, CD272) (56), T-cell immunoglobulin and ITIM domain (TIGIT) (57), and V-domain Ig suppressor of T-cell activation (VISTA) (58) have been described as hallmarks of T-cell exhaustion. Semaphorins and their receptors (particularly Nrp-1) have multiple roles in T-cell responses. Nevertheless, the potential role of semaphorin/Nrp-1 in regulating immunosuppressive receptors and CTL functions is complicated.

Sema3A and Sema3B

Recently, numerous lines of evidence indicate that the Sema3 family with Nrp-1 receptor play vital roles in inhibiting anti-tumor $CD8^+$ T-cell responses (59). Sema3A and Sema3B, constitutively distributed on immune cells, binding to Nrp-1, contribute to immune escape from anti-tumor effects of $CD8^+$ CTL (60).

Nrp-1 and Nrp-2 have been shown to be expressed on DCs, macrophages, and T-cell subpopulations and mainly exert pro-tumor effects (61, 62). Nrp-1, a transmembrane protein, is widely involved in cardiovascular and neuronal development, and can also regulate cancer immunology (59). Moreover, Nrp-1 is also co-receptor of VEGF and the Sema3 family (40). Nrp-1 has been characterized in different immune cellular phenotypes including macrophages, dendritic cells, and T-cell subsets, especially expressed on activated T cells and regulatory T-cell populations, but not on the resting T cells (63–65). $Nrp\text{-}1^+$ Tregs are highly expressed in both TME and peripheral blood, making Nrp-1 a potential immune checkpoint target for

immunotherapy (66). Those expressing high Nrp-1 CTL subset also express high PD-1$^+$, with the co-expression of other T-cell inhibitory receptors like CTLA-4, Tim-3, and LAG-3 in B16F10 melanoma (59). The combination of PD-1 antibody and Nrp-1 antibody is more efficient in repressing tumor growth *in vivo*. By contrast, Nrp-2, another isoform, is comparatively less studied in T cells. The expression pattern of Nrp-2 varied in the CD4/CD8-defined subsets. Nrp-2 was upregulated in the CD4$^+$CD8$^+$ DP T cells and downregulated in SP CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ cells as they gradually became lineage committed (63).

Sema3A secreted from activated DCs and T cells can bind to Nrp-1 on T cells and inhibit T-cell proliferation. However, Yang Zhi-Gang has reported that Sema3A was downregulated in acute leukemia, and exogenous Sema3A could inhibit the Nrp-1 expression on Tregs and promote apoptosis in leukemia cells (67). Those studies indicated that Sema3/Nrp-1 signaling was a novel target for tumor immunotherapy (65).

Sema4A

Sema4A, as a new class of immune regulatory molecules, is not expressed by resting T cells, but can be induced on activated T cells (14), constitutively expressed on APCs like dendritic cell and co-stimulates activation of CD4$^+$ T cells. Sema4A has been found to promote Th1-cell-mediated IFN-γ production in mice, but eliciting Th2-cell-mediated IL-4, IL-5, and IL-13 production in human by binding with immunoglobulin-like transcript 4 (ILT-4) receptor (68, 69).

Regulatory T cells (Tregs) have effects on limiting immunopathology, preventing autoimmune diseases, and maintaining immune homeostasis and also negatively regulating anti-tumor immunity (70). The deletion of Tregs can induce the reduction and elimination of tumors, but may induce uncontrolled autoimmunity and even death. Sema4A interacting with Nrp1 also promotes Treg cells' survival, stability, and function through modulation of the Akt-mTOR signaling and PTEN-Akt-FoxO axis (30). The deficiency of Nrp-1 on Tregs fails to limit autoimmunity and induces autoimmune diseases. Thus, the Nrp-1 receptor on Treg cells is dispensable for the suppression of autoimmunity and the maintenance of immune homeostasis. Sema4A–Nrp1 blockade *via* antibodies or soluble antagonists is possible to limit tumor growth by targeting Treg cells without triggering autoimmunity.

Sema4D

CD100 has two forms, soluble CD100 (sCD100) and membrane-bound CD100 (mCD100). Both mCD100 and sCD100 have vital roles in immune response. mCD100 is constitutively expressed on the resting T cells, and can be cleaved into sCD100 by matrix metalloproteases when T cells are activated (71, 72). The function of Sema4D on CD8$^+$ CTL is controversial. In HIV infection, the CTL is in lack of mCD100, leading to anti-virus capacity being disabled (73), while sCD100 enhances CTL function of virus clearance in HBV infection (74). Fan Fei-Fei found that MMP-14, sCD100 level decreased and mCD100 increased in non-small cell lung cancer (NSCLC) compared with healthy people, whereas recombinant CD100 or sCD100 upregulation by MMP-14 enhanced CTL activity by secreting IFN-γ and TNF-α. Moreover, the effect of sCD100 on CTL could be blocked by anti-CD72 antibody. Thus, it indicates that sCD100 shedding depends on the cleavage of MMP-14 and CD72 interaction and plays an important role in regulating CTL of NSCLC (75).

Evans has reported that Sema4D displays an immuno modulatory function. When Sema4D is highly expressed on the invasive margins of actively growing tumors, it influences the infiltration and distribution of leukocytes in the TME. Antibody neutralization of Sema4D disrupts this gradient of expression, enhances recruitment of activated monocytes and lymphocytes into the tumor, and shifts the balance of cells and cytokines toward a proinflammatory and antitumor milieu within the TME. This change in the tumor architecture was associated with durable tumor rejection in murine Colon26 and ERBB2(+) mammary carcinoma models (46). Recently, a Phase Ib/II study of pepinemab (anti-Sema4D) in combination with avelumab (anti-PD-L1) showed that the combination therapy was well tolerated and exerted antitumor activity in immunotherapy-resistant and PD-L1-low NSCLC patients (76). However, the function of Sema4D on Treg responses in cancer is still unknown. In ankylosing spondylitis, Sema4D inhibits Treg cell differentiation in the AhR pathway (77). Sema4D also promotes liver fibrosis in Schistosomiasis infection *via* TGF-β1 and IL-13 pathways. Sja-miR-71a in Sjaponicum egg-derived EVs can increase Treg and decrease Th1, Th2, and Th17 by directly inhibiting Sema4D (78).

Exhausted T cells not only highly express PD-1 and CTLA-4, but also highly express semaphorins and their receptors, especially Nrp-1; thus, tumor cells are compromised to immune checkpoint inhibitors and turn to self-tolerance. Moreover, the expression of semaphorins and Nrp-1 is positively correlated with PD-1 expression level. Therefore, concomitant blockade of semaphorins, Nrp-1, and PD-1 may reshape the anti-tumor function of CTL and abrogate tumor progression.

SEMAPHORINS AND TUMOR-INFILTRATING B CELL

T cells are not the only immune cells capable of fighting tumor cells. Tumor-infiltrating B cells (TIL-Bs) are also important for tumor immunity. Recent studies have found that bulk of B cells are enriched in tumor tissues including lung cancer, melanoma, renal cell carcinoma, breast cancer, and head and neck squamous cell carcinoma (HNSCC) (28, 79, 80), and B cells play a dual role in the progression of cancer. On the one hand, B cells can stimulate anti-tumor immunity by antigen presentation B cell (APC-B cell) and producing IgG (Plasma cell) to mediate antibody-dependent cytotoxicity; on the other hand, regulatory B cells (B-regs) inhibit CD8$^+$ T cell activity by secreting IL-10, PD-L1, and TGF-β, resulting in tumor immunosuppressive effects and tumor progression. TIL-Bs have prognostic

significance and promise to be a new target to complement T-cell-based immunotherapy. The expression of Sema4D on resting B cells is low, but upregulated upon activation. Sema4D has been found to promote the survival and activation of B cells and enhance antibody production (81), but the role of semaphorins on TIL-B is rarely reported.

SEMA4A

TIL-B mainly comprise naïve B cells, germinal center (GC) B cells, plasma cells, etc. The team of Jennifer A. Wargo from the University of Texas MD Anderson Cancer Center found that B cells and tertiary lymphoid structures play an important role in tumor immunity (82). In HNSCC patients with human papillomavirus infection (HPV^+) infection, GC TIL-Bs and tertiary lymphatic structure (TLS) are significantly increased, both of which correlate with a favorable outcome of HNSCC. Tullia C. Bruno found that the expression level of Sema4A was elevated in HPV^+ HNSCC by scRNAseq data analysis (28). Interestingly, Sema4A upregulation was associated with GC B-cell differentiation and TLS with GC. Sema4A promote transition from naïve to GC cells, consistent with the expression of CD38 and BCL-6, a key transcription factor that regulates GC. It indicates that Sema4A may regulate the formation of GCs within TLS and B-cell maturity in TME of HNSCC patients.

The current immunotherapy mainly aims to activate $CD8^+$ T cells, but the role of humoral immunity against tumor immunity is still unclear. As a component of the TME, TIL-Bs also play an important role in tumor progression (79). Sema4A is upregulated on GC TIL-Bs of HPV^+ HNSCC and drives naïve TIL-Bs towards activated and GC phenotypes, which can be one way to complement current $CD8^+$ T-cell-based immunotherapies.

SEMAPHORINS AND NATURAL KILLER CELLS

NK cells are defined as $CD3^-CD56^+$ leukocytes and can be subdivided into functionally distinct subgroups, namely, $CD56^{bright}CD16^{neg}$ and $CD56^{dim}CD16^{pos}$ (83). The pan-specific innate immune recognition and rapid killing mechanism of natural killer cells (NK cells) make them another sharp sword in anti-tumor therapy apart from T cells. Decreased NK cell toxicity with KIR and NKG2A upregulation is associated with increased cancer incidence (84). Cytokine-induced memory-like (CIML) natural killer cells are preactivated with interleukin-12 (IL-12), IL-15, and IL-18, followed by adoptive transfer into patients with active acute myeloid leukemia (AML) and exhibit enhanced responses against leukemia target cells weeks later, in the form of IFN-γ production and cytotoxicity, indicating that CIML NK cells represent potent antitumor effector cells for leukemia immunotherapies (85, 86). However, the molecular mechanism of CIML NK cell differentiation and reactivation remains unknown.

SEMA7A

Adoptive transfer immunotherapy of NK cells in solid tumor patients is not satisfactory. One of the main challenges is the transport and infiltration of NK cells to the tumor site. Sema7A can regulate the migration of immune cells including NKs. Sema7A is widely expressed in lymphocytes and myeloid cells including $CD56^{bright}$ NK cells. Stephanie Jost found that Sema7A is substantially upregulated on CIML NK cells after stimulation with cytokines (IL-12, IL-15, and IL-18), consistent with the expression of its ligand integrin-β1 and IFN-γ production (87). Strikingly, Sema7A blockade impairs substantial anti-tumor response mediated by CIML NK cells. These strongly indicate that Sema7A is a significant marker of NK cell maturation, and its ligand integrin-β1 contributes to CIML NK cell differentiation and activity.

NK cell-based tumor treatment strategies include strengthening activation of NK cell, blocking inhibitory signals on NK cell, and adoptive transfer of CAR-NK cell. Given that Sema7A/integrin-β1 interaction promotes CIML NK cell differentiation, Sema7A can be a potential biomarker of clinical outcomes for hematologic malignant patients involving CIML NK cell therapeutic interventions.

SEMAPHORINS AND DENDRITIC CELL

Dendritic cells are a group of antigen-presenting cells (APCs). Most of the DCs in the human body are immature, expressing low levels of costimulatory factors and adhesion receptors. DCs can control the activation or suppression of T cells through costimulatory molecules CD80 and CD86 interaction with CD28 or CTLA4, respectively, in cancer (88, 89). Tumor-infiltrating DCs have often been viewed as tolerogenic or immunosuppressive (90, 91). However, the biological function of semaphorins that regulate mature and migratory phenotype of DCs is poorly defined.

Sema3E

Sema3E has shown to modulate DC function in chlamydial infection. Relative to Sema3E wild-type mice, knockdown Sema3E expression exhibits higher bacterial burden by increasing Th2 response (IL-10), enhancing expression of PD-L1 and PD-L2 and reducing Th1/Th17 cytokine production (IL-12) (92). Another study found that Sema3E knockout exerted inhibitory effect on DC migration through regulation of CCR7 expression and augmenting PD-L2 expression, compared to Sema3E wild-type mice (93). These studies have shown that Sema3E can regulate the migration and function of DCs in

inflammation. However, the role of Sema3E in DCs has not been elucidated in the TME.

Sema4A

Sema4A is identified as a biomarker for DC activation status, especially in the human immune system (69). IL-33, as a candidate for cytokine therapies, can effectively enhance Sema4A expression and stimulate anti-tumoral cells including NK and $CD8^+$ T cells (29, 94), while the mechanism of IL-33 on anti-tumor effects remains unclear. Sema4A on DC interacting with its Plexin B2 receptor on CTL can promote INF-γ production, increase the cytotoxicity of CTLs, and repress tumor growth (29). *In vivo* syngeneic mouse models, Sema4A knockdown abolishes the antitumor activity of IL-33. These results suggest that Sema4A may be an intrinsic antitumor effector of IL-33 in mice.

Sema7A

DC migration is essential for host defense against tumor pathogens. The immature DCs have strong abilities to migrate. The study of Sonja I Buschow identified Sema7A as one of the most highly upregulated proteins upon DC maturation, adhesion, and migration in human and mouse by a large-scale proteome analysis (95). Sema7A-deficent DCs show an increased adhesion strength and lack the ability of migration in response to CCL21 by impairing the formation of actin-based protrusions. Sema7A knockdown impairs the actin cytoskeleton, resulting in enhancing the adhesion and attenuating migration ability of DCs (95).

Although Sema7A has a stimulating effect on the maturation and antigen presentation of DCs, which is beneficial for immune response, a growing number of studies have shown that Sema7A/integrin β1 is a promigratory signal and confers poor survival rate in glioma and breast cancer (96, 97). Therefore, Sema7A plays an anti-tumor effect in terms of DCs, but promotes tumor cells migration in the whole TME.

SEMAPHORINS AND MYELOID-DERIVED SUPPRESSOR CELLS

Myeloid-derived suppressor cells (MDSCs), distinctively expressing nitric oxide synthase (iNOS) and arginase-1 in the STAT3-dependent pathway (98), are bone marrow-derived immature heterogeneous myeloid cells in pathologic conditions such as chronic inflammation and cancer. MDSCs are progenitor cells of macrophages and DCs under normal circumstances, but exert immunosuppressive activity to T-cell function in the presence of maturation arrest (99). Semaphorin can regulate the polarization of MDSCs. Conejo-Garcia and Arindam Bhattacharyya found that semaphorins in exosomes derived from tumor mesenchymal stem cells promoted myeloid-derived suppressor cells (M-MDSCs) to differentiate to immunosuppressive M2-macrophages in breast cancer, but which kind of semaphorins was not mentioned in their research (100).

SEMA4D

MDSCs are major immunosuppressive cells in head and neck squamous cell carcinomas (HNSCCs), resulting in resistance to ICBs. However, the specific pathways of MDSC recruitment and infiltration remain to be investigated. In HNSCC, tumor cell-derived Sema4D inducing MDSC polarization corresponded with an inhibition in T-cell activation and an increase in arginase-1, TGF-β, and IL-10 production (101). Clint T. Allen found that Sema4D blockage improved responses to ICIs therapy for HNSCC patients due to repressing $Ly6G^{hi}Ly6C^{int}$ MDSCs (PMN-MDSCs) infiltration by reducing MAPK-dependent expression of chemokines (44, 102). Additionally, Sema4D mAb did not inhibit MOC1-tumor cell growth or tumor vascularity. These results indicated that anti-Sema4D antibodies enhance response of combination therapy by altering immune response not by inhibiting proliferation or angiogenesis, and highlighted that anti-Sema4D antibodies might be beneficial for patients with PD-1 inhibitor resistance.

CONCLUSION AND FUTURE PERSPECTIVES

Although immunotherapy has been considered a breakthrough for hematologic cancers and solid tumors, the survival duration and life quality of patients are compromised to tumor immune evasion. Immune evasion is one of the hallmarks of cancer, which is one of the main reasons for the poor prognosis of patients. Imbalance between pro-tumor and anti-tumor immune response leads to immune escape of tumor cells. The immunosuppressive responses are generally manifested as an increase in expression in inhibitory receptors and ligands in APC cells (DCs, macrophages, and B cells), CTLs, and NK cells; an increase in tumor-infiltrating immunosuppressive cell types (M2-Mϕs, Tregs, B-regs, and MDSCs); hypoxic and acidic conditions; and an increase in pro-tumor cytokine and chemokine production. Accumulating evidence shows that semaphorins are involved in tumor evasion and progression. Semaphorins are dysregulated in multiple types of tumors, making them not only tumor prognostic predictors but also therapeutic targets. However, the function and signal pathways of semaphorins in the tumor immune environment are intricate and not yet fully elucidated.

Semaphorins can act as attractants to elicit inflammation cells such as macrophages, dendritic cells, NK cells, B cells, and T cells to the TME (**Figure 2** and **Table 1**). For example, soluble Sema3A has opposite effects on the recruitment of macrophages in different types of cancer. In terms of the transmembrane Sema4 family, Sema4C and Sema4D promote macrophage recruitment and tumor progression. Sema4A promotes Treg survival and stability and accelerates tumor

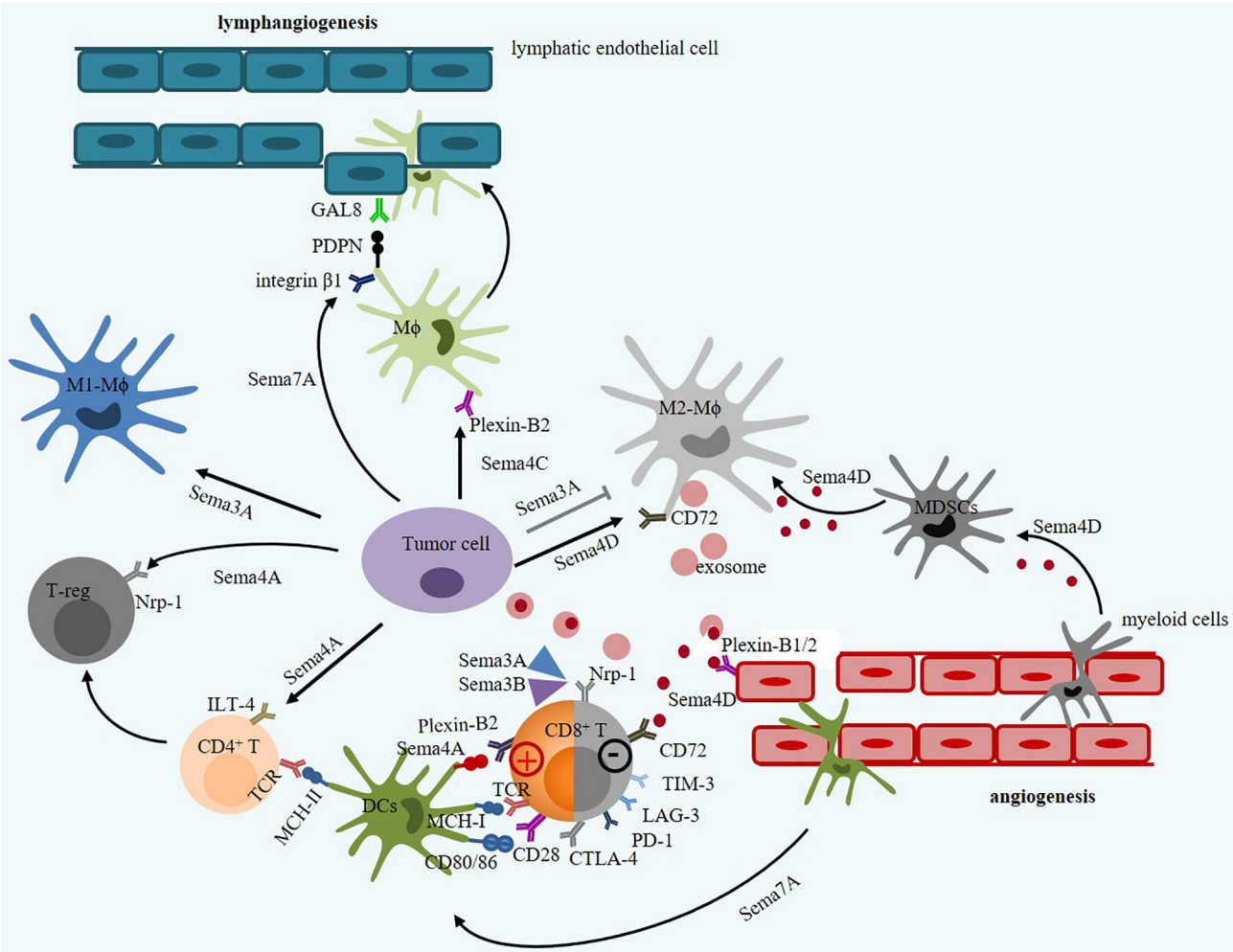

FIGURE 2 | The intricate roles of immune semaphorins and their receptors in tumor microenvironment. Sema3A and Sema3B contribute to decrease in toxicity of CTL by binding to Nrp-1. However, Sema3A promotes M1-Mϕ proliferation but inhibits M2-Mϕ proliferation. Sema4A promotes Treg activation and survival *via* Nrp1 receptor, but enhance CTL vitality *via* Plexin-B2 receptor. Sema4D derived from tumor cell or M2-Mϕ promote tumor angiogenesis *via* Plexin–B1/2 receptor on endothelial cells and inhibit immune response by promoting polarization of MDSCs and inhibiting T-cell function *via* CD72. Sema4D derived from Mϕ can secrete SDF-1 (CXCL12) that mediates tumor metastasis by binding to CXCR4. Sema7A can mediate macrophages and dendritic cell migration in integrin β1 signals, and mediate tumor lymphatic metastasis through upregulating PDPN expression.

growth. Sema7A is constitutively distributed on resting dendritic cells, is highly upregulated on mature DCs, and is a negative regulator of T-cell responses and plays a critical role in T-cell-mediated inflammation through α1β1-integrin (103, 104). Thus, those immune semaphorins provide valuable and novel insights into immunotherapy for cancer.

Combination immunotherapy is an effective way to reshape TME and improve the therapeutic effect, particularly for immunotherapy-resistant and PD-L1 negative/low tumors. Immune semaphorin-based mAb blockade therapy has become a research hotspot. For instance, the combination of Sema4D mAb with either CTLA-4 or PD-1 inhibitor abrogates tumor growth in murine oral cancer-1 mice by inhibiting MDSC recruitment and enhances CTL infiltration (44). Recently, the combination of lgG mAb targeting Sema4D (pepinemab) with PD-L1 inhibitor avelumab has been evaluated as a safe and tolerated synthetic therapy in phase II clinical trials of immunotherapy-resistant NSCLC patients (76). Another phase I trial (NCT03425461) has been registered on ClinicalTrials.gov. to evaluate the safety and tolerability of combination of anti-SEMA4D monoclonal antibody (VX15/2503) with nivolumab or ipilimumab in patients with stage III or IV melanoma who have progressed on anti-PD1/L1-based checkpoint inhibitors (**Figure 3**). Nevertheless, the serious toxic side effects of cancer immunotherapies mainly include cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (105). Advanced nanoparticle or exosome drug delivery system can transport semaphorin-based drugs to

TABLE 1 | The roles of representative immune semaphorins in tumor microenvironment.

Semaphorins	Tumor type	Receptors	Expression	Pathway	Functions	Marker on immune cell	Ref.
Sema3A	Breast cancer	Nrp1	Sema3A downregulated	CSF1-mediated phosphorylation of Akt and MAPK	M1-Mϕs increase; M2-Mϕs decrease	M1-Mϕs: $CD11b^{+}Ly6G^{-}$ $Ly6C^{low}MHCII^{high}$; M2-Mϕs: $CD11b^{+}Ly6G^{-}$ $Ly6C^{low}MHCII^{low}$	(27)
	Lewis lung cancer	Nrp1 PlexinA1 PlexinA4	Nrp1 downregulated in hypoxic areas	PlexinA1/PlexinA4-dependent VEGFR1 activation	Drive TAMs toward hypoxic niches	Mϕ: $F4/80^{+}$	(24)
	Glioblastoma	–	Sema3A upregulated	–	Elicit TAMs (microglial cell) accumulation	Microglial cell: Iba1	(38)
	Melanoma	Nrp1	Nrp1 upregulated on $CD8^{+}$ TILs	Inhibit T-cell migration toward CXCL12 gradient	Impair CTL functions	CTL: Nrp-1^{+}PD-1^{hi} $CD8^{+}$	(59)
Sema4A	HPV^{+} HNSCC	Nrp1 PlexinD1 Tim-2	Sema4A upregulated on TIL-Bs	Correlate with BCL6 expression	Enhance germinal center TIL-Bs infiltration	TIL-Bs: $CD38^{+}IgD^{-}$ $BCL6^{+}Sema4A^{+}$	(28)
	Melanoma, colon carcinoma	Nrp1	Nrp1 expressed on Tregs	Modulate the Akt-mTOR signaling axis	Potentiate Treg-cell function and survival	Tregs: $CD4^{+}CD25^{+}Foxp3^{+}$	(30)
Sema4C	Breast cancer	PlexinB2	Sema4C upregulated	Induce production of CSF-1 in plexin B2-dependent manner	Promote macrophage infiltration	Mϕ: $F4/80^{+}$	(25)
Sema4D	Gastric carcinoma	CD72	Sema4D upregulated	–	Promote macrophage infiltration	Mϕ: CD68	(26)
(CD100)	PanNET	CD72 PlexinB2	Sema4D upregulated	Modulate the SDF1/CXCR4 signaling axis	Anti-Sema4D antibody promotes tumor migration *via* TAMs	Mϕ: $F4/80^{+}$	(49)
	NSCLC	CD72	sCD100 decreased and mCD increased on CTLs	MMP-14 mediated CD100 shedding	Soluble Sema4D enhance CTL activity	$CD8^{+}$ T cell subsets depend on $CD45RA^{+/-}$, $CCR7^{+/-}$	(75)
	HNSCC	PlexinB1	Sema4D, PlexinB1 upregulated	Reduce MAPK-dependent CXCL1 expression	Induce MDSCs polarization	G-MDSCs: $Ly6G^{high}Ly6C^{int}$ M-MDSCs: $Ly6G^{low}Ly6C^{high}$	(44)
Sema7A (CD108)	Breast cancer	Integrin β1 PlexinC	Sema7A upregulated	Drive the expression of PDPN	Promote macrophage-mediated lymphangiogenesis	Mϕ: CD68, $F4/80^{+}$	(52)

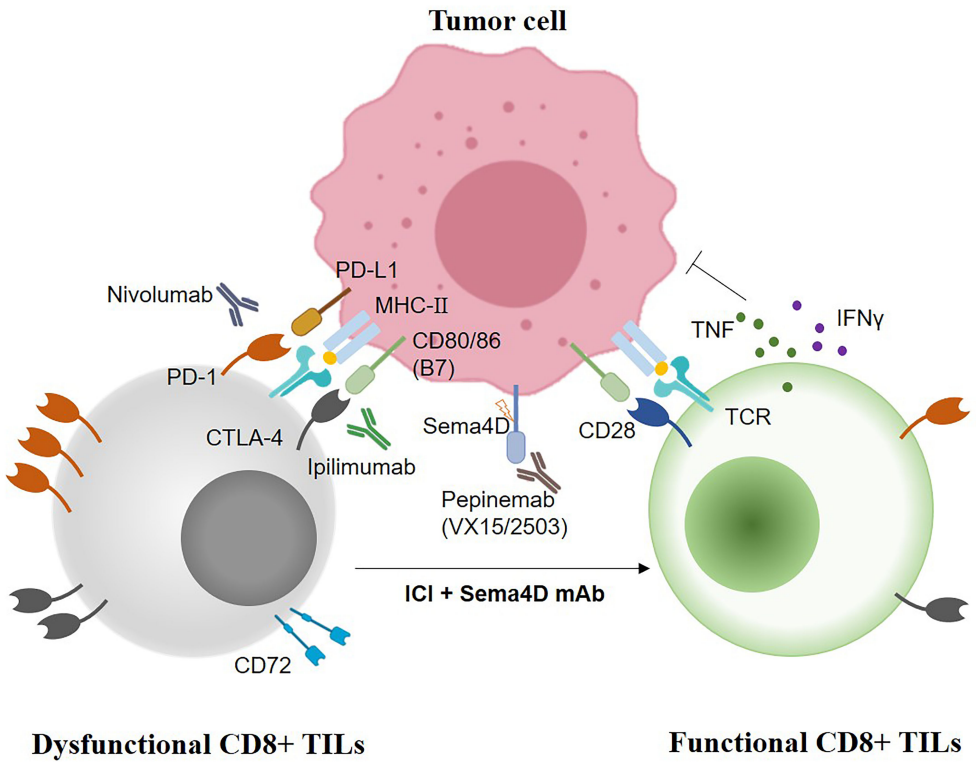

FIGURE 3 | The synergistic anti-tumoral strategies of combination Sema4D mAb with immune checkpoint inhibitors (Nivolumab, PD-1 inhibitor; Ipilimumab, CTLA-4 inhibitor, Pepinemab/VX15/2503, the humanized Sema4D mAb).

TME with specific antibody to potentially alleviate adverse effects (106). In addition to eliminating inhibitory signals in the TME, improving the immunogenicity of tumor cells to enhance CTL function is also an important strategy for immunotherapy. Adoptive transfer immunotherapy of CTL and NK cells and DC-based vaccines genetically engineered with semaphorins or their receptors of tumor cells may also be a promising cancer treatment modality. Thus, more *in vitro* studies, tumor models, and clinical trials are urgently needed to verify the effectiveness of reshaping TME and modulating immune cells by combination immunotherapy and adoptive transfer immunotherapy of immune effectors.

AUTHOR CONTRIBUTIONS

JJ prepared and wrote the original manuscript. FZ, YW, KF, and Z-DY edited the manuscript. RZ and X-LR revised and approved the manuscript. All authors contributed to this work and approved the submitted version.

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Identification of Tumor Antigens and Immune Subtypes of Glioblastoma for mRNA Vaccine Development

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The use of vaccines for cancer therapy is a promising immunotherapeutic strategy that has been shown to be effective against various cancers. Vaccines directly target tumors but their efficacy against glioblastoma multiforme (GBM) remains unclear. Immunotyping that classifies tumor samples is considered to be a biomarker for immunotherapy. This study aimed to identify potential GBM antigens suitable for vaccine development and develop a tool to predict the response of GBM patients to vaccination based on the immunotype. Gene Expression Profiling Interactive Analysis (GEPIA) was applied to evaluate the expression profile of GBM antigens and their influence on clinical prognosis, while the cBioPortal program was utilized to integrate and analyze genetic alterations. The correlation between antigens and antigen processing cells was assessed using TIMER. RNA-seq data of GBM samples and their corresponding clinical data were downloaded from the Cancer Genome Atlas (TCGA) and the Chinese Glioma Genome Atlas (CGGA) for further clustering analysis. Six overexpressed and mutated tumor antigens (ARHGAP9, ARHGAP30, CLEC7A, MAN2B1, ARPC1B and PLB1) were highly correlated with the survival rate of GBM patients and the infiltration of antigen presenting cells in GBMs. With distinct cellular and molecular characteristics, three immune subtypes (IS1-IS3) of GBMs were identified and GBMs from IS3 subtype were more likely to benefit from vaccination. Through graph learning-based dimensional reduction, immune landscape was depicted and revealed the existence of heterogeneity among individual GBM patients. Finally, WGCNA can identify potential vaccination biomarkers by clustering immune related genes. In summary, the six tumor antigens are potential targets for developing anti-GBMs mRNA vaccine, and the immunotypes can be used for evaluating vaccination response.

Keywords: mRNA vaccine, glioblastoma, tumor immune microenvironment, immunotyping, WGCNA, landscape

INTRODUCTION

Gliomas are intrinsic brain tumors arising from glial or precursor cells. They are classified into grades I to grade IV based on the degree of undifferentiation, anaplasia, and aggressiveness (1). Glioblastoma multiforme (GBM, grade IV glioma) accounts for 82% of all malignant gliomas and is characterized histologically by considerable vascular proliferation, cellularity and mitotic activity, and necrosis (2). Despite the availability of standard treatment options for GBM including surgical resection, radiotherapy and chemotherapy, the median survival time of GBM patients is only 12–15 months after diagnosis (3, 4). There has been growing evidence supporting the dynamic interaction between the central nervous system (CNS) and the systemic immune system. As a result, several studies have explored the efficacy of immunotherapy in the treatment of glioblastoma (5).

Currently, immunotherapies for gliomas include chimeric antigen receptor T cell therapy (CAR-T), immune-checkpoint inhibitors, oncolytic viral therapies, and vaccines (6). Cancer vaccines are classified into three major categories based on content and format, such as cell vaccines (tumor or immune cells), nucleic acid vaccines (viral vector, RNA or DNA), protein/peptide vaccines (7). Clinical trials evaluating the efficacy of immune cells (Dendritic cells e.g.), pulsed with TAAs (tumor-associated antigens) have revealed promising results. This is despite the heterogeneity in dose, route, and location of administration as well as the adjuvant used among different trials. HSP (Heat Shock Proteins) vaccines are a subclass of protein vaccines that are composed of HSPs bound to tumor peptides. Phase I HSP vaccine trial in patients with recurrent GBM appeared safe and tolerable (8). Several peptide vaccine trials targeting EGFRvIII showed that these peptide vaccines had a degree of effect with no significant toxicities encountered (9). Recently, a peptide vaccine targeting mutant IDH1 in newly diagnosed glioma has been evaluated in clinical trials and found to be safe, immunogenic, and efficacious (10). However, these vaccines still face with many challenges including: potential of tumor antigens escape, limited repertoire of using defined antigens, and high variability in the physicochemical properties (11, 12). Nucleic acid vaccines are a promising alternative that allow protein and peptide antigen to be expressed with the correct protein modifications in cells (13, 14).

DNA and mRNA vaccines are types of nucleic acid vaccines. The mRNA vaccines have several advantages over the DNA vaccines in safety and efficacy since mRNA does not need to enter the nucleus and be incorporated into the genome (15). Recently, there have been several studies evaluating the efficacy of mRNA vaccines in different types of tumors with varied outcomes being achieved. In gastrointestinal cancer, mutation-specific T cell responses were elicited against predicted neoepitopes and T cell receptors targeting $KRAS^{G12D}$ mutation could be isolated after mRNA vaccines application (16). Two mRNA vaccines (CV-9103 and CV-9104) based on four prostate-specific antigens (STEAP, PSCA, PSMA, and PSA), have showed good tolerability and favorable immune-activation in phase I/II clinical trials in prostate cancer patients (17). Another mRNA vaccine targeting Trp2 induced antigen-specific T cell response and suppressed melanoma proliferation in preclinical trials (18). Angelique et al. reported that dendritic cells (DCs) transfected with CD133 mRNA (cancer stem cell marker) activated T cells, produced an effective and long-lived immune response, and suppressed the proliferation of $CD133^{+}$ glioma stem cells (GSCs) and tumor growth in mice (19). Although there are very few studies that have evaluated the efficacy of mRNA vaccines in GBM, vaccination for GBMs TAA remains a viable concept and current trials are under way for several other targets.

The purpose of our study was to identify novel antigens of GBM that can be used as targets for mRNA vaccine development. In our study, we analyzed fraction alteration and gene expression data of GBMs and identified six candidate genes associated with poor prognosis and robust stimulation of the infiltration of antigen-presenting cells (APC). In addition, due to tumor complex immune microenvironment (TIME) and tumor heterogeneity, some tumor patients may be more likely to benefit from mRNA vaccines (20). We then developed a tool to identify GBM patients who might be more suitable for vaccination. To achieve this, we carried out cluster analysis of immune related genes, and identified three immune subtypes with distinct clinical, cellular, and molecular characteristics. The results were validated using an independent cohort. Finally, we used the immune landscape and immune gene co-expression modules to analyze the distribution of immune related gene characteristics in GBMs. The employed screening workflow was depicted in **Supplementary Figure 1**.

METHOD AND MATERIAL

Identification of Tumor Associated Antigens of Glioblastoma Multiforme (GBM) for Vaccination

Cancer cells harbor unique mutant genes that theoretically create corresponding unique tumor-specific antigens (21). Besides, CNV (copy number variation) burden play significant role in tumors' recurrence and death, indicating that CNV should be considered to be an antigen factor (22). With a deeper understanding of the immune system, the abberant expression of some gene products by tumor cells can be used to develop a variety of antigen-specific vaccination strategies and activate tumor antigen-specific T cells (23). Therefore, we analyzed the gene expression levels and gene alteration status in GBMs to identify tumor associated antigens. Gene Expression Profiling Interactive Analysis is a web-based tool used to process and deliver gene expression profiles based on the samples from GTEx (Genotype-Tissue Expression) and TCGA (the Cancer Genome Atlas) (24). In this study, GEPIA2 (http://gepia2.cancer-pku.cn) was employed to detect the differentially expressed genes between normal brain tissue and GBM ($|Log2FC|>1$, $q<0.05$) and explore their prognostic value in GBMs. Then, we applied the cBioPortal (http://www.cbioportal.org) for exploring, visualizing, and analyzing the genome alteration status of GBM antigens with multidimensional cancer genomics data (25).

Last but not least, effectively uptake, processing, and presentation of antigens by APCs (antigen-presenting cells) can initiate antitumor immune responses through the activation of both $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes (26–28). Tumor Immune Estimation Resource (TIMER, https://cistrome.shinyapps.io/timer/) can be used for systematically evaluating the clinical impact of different immune cells (dendritic cell, macrophage, neutrophil, $CD8^+$ T cell, $CD4^+$ T cell and B cell) in the tumor microenvironment of different tumor types. Therefore, we utilized TIMER to explore the relationship between expression of the identified potent antigens in GBMs and the degree of antigen-presenting cell (APC) infiltration. Spearman correlation analysis between APC cells and tumor purity was calculated using TIMER (29).

Data Preprocessing

From The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/), we downloaded FPKM RNA-seq dataset and mutect2-processed mutation dataset of 167 GBMs as well as their corresponding clinicopathological information. In addition, the RNA-seq data of 369 GBM cases and their corresponding clinicopathological information were obtained from the mRNAseq_325 and mRNAseq_693 datasets of the Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/) and used as the validation dataset. The gene expression levels of the GBM samples were transformed using log2 for further analysis.

Identification of Immune Subtypes and Their Cellular Characteristics

Patient stratification based on tumor immune subtypes can be used to distinguish patients who are suitable for vaccination therapy. After preprocessing the expression data of immune-related genes, we carried out consensus clustering to stratify GBM samples using the "ConsensusClusterPlus" package in R (30). Consensus Cumulative Distribution Function (CDF) was calculated to determine the optimal cluster number. Since the correlation between immune-related signatures and cancer markers is useful for tumor prognosis, we assessed the correlation between immune subtypes and 68 signatures from Wolf et al. (31). We then evaluated 28 immune cell signatures and LM22 signatures to assess the abundance of immune cells in each GBM tumor sample and compare the results among immune subtypes (32, 33). "ESTIMATE" package was employed to estimate Tumor purity scores (tumor purity, stromal score, immune score and estimate score) (34).

Immune Landscape Analysis

Dimension reduction analysis is the commonly used analysis method to increase the understanding of biological systems in tumor. It maps high-dimensional space to low-dimensional space through orthogonal transformation and extracts the largest amount of data information by preserving the a few components (35). In the present study, dimension reduction analysis was conducted using the "monocele" package in R with Gaussian distribution (36, 37). Subsequently, the immune landscape was visualized using a functional map cell trajectory where different immune subtypes were identified by different colors.

Gene Co-Expression Network

We constructed a scale-free network using the "blockwiseModules" function of the WGCNA package in R. The soft threshold of adjacency matrix was set as a continuous value between 0 and 1 so that the constructed network can be closer to the state of the real biological network (38). The "clusterProfile" package in R is a universal enrichment tool which integrates statistical analysis and visualization of functional profiles for genes and gene clusters (39). Then, the GO and KEGG functional components in the "clusterprofile" package were employed to analyze the biological function and pathways of modules associated with GBM prognosis.

Statistical Analysis

The Wilcox test was used to compare data between the two groups, while the Kruskal Wallis test was used to compare three or more groups. Kaplan–Meier curves were used for OS analysis. The cut-off value was set as the best cut-off value from the "survminer" package in R. $P<0.05$ was regarded as statistically significant.

RESULT

Exploring Potential Tumor Antigens of Glioblastoma Multiform (GBM)

At the beginning, results of GEPIA2 analysis revealed 7664 differentially expressed genes where 5221 genes were overexpressed in GBM when compared to normal brain tissue (**Figure 1A**). Using the cbioportal analysis algorithm, we identified 7832 amplified genes as potential tumor antigens of GBM (**Figure 1B**). In the fraction genome altered group, ten genes with the highest alteration frequency included COMMD10, DES, TUBA4A, HLA-DRA, EGFR-AS1, SEC61G-DT, ELDR, EGFR, SEC61G and DIS3 (**Figures 1C, D**). Moreover, a total of 14363 mutated genes were identified by analyzing their mutation count in GBMs (**Figure 1E**). Among them, we displayed ten genes with the highest mutation frequency in GBM, including ACE2, ADAM10, ADGRB3, AMER1, ANXA7, ARNT, ASIC2, ATPGV1E1, BPIFB6 and CASP9 (**Figure 1F**). Overall, 1101 amplified, mutated, and overexpressed genes were identified for further analysis (**Figure 1G**).

Identification of Tumor Antigens Associated With GBM Prognosis and Antigen Presenting Cells

Then, we analyzed the role of 1101 aforementioned genes in the survival and immune response of GBM patients. Results of survival analysis showed that the expression of 14 genes could predict OS (Overall Survival) and PFS (Disease Free Survival) of GBM patients (**Figure 2** and **Supplementary Figure 2**). More importantly, the expression levels of six genes (ARHGAP9, ARHGAP30, CLEC7A, MAN2B1, ARPC1B, and PLB1) were

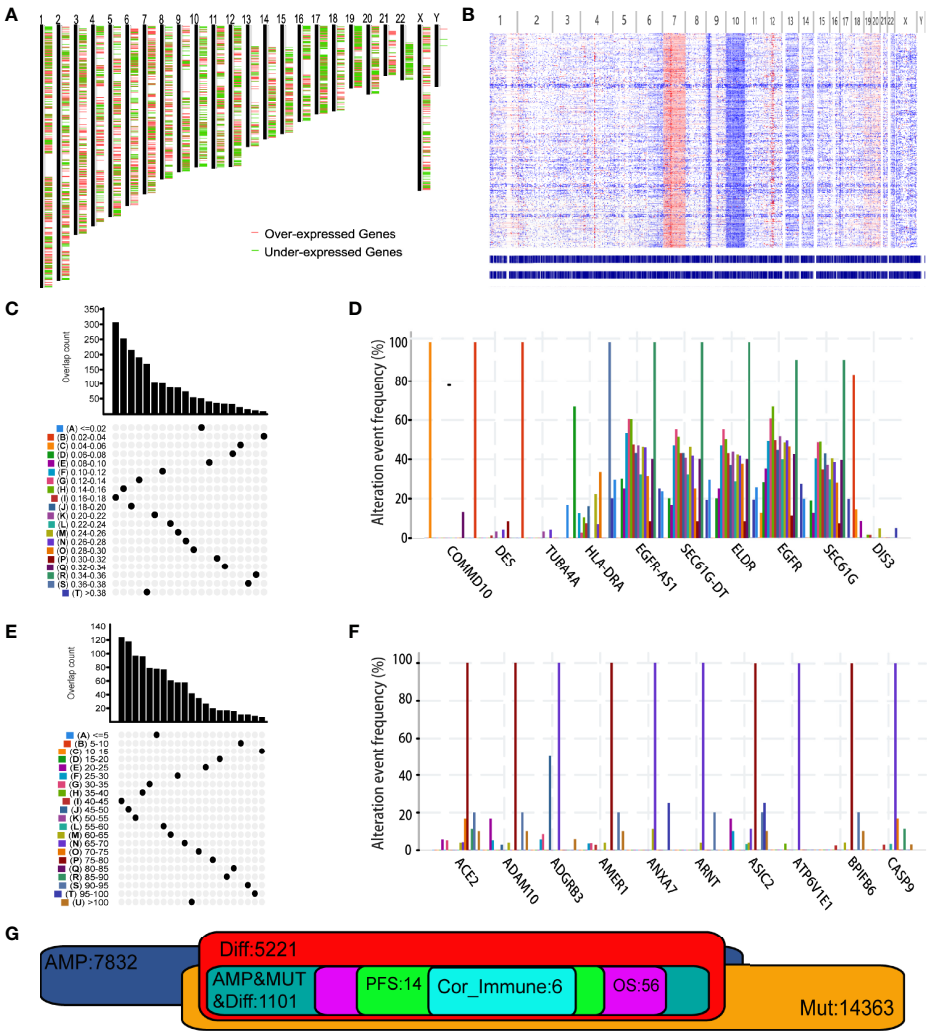

FIGURE 1 | Identification of potential tumor antigens of Glioblastoma multiforme (GBM). **(A)** Chromosomal distribution of up- and down-regulated genes in GBMs. Red plot: overexpressed genes; Green plot: under-expressed genes. **(B)** The chromosomal distribution of the aberrant copy number genes in GBMs. Red plot: amplified genes; Blue plot: deleted genes. **(C)** Samples overlapping in altered genome fraction groups. **(D)** Top ten genes with highest frequency in altered genome fraction. **(E)** Samples overlapping in mutation count groups. **(F)** Top ten genes with highest frequency in mutation count groups. **(G)** Potential tumor antigens (total 1106) with overexpression, mutation, and amplification in GBM, and significant association with OS, RFS and immune infiltration (total 6 candidates).

positively correlated with the level of abundance of B cells, macrophages, and dendritic cells (DCs) and were thus considered suitable targets for vaccine use (**Figure 3**). In general, their upregulation was related to poorer GBM prognosis and more APCs infiltration. Hence, these tumor antigens play key roles in the development and progression of GBM and could be directly processed and presented by the APCs to T cells or recognized by the B cells to trigger an immune response.

Immune Subtypes of GBM

Next, we analyzed the immune status of GBM and identified patients likely to benefit from vaccination by conducting immunotyping. With expression profiles of 1658 immune related genes, we constructed consensus clustering of 167 GBMs from TCGA cohort and validated the stratification using GBM samples from the CGGA cohort. Due to the relatively small sample size of discovery cohort, we chose k = 3 to group the samples into three immune subtypes (IS) (**Figures 4A–C**). The GBM patients in the IS2 group had the best survival prognosis, followed by GBM patients in the IS1 group; GBM patients in the IS3 group had the worst prognosis (**Figure 4D**). We then explore the relationship between the six pan-cancer immune subtypes (C1-C6) and the three immune subtypes identified in this study (40). The immune subtypes identified in this study mainly clustered into C1, C4 and C5 pan-cancer immune subtypes (**Figure 4E**). With differential response to radiotherapy and chemotherapy, four GBM subtypes (proneural, neural, mesenchymal, and classical) were identified before (41–43). Among them, GBMs from IS2 were overlapped by proneural subclass, those from IS3 were covered by

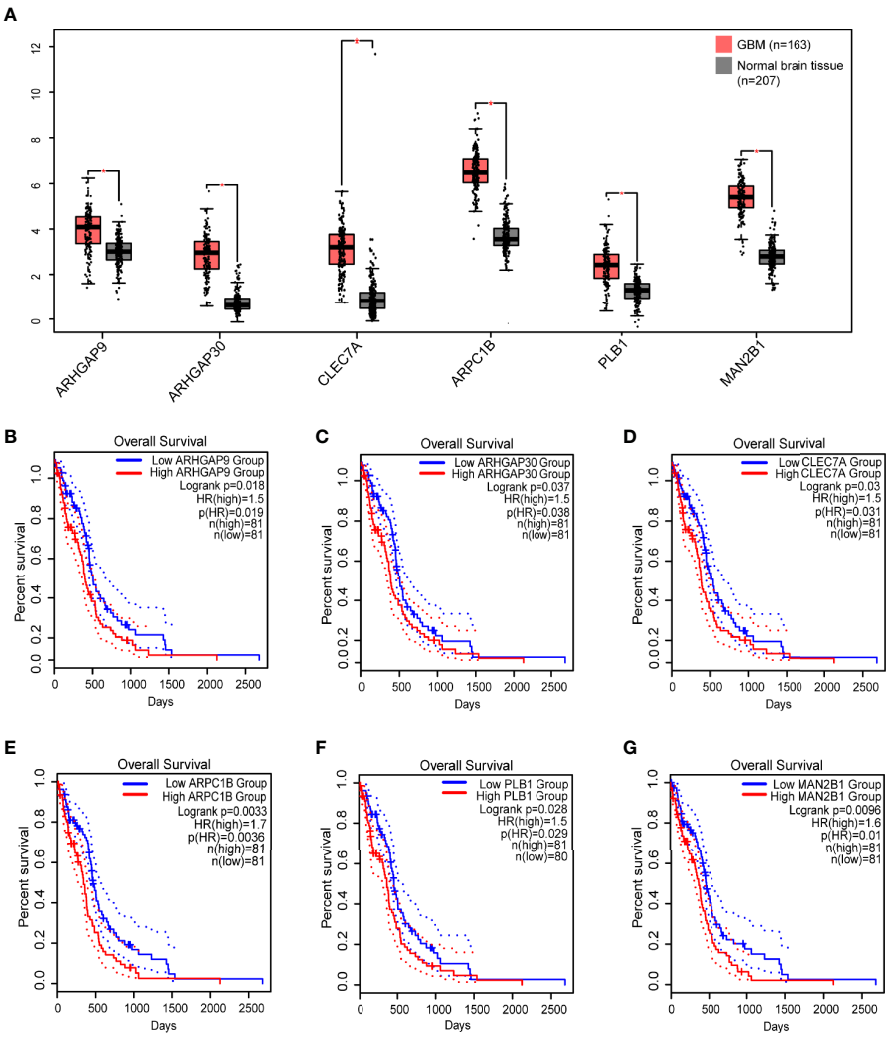

FIGURE 2 | Identification of tumor antigens associated with GBM prognosis. **(A)** Differential expression of six candidates in normal brain tissue and GBMs. *, significant difference. Kaplan-Meier curves showing OS of GBM patients stratified on the basis of **(B)** ARHGAP9, **(C)** ARHGAP30, **(D)** CLEC7A, **(E)** ARPC1B, **(F)** PLB1 and **(G)** MAN2B1 expression levels. 50% (Median) cutoff was set up for dividing low and high expression groups. Log-rank test was used for hypothesis testing, and a *p*-value <0.05 was considered statistically significant.

mesenchymal subclass and GBMs from IS1 were mainly overlapped by classical subclasses (**Figure 4F**) The immune subtypes in the CGGA cohort were associated with prognosis, which was consistent with the results obtained from the TCGA cohort (**Figure 4G**). Findings from our study suggest that our classification of GBMs might be more detailed than the classification according to the pan-cancer immune subtypes. Thus, our classification provides more useful guideline for development and execution of the GBM immunotherapy strategy.

The Association Between Tumor Mutational Burden (TMB) and Immune Subtypes

Tumor mutation burden is significant for the efficacy of vaccines since the abundance of antigens and neoantigens affects the immunogenicity of the tumor (44). We assessed the TMB and mutations data of the GBMs from the TCGA cohort based on the three subtypes. Ten most frequently mutated immune-related genes (EGFR, PIK3CA, PIK3R1, PIK3CG, ANK1, PDGFRA, SEMA3C, TG, TMPRSS6 and L1CAM) was showed with waterfall plot (**Supplementary Figure 3A**). We found the number of mutated genes and the tumor mutational burden was significant different among three subtypes (**Supplementary Figures 3B, C**). These findings indicate that the three immune subtypes expressed distinct amounts of tumor antigens from mutated genes and that the IS3 subtype had the least immunogenicity among these subtypes.

The Association Between TIME and the Immune Subtypes

The ssGSEA method was used to identify the 28 immune signatures previously reported in both TCGA and CGGA

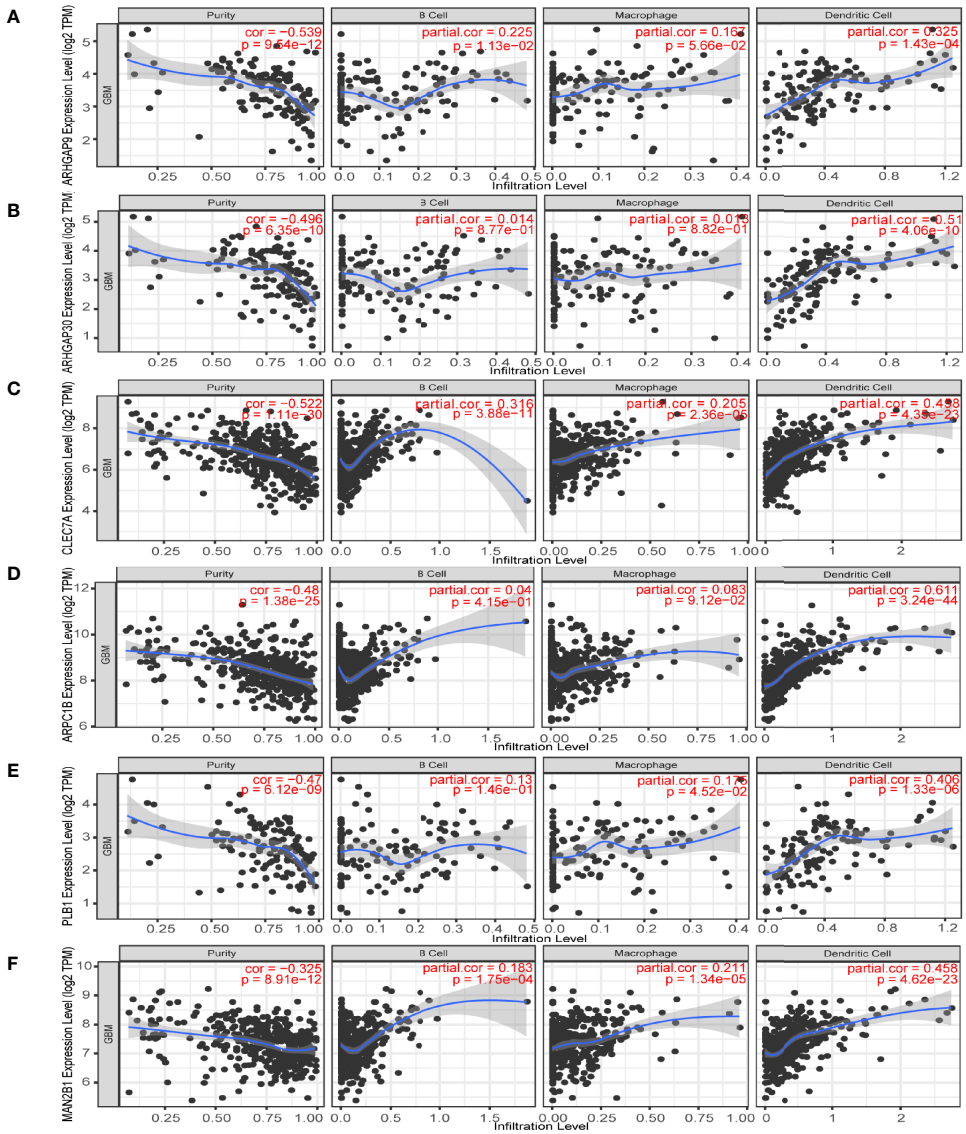

FIGURE 3 | Identification of tumor antigens associated with antigen-presenting cells (APCs). Correlation between the expression levels of **(A)** ARHGAP9, **(B)** ARHGAP30, **(C)** CLEC7A, **(D)** ARPC1B, **(E)** PLB1 and **(F)** MAN2B1 and infiltration of APCs (macrophages, dendritic cells, and B cells) in GBM.

cohorts where immune subtypes showed different proportions of immune cell components (**Figures 5A, B**). For example, the scores of mast cells, MDSC, activated dendritic cells and macrophages were significantly higher in IS3; eosinophils, type 2 T helper cells and activated CD4 T cells were higher in IS2; while central memory CD4 T cells, plasmacytoid dendritic cells and NK T cells were more abundant in IS1. Analysis of CIBERSORT algorithm showed that there were more immunosuppressive regulatory immune cells (macrophage M2 and gamma delta T cells) but less cytotoxic immune cells (activated NK cells) in the IS3 subtype compared to the IS1 and IS2 subtypes (**Figure 5C**). Thus, the IS2 subtype can be considered to be immunologically "hot", the IS1 to be in an intermediate state, and the IS3 subtype to be immunologically "cold". A similar trend was seen in the CGGA cohort (**Figure 5D**). Besides, the expression of most immune signatures was higher in the IS3 subtype compared to the IS2 and IS1 subtypes (**Figure 5E**). Immune checkpoints (ICPs) (e.g., PD1 and PDL1) and immunogenic cell death modulators (e.g., HGF and IFN) are crucial for modulating the immune responses of effectors and maintaining the self-tolerance of tumor to minimize tissue damage (45, 46). PD1 and PDL1 are the most common ICPs for blockage therapy. The IS2 subtype had the least expression of PD1 and PDL1 among the three subtypes; meanwhile, IS3 has highest level of PD1 expression which suggested they might show good response rates for immune

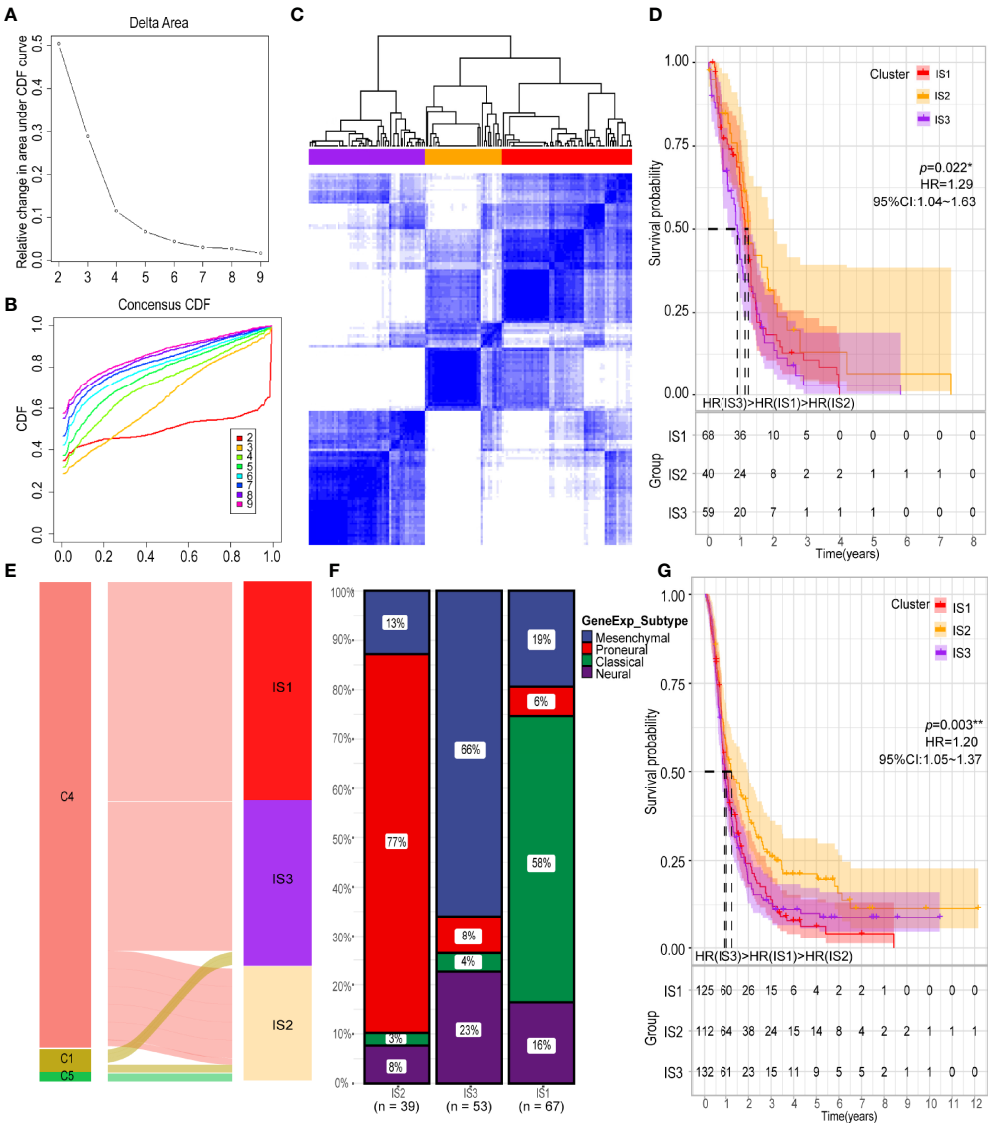

FIGURE 4 | Identification of potential immune subtypes of GBM. **(A)** Cumulative distribution function curve and **(B)** delta area of immune-related genes in TCGA cohort. **(C)** Sample clustering heat map. **(D)** Kaplan-Meier curves showing OS of GBM immune subtypes in TCGA cohort. **(E)** Overlap of GBM immune subtypes with three pan-cancer immune subtypes (NC1 = 2, NC4 = 158, NC5 = 1). **(F)** Distribution of four gene expression subtypes across IS1-IS3 in TCGA cohort. **(G)** Kaplan-Meier curves showing OS of GBM immune subtypes in CGGA cohort. *$p < 0.05$, **$p < 0.01$.

checkpoint blockade (**Figures 6A–D**). Subsequently, we assessed the differential expression of and ICD modulators and ICPs between the three immune subtypes. We found that 34 ICPs had distinct expression patterns among the three subtypes. There was significant upregulation of CD28, CD244, CD200R1, CD27, CD40, CD40LG, CTLA4, CD48, CD86, CD80, CD70, HAVCR2, ICOS, ICOSLG, IDO1, NRP1, TIGIT, TNFRSF14, TNFRSF18, TNFRSF8, TNFRSF9, TNFRSF14, TNFSF4, and TNFSF9 in the IS3 subtype (**Figures 6E, F**). In addition, almost all ICDs were differentially expressed among the three subtypes (**Figures 6G, H**). Taken together, immunotyping can be considered a biomarker of immune status in GBMs and can be used to predict the response of the patients to vaccination.

The Immune Landscape of GBM

We further explored the immune characteristics of GBM by integrating the immune-related gene expression profiles from TCGA cohort to construct the immune landscape where the immune distribution of each GBM were visualized (**Figure 7A**). The expression profiles were aggregated and visualized in a two-dimensional scatter plot with several branches using the DDRTree algorithm (a manifold learning approach) after dimensionality reduction. Component 1 was highly correlated with natural killer cells, activated CD4 T cells and type 17 T helper cells, while component 2 was positively correlated with central memory CD8 T cells, central memory CD4 T cells, type 1 T helper cells, and natural killer cells (**Figure 7B**). Further prognostic analysis of the

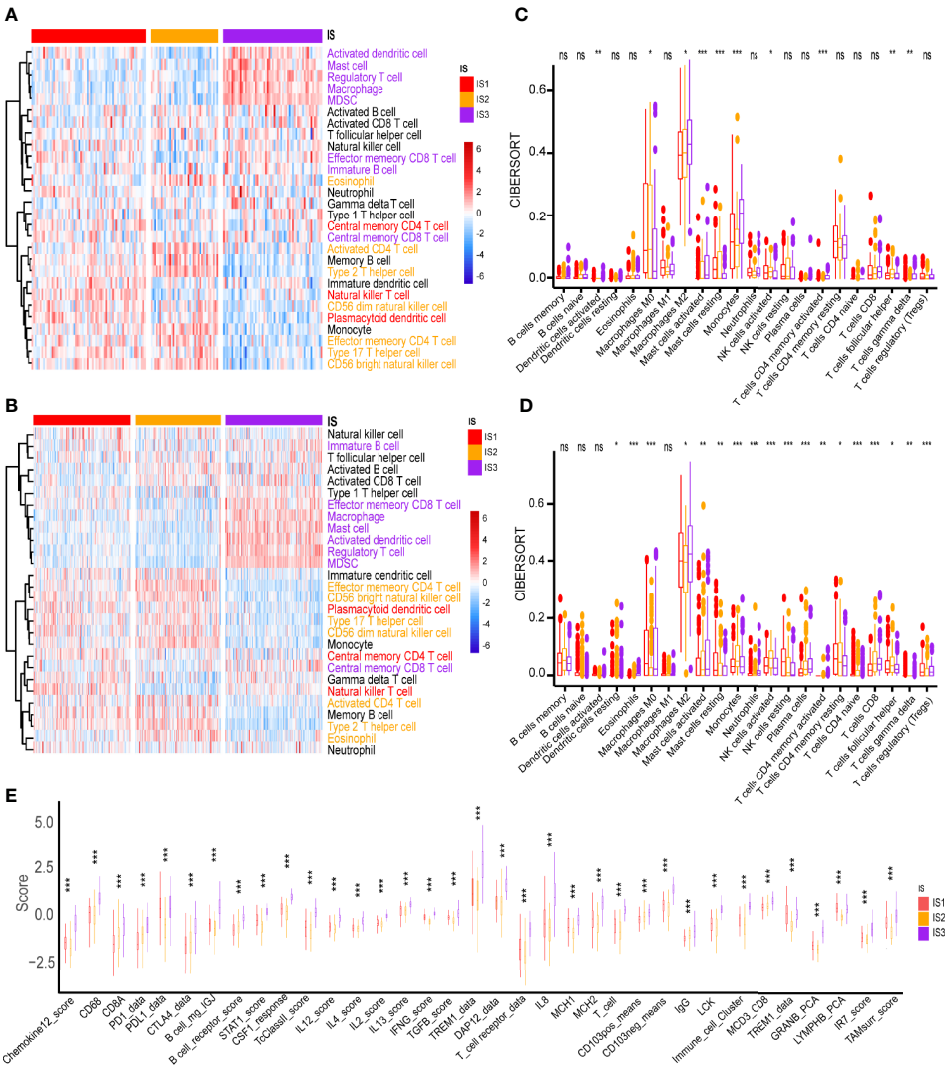

FIGURE 5 | Cellular and molecular characteristics of immune subtypes. Differential enrichment scores of 28 immune cell signatures among GBM immune subtypes in **(A)** TCGA and **(B)** CGGA cohorts. Different colors of the text on the right represents the immune cells were more enriched in the corresponding subtypes. Differential enrichment scores of CIBERSORT 22 immune cell signatures in **(C)** TCGA and **(D)** CGGA cohorts. **(E)** Differential enrichment scores of 56 immune signatures among GBM immune subtypes. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ns, no significance.

GBMs distributed on the extreme ends of the branches showed that patients in group 3 had poorer prognosis than those in group 1, indicating that the immune landscape can be used to discriminate the patients and predict their prognosis (**Figures 7C, D**). The GBMs of the IS3 subtypes were further stratified into three immune subtypes, IS3A, IS3B and IS3C, based on the distribution of the individual GBM samples in the immune landscape (**Figure 7E**). Among them, IS3A had better prognosis than the other types (**Figure 7F**). The estimate score and expression of ICP and ICD were significantly different among these immune subtypes (**Figures 7G–J** and **Supplementary Figures 4A, B**). Interestingly, more activated B cells, cytotoxic T cells and NK cells were located in IS3A than that of IS3B and IS3C, indicating the GBMs of IS3A showed more inflamed microenvironment (**Supplementary Figure 4C**). These findings suggest that the immune landscape is an important complement to immunotyping.

WGCNA Analysis of Immune Related Genes in GBM

Weighted gene co-expression network analysis (WGCNA) was employed to conduct co-expression analysis of the identified immune related genes, and the results were visualized using a dendrogram (**Figure 8A**). In the scale-free network, the optimal soft threshold was set at 4 (**Figures 8B, C**). The colors of the dendrogram branches indicate different gene clusters (min module size = 20, deep split = 4 and height = 0.25) (**Figure 8D**). In the end, 13 gene modules were clustered except for the grey module (**Figure 8E**). The module eigengenes of IS2 were more abundant in the black, green, red,

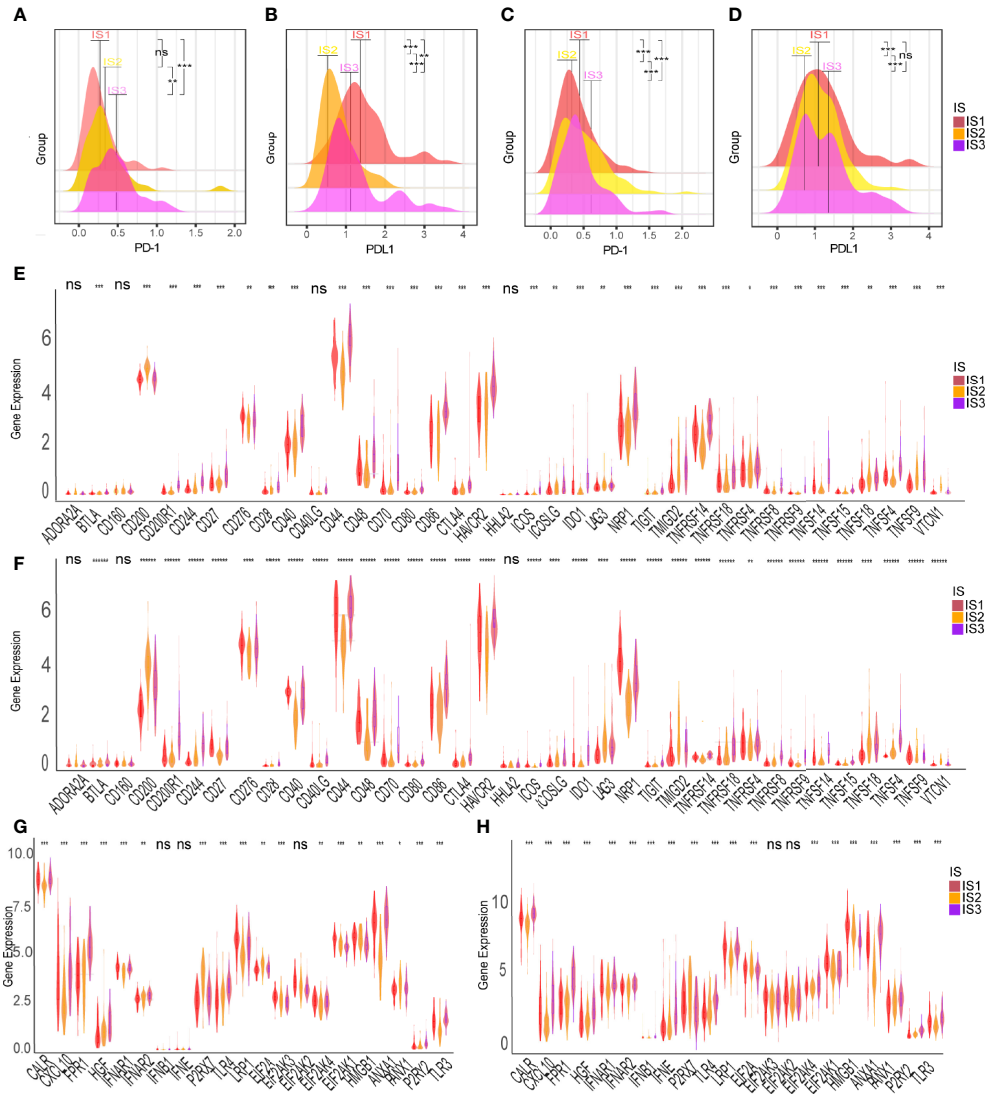

FIGURE 6 | Association between immune subtypes and ICPs and ICD modulators. Differential expression of PD-1 among the GBM immune subtypes in **(A)** TCGA and **(C)** CGGA cohorts. Differential expression of PD-L1 among the GBM immune subtypes in **(B)** TCGA and **(D)** CGGA cohorts. Differential expression of ICP genes among the GBM immune subtypes in **(E)** TCGA and **(F)** CGGA cohorts. Differential expression of ICD modulator genes among the GBM immune subtypes in **(G)** TCGA and **(H)** CGGA cohorts. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$, ******$p<0.000001$. ns, no significance.

yellow, and purple modules, while the brown, pink and turquoise modules had more eigengenes of IS3 (**Figure 8F**). Moreover, this study revealed that the module eigengenes of the green and brown modules were significantly associated with the prognosis of GBMs (**Figures 9A–C**). Interestingly, the modules eigengenes of brown and green modules were positively correlated with component 2 and component 1, respectively (**Figures 9D, F**). Biological functions analysis of the prognosis-related modules showed that genes of the brown module were enriched in macrophage activation, IL-17 signaling pathway and cytokine-cytokine receptor interaction (**Figure 9E**). The genes in the green module were enriched in antigen processing, B cell receptor signaling pathway and T cell receptor signaling pathway (**Figure 9G**). In the end, thirteen genes including PLAUR, F13A1, THBD, CD300E, HK3, FPR2, SOCS3, NDUFB9, PSMD6, PSMD10, CACYBP, GEMIN6 and PSMD14 were identified as hub genes with >80% relevance in the brown and green modules. These genes were considered to be potential biomarkers for the mRNA vaccine.

DISCUSSION

Glioblastoma multiforme (GBM) is characterized by high degree of malignancy and poor prognosis. The standard treatment plan for GBM includes maximal safe resection, radiotherapy, and concurrent administration of temozolomide (TMZ) (1). However, the therapeutic outcomes for these strategies are

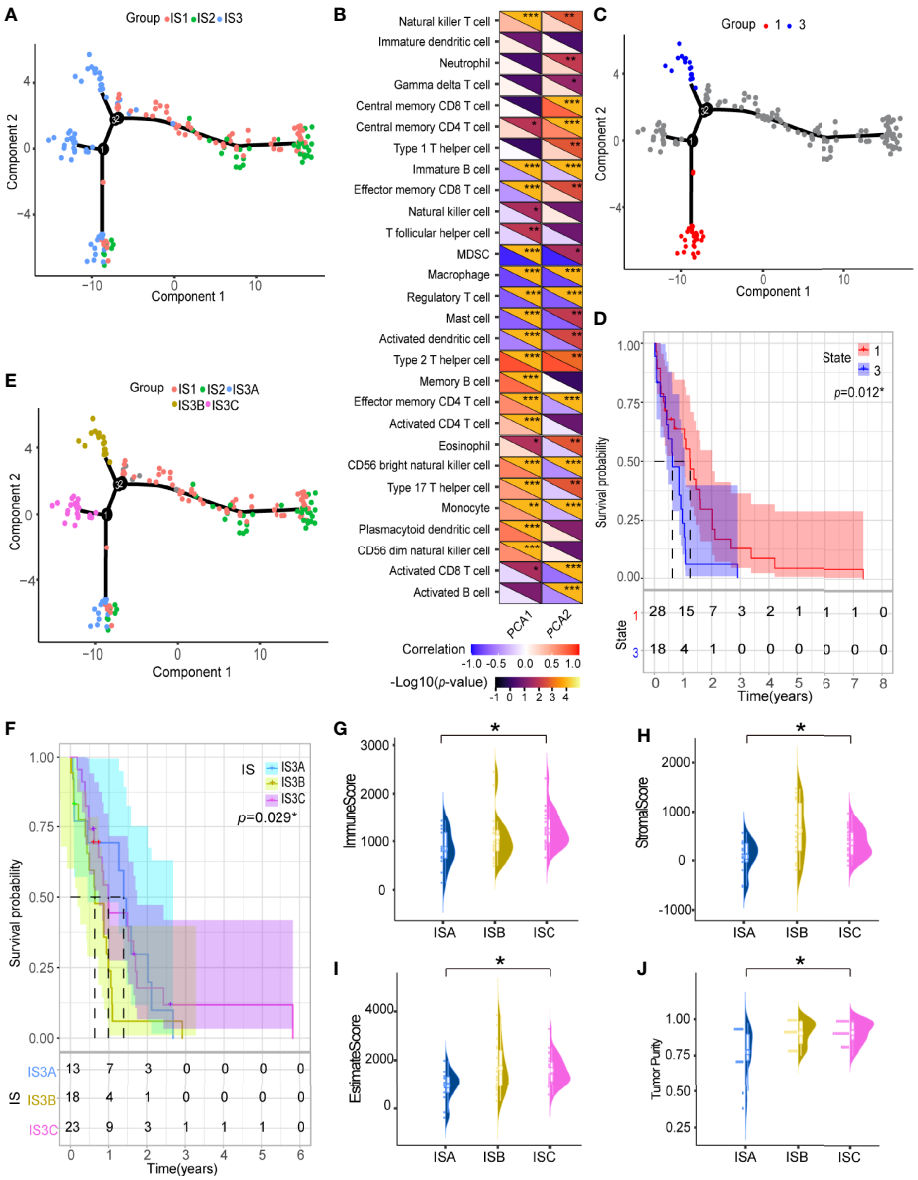

FIGURE 7 | Immune landscape of GBM. **(A)** Immune landscape of GBMs where each point represents a patient, and the immune subtypes are color-coded. The horizontal axis represents the first principal component, and the vertical axis represents the second principal component. **(B)** Heat map of two principal components with 28 immune cell signatures. Immune landscape of samples from **(C)** two extreme locations and **(D)** their prognostic status. **(E)** Immune landscape of the subsets of GBM immune subtypes. **(F)** Different subsets in IS3 associated with different prognoses. Immune assessment of different subsets in IS3, represented by **(G)** immune score, **(H)** stromal score, **(I)** estimate score and **(J)** tumor purity. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

poor with a median OS of just 12-14 months (47). There is therefore need for novel and improved therapeutic alternatives for GBM. Cancer vaccines and other immunotherapies (CAR-T, ICP blockage and so on) are promising alternative strategies for the treatment of GBMs, although they are influenced by immune escape or immunosuppression of the microenvironment (48). Tumor associated antigens are antigens preferentially expressed in cancer cells which arise due to somatic mutations or growth-related factors (49). These antigens are good candidates for cancer vaccine development. The detection and analysis of these antigens can quickly lead to the identification of suitable human mRNA vaccine targets. Recently, analysis of potential tumor antigen in pancreatic adenocarcinoma and cholangiocarcinoma provided novel insights for mRNA vaccine development of these tumors (50, 51). However, there have been no comprehensive analysis of potential GBM antigens that can be used for the development of an anti-GBM vaccine.

In this study, we identified six tumor antigens (ARHGAP9, ARHGAP30, CLEC7A, MAN2B1, ARPC1B and PLB1) that had mutations, amplification and were overexpressed in GBMs, as

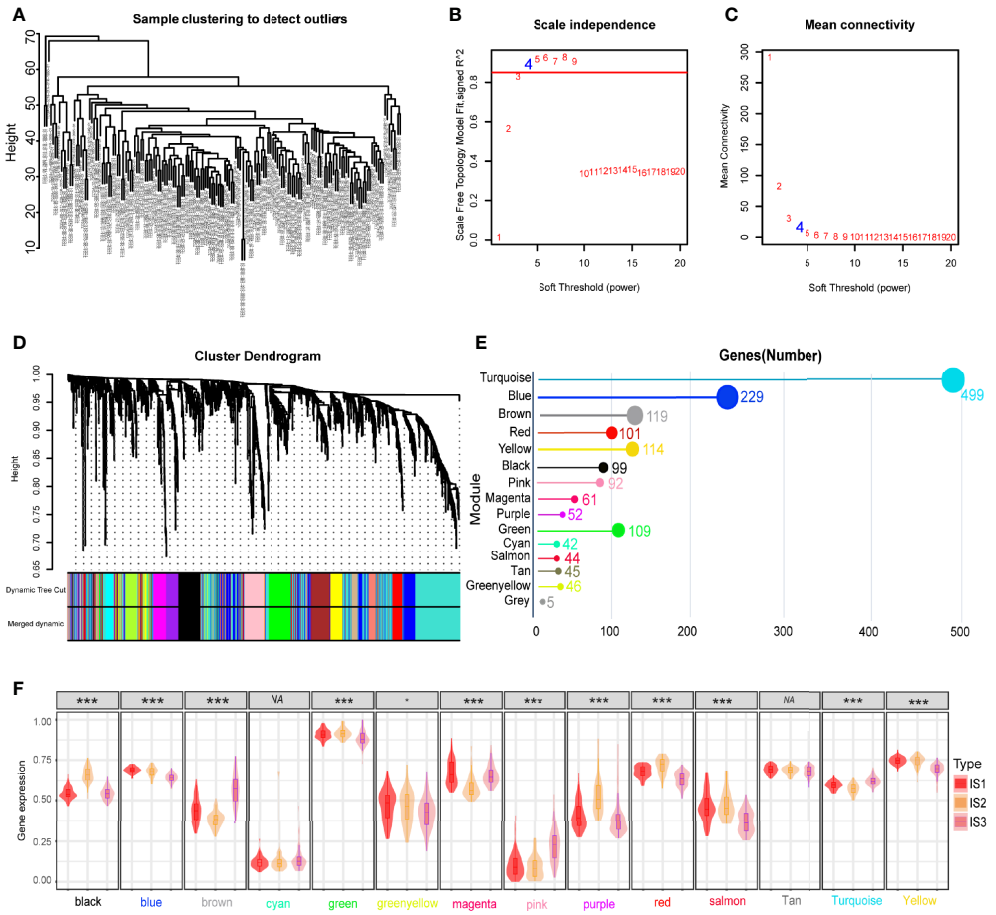

FIGURE 8 | Identification of immune gene co-expression modules of GBM. **(A)** Sample clustering. **(B)** Scale-free fit index for various soft-thresholding powers (β). **(C)** Mean connectivity for various soft-thresholding powers **(D)** Dendrogram of all differentially expressed genes clustered based on a dissimilarity measure (1-TOM). **(E)** Gene numbers in each module. **(F)** Differential distribution of feature vectors of each module in GBM subtypes. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

promising candidates for mRNA vaccine. ARHGAP30 (Rho GTPase activating protein 30) and ARHGAP9 (Rho GTPase activating protein 9) are proteins containing a Rho-GTPase activating (Rho-GAP) domain. These proteins are critical in the modulation of several tumorigenic pathways (p38/MAPK, FOX, Wnt/β-caterin pathways) and are significantly associated with the prognosis of breast tumor, bladder carcinoma and other cancers (52–56). CELC7A is a pattern recognition receptor that detects glucan-like structures to trigger the phagocytic activity of macrophages (57). CELC7A (C-type lectin domain family 7, member A) ligates galectin-9 in the tumor microenvironment of pancreatic ductal adenocarcinoma, resulting in the suppression of T cell immunogenicity and reprogramming of tolerogenic macrophages (58). ARPC1B is a constituent of the actin-related protein 2/3 (ARP2/3) complex which binds and activates Aurora A to regulate centrosome integrity (59). Since ARPC1B is required for actin reorganization and lamellipodia formation, ARPC1B mutations induce dysfunction of cytotoxic T cells (60). There are several reports indicating that mutant ARPC1B plays a significant role in immunodeficiency diseases (61–63). It was reported mutations of rs117512489 in PLB1 (phospholipase B1) was associated with the prognosis of the patients with non-small cell lung cancer (64). Results of next generation sequencing validated the role of PLB1 as a biomarker for lung cancers (65, 66). In the present study, the expression of these six antigens was positively associated with the abundance of antigen presenting cells (APCs), suggesting that they might have potential as vaccine targets and stimulate APC activation (67, 68). However, there is need for further research to determine their mechanisms of action against tumors.

In our study, we analyzed the characteristics of GBMs based on the pan-cancer immune categories and found that a majority of the GBMs had characteristics corresponding to the C4 (Lymphocytes Depleted) category. A few GBMs had characteristics corresponding to the C1 (Wound Healing) and C5 (Immunologically Quiet) categories. In fact, only a small population of tumor patients responds to the vaccine treatment, although there is a lack of tools to select patients likely to benefit from the treatment and evaluate immune response. Therefore, we clustered GBMs based on the expression of integrated

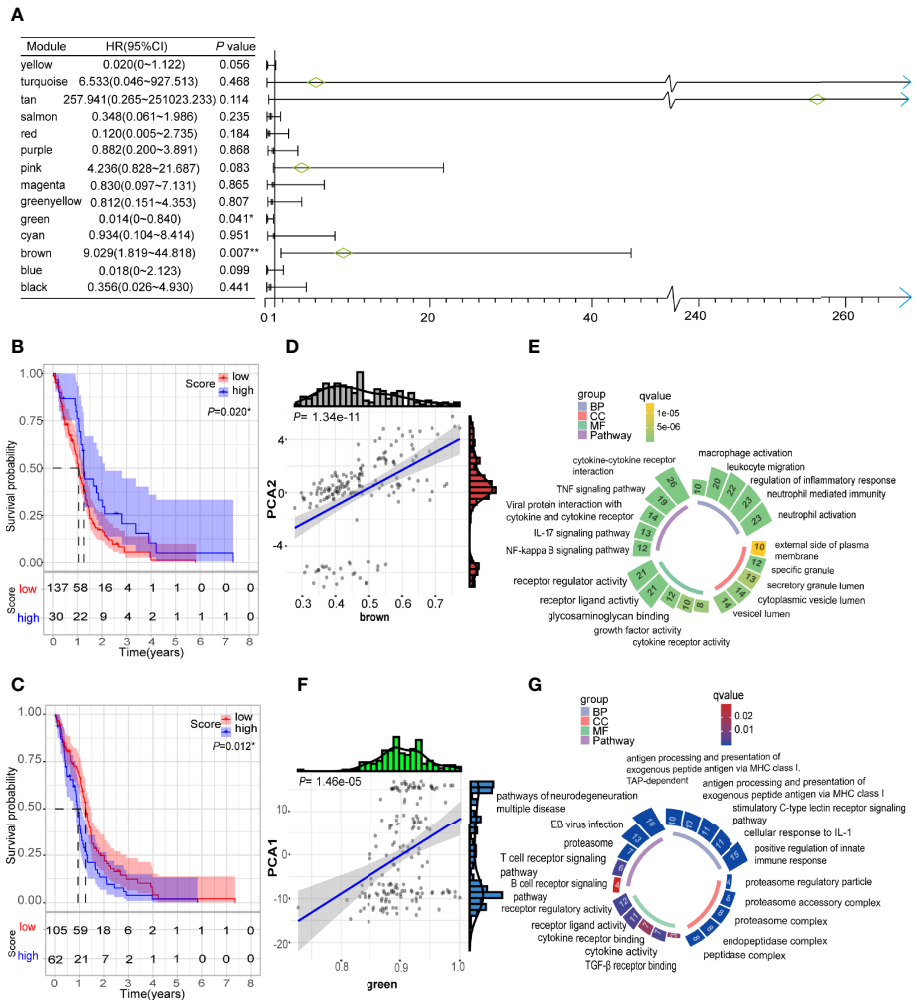

FIGURE 9 | Identification of immune hub genes of GBM. **(A)** Forest maps of single factor survival analysis of 14 modules of GBM. **(B)** Differential prognosis in brown module with high and low mean. **(C)** Differential prognosis in green module with high and low mean. **(D)** Correlation between brown module feature vector and second principal component in immune landscape. **(E)** Circular barplot showing GO term (BP: biology process; CC: cellular component; MF: molecular function) and KEGG term (pathway) in the brown module. The barplot size and color intensity represent the gene count and enrichment level respectively. **(F)** Correlation between green module feature vector and first principal component in immune landscape. **(G)** Circular barplot showing GO term (BP, biology process; CC, cellular component; MF, molecular function) and KEGG term (pathway) in the green module. The barplot size and color intensity represent the gene count and enrichment level respectively. *$p < 0.05$.

immune related gene profiles, so as to provide a guideline for the application of anti-GBM mRNA vaccine. The GBM samples were clustered into three immune subtypes with different clinical prognosis and immune profiles. GBMs in the IS3 subtype had the poorest survival compared to the IS1 and IS2 subtypes, indicating that immunotyping is a potential prognostic biomarker for GBMs. More importantly, there may be distinct mechanisms involved in the modulation of the tumor immune environment among the three GBM subtypes, suggesting that different therapeutic strategies are required for each subtype. GBMs with IS1 and IS2 represent the lack of regulatory immune cells and immunosuppressive antigen-presenting cells, resulting in T cell activation and survival advantage. Therefore, the use of immunotherapy in these patients can induce a stronger immune response. On the contrary, the immune-cold subtype (IS3) had a highly complex and thornier tumor microenvironment. Generally, macrophages are recruited into the tumor in response to inflammation-mediated chemokines, where they engulf tumor cells and present antigens to adaptive immune cells (69). GBM cells can utilize paracrine metabolites or surface signals to polarizes these macrophages toward the anti-inflammatory M2 phenotype (70). These tamed M2 macrophages expressed immune checkpoint molecules to suppress adaptive immune anti-tumor response, that supports the survival of cancer stem cells (71). The high expression of ICP (PD-1, PD-L1, LAG-3, etc.), in the GBM samples of the IS3 subtype was an indication that there was severe suppression of lymphocyte activation but exhaustion of existing T cells (72–74).

Furthermore, several immunosuppressive factors such as TGF-β and IL-10 released by GBM cells, Tregs, microglia, macrophages, also lead to local immunosuppression (75, 76). These results indicated that GBMs of the IS3 subtype were more likely to benefit from vaccination in combination with immune checkpoint blockage. In addition, the use of CAR T-cell therapy deserves to be explored.

Gliomas have poor clinical outcomes, due to the high degree of intratumoral heterogeneity (ITH) and low efficacy of immunomodulator (IM) therapy compared with other tumor types (77). Several studies indicated that the ITH of GBM contributes to the development of chemoradiotherapy resistance and different subpopulations of GBM respond differently to treatment, resulting in the generation of treatment-refractory recurrent tumors (78, 79). The machine learning-based analysis led to dimensionality reduction of the expression profile of GBMs, revealing the intra-cluster heterogeneity in GBMs. From this analysis, GBMs in the IS3 subtype were further subdivided into three subtypes. Among the three subtypes, IS3A showed significantly better survival than the other two subtypes. Estimate score analysis of the GBMs in the IS3 subtype reflected various immune factors while therapeutic estimation and application of combination therapy on these patients was required. It has been reported that combined immunotherapy involving the use of anti-CTLA-4 monoclonal antibody and vaccine can modulate the tumor microenvironment and enhance anti-tumor immune response compared to the vaccine or monoclonal antibody alone in triple negative breast cancer (80). Integrating clustering and immune landscape can provide more detailed immune profile of GBM and assist clinicians in designing accurate immunotherapy strategies.

Despite the previous reports on vaccine showed potential of GBM vaccination treatment, there is great limit of current peptide vaccines' efficacy. For example, IDH mutation frequencies were less than 10% of primary GBM which influence the efficacy of IDH targeting vaccine in primary GBMs (81). As for EGFR*vIII* targeting vaccine, it was proven for safety but no benefit to overall survival because of immunoediting under immunologic pressure (82). With more flexibility, mRNA vaccine is equipped with several advantages: not require prior knowledge, not restricted by the patient's HLA type (14). Recently, several clinical trials on mRNA vaccine therapy are ongoing (NCT02649582, NCT02808364 and NCT02709616). Interestingly, the results of a preclinical trial by Duane A showed that TMZ enhances vaccine-driven immune responses and significantly reduces GBM growth in a murine model, which suggested that vaccines therapy can act synergistically with chemotherapy in furthering a therapeutic effect (83). GBM patients who underwent vaccine therapy targeting CMV pp65 exhibited unexpectedly prolonged progression free survival (PFS) and overall survival (OS) (84). Notably, GBMs hampers immunotherapy with low infiltration of lymphocytes but high fractions of macrophages and there is need for further research on the anti-GBM mRNA vaccine and combined protocol (85). Our study comprehensively analyzed the potential antigens and the conditions for the application of the mRNA vaccine in GBMs. The results from this study provide a theoretical foundation for mRNA vaccine development and combined immunotherapy for GBMs.

CONCLUSION

ARHGAP9, ARHGAP30, CLEC7A, MAN2B1, ARPC1B and PLB1 were identified as potential GBM antigens for mRNA vaccine development. GBM patients in the IS3 subtype are more likely to benefit from vaccination.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available in The Cancer Genome Atlas database (TCGA, https://portal.gdc.cancer.gov/) and Chinese Glioma Genome Atlas (CGGA, http://www.cgga.org.cn/).

AUTHOR CONTRIBUTIONS

HL and KW designed the study, checked the data and prepared the manuscript. YX and LZ performed data collection. GL, YY, and RM searched the literature and took part in the manuscript preparation. PX, SC, and YZ conduct statistical analysis. PW and DZ supervised this project. All authors read and approved the final manuscript.

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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.2022.773264/full#supplementary-material

Supplementary Figure 1 | Procedures for the analysis of antigen detection and GBM immune subtypes identification.

Supplementary Figure 2 | Association between the expression of tumor antigens with GBM RFS. Kaplan-Meier curves showing PFS of GBM patients stratified on the basis of **(A)** ARHGAP9, **(B)** ARHGAP30, **(C)** CLEC7A, **(D)** ARPC1B, **(E)** PLB1 and **(F)** MAN2B1 expression level. p-value <0.05 was considered statistically significant.

Supplementary Figure 3 | Association between immune subtypes and TMB and mutation. **(A)** Ten highly mutated genes in GBM immune subtypes. **(B)** mutation number and **(C)** TMB in GBM IS1-IS3. $^{*}p < 0.05$.

Supplementary Figure 4 | Association between immune subtypes and immune molecules and immune cells signatures. Differential expression of **(A)** ICP genes and **(B)** ICD genes among the GBM immune subtypes. **(C)** Differential enrichment scores of 28 immune cell signatures in the above subsets. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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Bryostatin Activates CAR T-Cell Antigen-Non-Specific Killing (CTAK), and CAR-T NK-Like Killing for Pre-B ALL, While Blocking Cytolysis of a Burkitt Lymphoma Cell Line

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The advent of CAR-T cell therapy has changed the face of clinical care for relapsed and refractory pre-B-acute lymphocytic leukemia (B-ALL) and lymphoma. Although curative responses are reported, long-term cures remain below 50%. Different CAR T-cell leukemia targets appear to have different mechanisms of CAR-T escape. For CD22, therapeutic evasion is linked to down-modulation of the number CD22 proteins expressed on the extracellular aspect of the leukemia cell plasma membrane. Recently, pharmacologic agents known to induce cellular differentiation or epigenetic modification of leukemia have been shown to impact CD22 and CD19 expression levels on B-ALL, and thereby increase sensitivity to CAR-T mediated cytolysis. We explored the impact of epigenetic modifiers and differentiation agents on leukemia cell lines of B cell origin, as well as normal B cells. We confirmed the activity of bryostatin to increase CD22 expression on model cell lines. However, bryostatin does not change CD22 levels on normal B cells. Furthermore, bryostatin inhibited CAR-T mediated cytolysis of the Raji Burkitt lymphoma cell line. Bryostatin increased the cytolysis by CD22 CAR-T for B-ALL cell lines by at least three mechanisms: 1) the previously reported increase in CD22 target cell numbers on the cell surface, 2) the induction of NK ligands, and 3) the induction of ligands that sensitize leukemia cells to activated T cell antigen-non-specific killing. The opposite effect was seen for Burkitt lymphoma, which arises from a more mature B cell lineage. These findings should caution investigators against a universal application of agents shown to increase killing of leukemia target cells by CAR-T in a specific disease class, and highlights that activation of non-CAR-mediated killing by activated T cells may play a significant role in the control of disease. We have termed the killing of leukemia targets, by a set of cell-surface receptors that does not overlap with NK-like killing "CTAK," CAR-T Cell antigen-non-specific killing.

Keywords: CAR-T cell, CD22, adoptive immunotherapy, antigen density, innate immunity, T cell, cellular cytotoxicity, acute lymphocytic leukemia (B-ALL)

INTRODUCTION

Adoptive immunotherapy with chimeric antigen receptor (CAR)-mediated T cells has opened a new chapter in the treatment of relapsed and refractory pre-B cell acute lymphocytic leukemia (B-ALL) in pediatric patients as well as for leukemia and lymphomas of B cell lineage in adults (1). Targets include B-cell restricted antigens expressed early in lineage commitment such as CD19 and CD22, later in development such as CD20, and also in more terminal stages of B cell differentiation such as BCMA (2–8). To overcome antigen loss variation, CAR-T targeting multiple antigens have been proposed, including CD19/CD20 and CD19/CD22 Tandem CARs and HIV-Specific DuoCARs which express three binding moieties (9–11). Unlike the escape from CAR-T immunosurveillance by CD19-CAR, which seems to be primarily due to splice variations and thereby the loss of the CAR-binding epitope, CD22-CAR-T evasion is different (12). Leukemic escape from CD22-targeting CAR-T has been demonstrated clinically to be associated with a down-regulation of the number of CD22 molecules expressed on the cell surface (2). In 2019, Ramakrishna et al., demonstrated that inclusion of bryostatin augmented anti-CD22 CAR activity in murine model systems by increasing CD22 antigen expression on the ALL cell lines NALM6 and KOPN8, two model leukemia cell lines, as well as a patient derived xenograft, building on earlier work in chronic lymphocytic leukemia (CLL) (13, 14).

The expression of cell surface glycoproteins, such as CD22, can be regulated at the level of increased mRNA and protein expression, changes in membrane residence, or alterations in recycling of membrane proteins from endocytic vesicles. The use of epigenetic modifiers or differentiation agents has the ability to regulate each of these processes. Until recently, endocytic recycling was regarded as a largely passive process, and that resident proteins were sorted either for degradation or followed bulk membrane flow back to the surface (15). The endocytic process is now known to feature fast recycling through the early endosome, slow recycling through the endocytic recycling compartment, and in some cases retrograde transport to the Golgi apparatus. Degradation is also a carefully regulated sorting process carried out in the endolysosome, which then later fuses to form a mature lysosome (15). In an detailed study, epidermal growth factor receptor (EFGR) was found to internalize the endosome-associated transcriptional regulatory factor RNF11 which translocates to the nucleus where it regulates endoplasmic reticulum export machinery to promote the movement of newly synthesize EGFR through the Golgi to the cell surface (16). The full control of CD22 membrane residence is still under investigation and will likely change depending upon the differentiation state of the B cell.

We show that exposing leukemia cell lines to anti-CD22 CAR-T also changes CD22 surface expression. CAR-T directly and rapidly modulates CD22 surface expression. Surprisingly, the exposure to CD22 CAR-T also modulated CD19, indicating a generalized mechanism of cell surface membrane regulation that can be used to escape CAR-mediated immune surveillance. Thus, rapid modulation is part and parcel of the CAR-T interaction process with transformed B cells, that can potentially be modulated by epigenetic modifiers. Unexpectedly, we also discovered that bryostatin induces changes in immortalized B cell lines that are dependent on the differentiation state (disease origin) of the transformed cell. For pre-B-ALL model cell lines, not only was the number of CD22 molecules on the surface upregulated, two other types of innate immune targeting molecules or activities were induced. The first activity induced can be classified as sensitization to NK-killing, which can be blocked by the presence of the K562 cell lines. Here, we also describe a non-classical innate immune receptor activity that operates similarly to NK-like killing for activated human T cells, but is not blocked by K562. We refrained from the terminology "LAK cell" as this is reserved for a specific type of immune cell driven by high levels of cytokine alone (17). We refer to this second set of receptors as "activated T cell antigen-non-specific" cell ligands, that engage in "CAR T-cell antigen non-specific killing" (CTAK). This activity is induced by the unique properties of CAR-T manufacturing, and is recognized upon bryostatin treatment of ALL. In direct opposition to the effect on ALL lines, we found that bryostatin profoundly inhibits killing of the Raji Burkitt lymphoma cell line, indicating an essential dependence on B cell differentiation for sensitization to CAR-T cell mediated killing.

MATERIALS AND METHODS

Cell Lines and Culture Media

Three CD22 positive leukemia cell lines were used in this study: Raji, NALM6 and REH. The K562 cell line was used as a negative control. For Luciferase-based cytotoxicity assays, Raji-Luc, NALM6-Luc, REH-Luc and K562-Luc were used as target cells. B-LCL cell lines were used for anti-CD22 CART cells rapid expansion protocol (REP). Raji, NALM6, REH, K562, LCL, Raji-Luc and K562-Luc were provided by Dr. Michael Jensen, Seattle Children's Research Institute. NALM6-Luc and REH-Luc were produced by transducing NALM6 or REH cells with a Luciferase-expressing lentiviral vector (LV), then positive clones were selected and expanded. STR fingerprinting was conducted to verify the identity of cell lines, and each cell line was validated to be *Mycoplasma* free by qPCR. Cell lines were cultured in RPMI 1640 supplemented with 2 mM l-glutamine, 10 mM HEPES (Invitrogen), and 10% heat-inactivated FBS (VWR). Human PBMCs from healthy donors were obtained from Bloodworks Northwest and isolated with SepMate™ PBMC Isolation Tubes and Lymphoprep (Stemcell Technologies). CART and un-transduced control (UTD) cells were cultured in TexMACS™ medium (Miltenyi Biotec) with recombinant IL-2 (premium grade, Miltenyi Biotec). B cells were cultured in B cell culture media (BCM), including RPMI-1640, 10% FCS, 55 mM 2-ME, 1% Pen Strep, 10 mM HEPES, 1 mM Sodium Pyruvate and 1% MEM NEAA, supplemented with recombinant human IL-2 (50 ng/ml, Miltenyi Biotec), IL-4 (10 ng/ml, PeproTech), IL-21 (10 ng/ml, Miltenyi Biotec), and BAFF (10 ng/ml, PeproTech). NK92 were culture in RPMI medium with 10%

heat-inactivated FBS (VWR), 1% NEAA, 1% Sodium Pyruvate, 200U/mL IL-2, 2 mM L-glutamine and 25 mM HEPES.

Primary B Cell Culture and Expansion

Primary B cell expansion was carried out as per Su, K.Y., et al., with the following modifications (18). Six-well plates were pre-seeded overnight with the MS5-based stromal cell line, CD40L-low ($MS40L^{low}$), kindly provided by Dr. Garnett Kelsoe, Duke University, Durham, NC (19) in BCM. B cells were isolated from 3 individual donors using immunomagnetic bead separation (B cell isolation kit, Miltenyi Biotec), cultured in coated six-well plates, $1x10^3$ per well, in BCM for 8 d, and expanded B cells subsequently harvested and cryopreserved in 90% FBS/10% DMSO until use.

CAR-T Production

CD22 chimeric antigen receptor (CAR) used in this study consists of a single chain fragment variable (ScFv) sequence derived from m971, CD8a hinge and transmembrane domain, 4-1BB(CD137) and CD3- ζ chain signaling domains, as previously described (20). CD22 CAR-encoding lentiviral vector (LV) was produced by transient transfection of the HEK293T/17SF cell line. 2×10^8 HEK293T/17SF cells were seeded into 1L flask (Cole Palmer #EW-06019-30) with 200mL FreeStyle293 expression medium (Gibco). The following day, HEK293T/17SF cells were transfected by PEIpro (Polyplus) with plasmids encoding CD22 CAR, gag-pol, rev and VSV-G envelope protein, and sodium butyrate (MiiliporeSigma) was added at 24 h. After 2 days, supernatant was collected and filtered by 0.45uM filter, LV was concentrated by centrifugation at 10,000 xg for 4hr. Pelleted LV was resuspended in serum-free RPMI medium and stored at -80°C. PBMC were activated with TransAct activation reagent in TexMACS medium (Miltenyi Biotec) supplemented with 40 IU/mL IL-2 at density of 1 $x10^6$ cells/ml. Activated T cells were transduced with CD22-CAR LV in the presence of 8 μg/mL protamine sulfate on Day 2 in TexMACS medium supplemented with 40 IU/mL IL-2, and volume increased day 3 with IL-2 containing media. On day 4, cultures were harvested and re-seeded in TexMACS with 200 IU/ml IL-2 and expanded until harvest on day 10–13.

Rapid Expansion Protocol (REP)

Based on protocols established to expand T cell clones, CAR-T or untransduced control T cells (UTD) were co-incubated with irradiated B-LCL (8000 rads) at a 1:7 ratio in complete RPMI supplemented with IL-2 (50 U/ml), IL-7 (5 ng/ml), and IL-15 (0.5 ng/ml). Cells were passaged every 2-3 days and harvested after 10-13 days of expansion (21) (Riddell S and Greenberg P, US Patent 5,827,642). The REP maintains the original phenotype of expanded CAR-T and T cells clones, and CAR-T and UTD remain CD56 negative (**Supplementary Figure S7N**).

Biochemical Reagents, Antibodies and Recombinant Proteins

5-azacytidine, Vorinostat, Panobinostat, All-Trans Retinoic Acid (ATRA) and Bryostatin 1 (Sigma) were used to treat Raji, NALM6 and REH cell lines for 48 hours, or 24 hours in the case of Bryostatin. Viability, CD19 and CD22 expression levels were assayed at the end of treatment.

Flow cytometry was performed on a Fortessa (BD Biosciences) and data analyzed with FlowJo software (BD Biosciences). Expression levels of CD19 and CD22 on leukemia lines were measured using Quanti-Brite PE beads (BD Bioscience) and PE-labeled anti-CD19 (BioLegend, clone HIB19) and anti-CD22 (BD Bioscience, clone HIB22) antibodies. To determine antigen copy number per tumor cell, cellular MFI was compared with a linear plot of bead MFI versus the number of PE molecules per bead. All staining was performed in 100 μl FACS buffer (PBS + 2% BSA). T cells were phenotyped with: anti-CD3 (BioLegend, clone HIT3a, PB), CD4 (BioLegend, clone SK3, FITC), CD8 (BD Biosciences, clone RPAT8, BUV395), biotinylated CD22 protein (Sino Biological, for CAR detection) and SA-PE (BioLegend). NK92, un-transduced PBMCs and CD22 CAR-transduced PBMCs were phenotyped with: anti-NKG2D (Biolegend, clone 1D11, APC), DNAM-1 (Biolegend, clone 11A8, APC), NKp30 (Biolegend, clone P30-15, PE), Nkp44 (BD Biosciences, clonep44.8.1, PE), NKp46 (Biolegend, clone 9E2, PE), TRAIL (Biolegend, clone RIK-2, PE), FasL (BD Biosciences, clone NOK-1, APC), KIR2DL1/DS1 (Beckman Coulter, catalog A09778, PE), KIR3DL1/DS1 (Beckman Coulter, catalog A60795, PE), NKG2A (Biolegend, clone S19004C, PE), ICAM1 (Biolegend, clone HA58, PE), ICAM2 (Biolegend, clone CBR-IC2/2, PE), LFA-1 (Biolegend, clone m24, APC), CD56 (BD Biosciences, clone R19-760, PE).

CAR-T and Leukemia Cell Co-Culture and Separation

Anti-CD22 CART cells and leukemia targets (Raji, NALM6 and REH) were cultured at an effector to target ratio (E:T) of 4:1, 2:1, 1:1 or 0.5:1 for 24 hours with or without bryostatin, at which time CD19 and CD22 expression levels were quantified. To assess surviving leukemia target cells, co-cultures from the 1:1 ratio were harvested at 24 hours, cell populations separated by CD3-positive immunomagnetic bead selection (Stemcell Technologies), depletion verified by flow cytometry and CD3 negative cells (leukemia) cultured over time to assess antigen expression.

Cut and Tag Analysis

Fresh cells ($2x10^5$ to $5x10^5$ per treatment) were harvested and washed twice in 1.5 mL wash buffer (20mM HEPES pH 7.5; 150mM NaCl; 0.5mM Spermidine (Sigma S2501); 1× Protease inhibitor cocktail, Roche), and Cut&Tag libraries generated, following the protocol "Bench top CUT&Tag V.2" (22).

Cut and tag DNA libraries were sequenced on a HiSeq instrument (Novogene, Sacramento, CA), paired-end 150, with read depth of 17M per sample. The quality of sequencing data was checked by FastQC (23). FastQC: A Quality Control Tool for High Throughput Sequence Data). Sequencing adaptors identified and trimmed by TrimGalore [Trim Galore (RRID : SCR_011847)]. Sequencing reads were aligned to the UCSC Hg38 using the Bowtie2 package (24). Alignment results were normalized by the RPKM (Reads Per Kilobase of transcript, per Million mapped reads) method and methylation heatmaps

around gene regions were plotted by DeepTools2 (25). Peak calling analysis was done by SEACR (26). Normalized bigwig results were visualized in UCSC genome browser. Differential peak analysis was done by DESeq2 (27) and peaks were annotated by GSCA (Ji Z and Ji H (2014), GSCA: Gene Set Context Analysis. R package version 1.4.0.).

Cytolysis and Inhibition Assays

5 x 10^3 target cells (Raji-Luc, NALM6-Luc, REH-Luc or K562-Luc) were co-cultured with UTD control or anti-CD22 CAR-T cells at various effector to target ratios (16:1, 8:1, 4:1, and 2:1) in 96-well plates and incubated overnight at 37°C, 5% CO_2 in 100 µL of complete RPMI medium without cytokines. Twenty-four hours later, 100 µL of SteadyGlo reagent (Promega) was added to each well and incubated for 10 minutes at room temperature followed by quantification of luminescence using an Enspire plate reader (Perkin Elmer). The luminescence was captured as counts per second (CPS) for each experimental well containing the indicated E:T ratio (sample CPS), target cells alone (target CPS) and tween-20 treated target cells (negative CPS). Percent specific lysis presenting luciferase reduction was calculated as: (1- (sample CPS-negative CPS)/(target CPS-negative CPS)) x 100%.

For ligand-based cytolysis blocking assays, 5 x 10^3 target cells (NALM6-Luc or REH-Luc) were plated in a 96-well plates in 50uL complete RPMI medium. Recombinant protein (DNAM-1-his, Acro Biosystems, DN1H52H6; NKG2D-his, Acro Biosystems, NKDH5245; NKp30, Acro Biosystems, NC3H5228) or anti-ICAM1 antibody (Biolegend, 322721) was added to target cells at 10ug/mL and incubated at 37°C for 30min. 5 x 10^4 effector cells (NK92, UTD or CD22 CART) were added to target cells and treated with 1nM Bryostatin at 37°C overnight.

Reverse Transcription Droplet Digital PCR (RT-ddPCR)

Cells from each condition were collected and RNA was isolated by RNeasy mini kit (Qiagen, Catalog#74104). RNA quality was checked by high sensitivity RNA ScreenTape assay (Agilent, 4200). RNA quantity was determined by Qubit RNA RS kit (Thermo Fisher). RNA samples were mixed with one step RT-ddPCR advanced kit for probes (Bio-Rad), together with ddPCR GEX primer/probe for CD19 or CD22 (Bio-Rad) in a 96 well plate to generate RT-PCR reaction mix. Reaction droplets were generated by QX200 AutoDG droplet generator, PCR reaction was performed by C1000 Touch Cycler (Bio-Rad). RT-PCR droplets were read by QX200 droplet digital PCR system and data was analyzed using Quantasoft (Bio-Rad).

Western Blot

One and a half million cells from each treatment were washed twice in cold PBS, lysed in 100 ul cold RIPA buffer (Bio-Rad) containing protease inhibitor cocktail (Roche). The lysate was incubated for 1 hour on ice, pelleted at 15000 RPM at 4°C for 20 min, and supernatants collected and mixed with 200 µl Laemmli Sample Buffer (Bio-Rad), then boiled for 3 min at 100°C. Protein concentrations were determined by Nanodrop and 20µg of each sample resolved by PAGE and proteins transferred to 0.45 µm nitrocellulose transfer membrane (Bio-Rad) and probed with primary antibodies against CD19 or CD22 with β-actin (Odyssey Li-Cor, Lincoln NE) overnight at 4°C, and secondary IRDye 800CW antibody at room temperature for 1 hour. Bands were visualized and quantified on an Odyssey imaging system with Image Studio lite software (LI-COR). Relative band intensity of CD19 and CD22 was calculated and normalized to β actin.

Statistical Analyses

Plots show average of three replicate wells, standard deviation, and p-value as calculated by nonparametric t test, unless otherwise noted. All plots and analyses were analyzed using Prism software (v. 9.2.0, GraphPad Software, LLC) and are representative of three experiments, unless otherwise noted.

RESULTS

Impact of Differentiation Agents on CD22 and CD19 Surface Expression

The modulation of CD22 expression levels on the surface of leukemia cells is of great interest to the immunotherapy field. To explore mechanisms to increase CD22 expression we tested whether differentiation agents or epigenetic modifiers that are well-studied in human clinical trials are able to impact the expression of CD22 on the surface of model cell lines as well as normal B cells. The B cell leukemia lines tested were the Burkitt lymphoma cell line Raji, and the B cell acute lymphocytic leukemia (ALL) cell lines NALM6 and REH. Panobonistat was tested for impact on cell viability and target antigen expression from 0.5 to 100 nM, at 48 hours. No impact on viability was seen up to 5 nM (**Figure 1**). No increase in CD22 expression was seen in this concentration range (Summarized in **Supplementary Table 1**). Vorinistat (SAHA) was also tested at 48 hours, at concentrations ranging from 0.1 to 20 uM. Impact on viability was seen at 1 or 5 uM, and at or below these ranges no increase in CD22 expression was seen. ATRA was tested between 0.1 and 100 uM at 48 hours, and no impact on cell viability was seen at 10 uM or below. Notably, ATRA increased CD22 expression on Raji cells, while NALM6 and REH levels remained constant. 5-Azacytidine was also tested at 48 hours at concentrations between 0.1 and 100 uM. No impact on viability was seen at 5 uM or below. While a slight rise in CD22 expression was seen at 0.1 mM 5-Azacytidine, this difference did not reach statistical significance. Because of the rapidity of effects seen with bryostatin, experiments were carried out for 24 hours. Bryostatin has no impact on cell viability from 1 nM up to 200 nM, and increased CD22 expression in each cell lines tested, although the change in REH was not statistically significant. Assays were also carried out in a similar manner to assess the impact of each agent on the number of surface CD19 molecules expressed per cell. CD19 was far less amenable to modulation by epigenetic or differentiation agents. Only with bryostatin, and

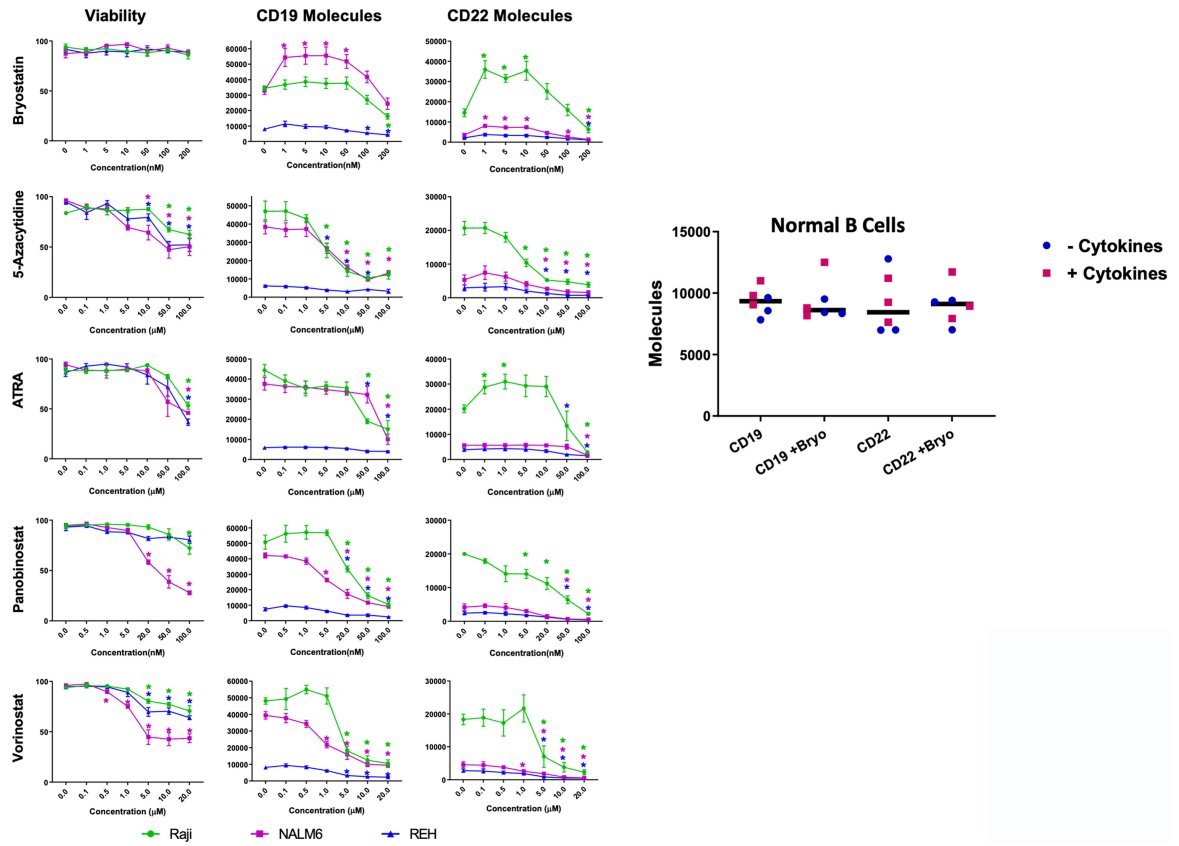

FIGURE 1 | Viability and Surface Expression of CD19 and CD22 in treated B-cell leukemia cell lines and normal B cells. Left panel: Epigenetic modifiers/ differentiation agents (Bryostatin, 5-Azacytidine, ATRA, Panobinostat or Vorinostat) were added at increasing concentrations (x-axis, as indicated) to the culture media of B cell lines (Raji-green circle, NALM6-magenta square, REH-blue triangle) for 48 hours (or 24 h for bryostatin). Following drug exposure, cell viability was calculated and plotted (column 1). Each agent adversely affected viability as concentration increased, except for bryostatin. Surface expression of CD19 (column 2) and CD22 (column 3) in leukemia cell lines was qualified by flow cytometry using Quanti-Brite PE beads. Average of triplicate wells is shown, values differing from untreated controls are indicated, * indicates $p<0.05$. Right panel: Expanded peripheral blood B cells from three donors, cultured on CD40L expressing feeder cells in media supplemented with (squares) or without (circles) B cell growth factors (IL-2, IL-4, IL-21, BAFF, see *Materials and Methods*), were tested for changes in cell surface expression induced by bryostatin. The number of CD19 and CD22 molecules differed between donors to a degree, but was not significantly impacted by bryostatin, paired t-test $p>0.05$, grand median, solid bar, shown for reference.

only in the NALM6 cell line, were statistically significant increases noted.

To explore the impact of bryostatin on CD22 and CD19 expression on normal B cells, B lymphocytes were purified by negative selection (untouched) and cultured on a CD40L-expressing feeder cell line, with or without the supporting cytokines, IL-2, IL-4, IL-21 and BAFF, as reported by Su et al., for seven days (18). Expanded B cells were cultured in the presence of 1 nM bryostatin for 24 hours and the number of CD19 and CD22 molecules per cell analyzed. Expression of CD22 and CD19 on the expanded normal B cell population was not affected by bryostatin, **Figure 1**. This implies that the response of Raji more closely resembles normal B cells, in keeping with the more developmentally mature status of Burkitt lymphoma in comparison to pre-B-ALL. We further explored the mechanism by which bryostatin impacted the level of CD22 antigen expression by quantifying the RNA and protein.

Up-Regulation of CD22 by Bryostatin Includes Minor Increases in Transcriptional Activation

Bryostatin had the broadest effect (with respect to degree of increase in CD22 and consistency across cell lines) on CD22 surface expression. We therefore sought to establish if this effect was due to a concomitant increase in total CD22 protein, as well as measuring the amount of CD22 mRNA. Although bryostatin appeared to increase the amount of total protein for both CD22 and CD19, these differences were not statistically significant when assessed by Western blot, **Figure 2**. Likewise, when the amount of mRNA encoding these surface markers was quantified, no significant differences were seen, except for CD22 expression in Raji.

In addition to the known modulation of protein kinases (PKC delta and epsilon) and c-Jun, we sought to determine if bryostatin induces changes in the epigenome of treated cells (28).

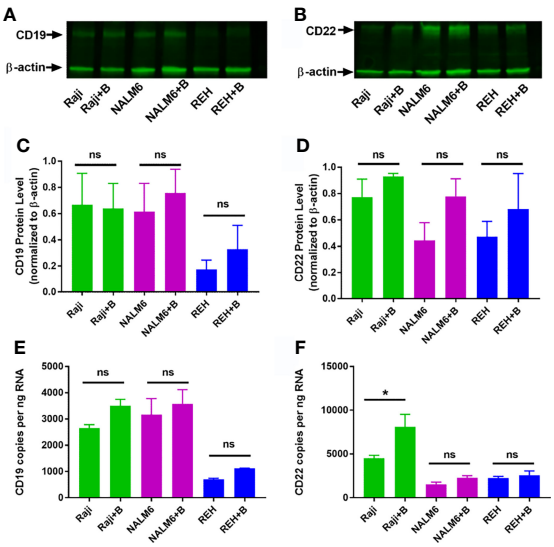

FIGURE 2 | Total RNA and protein levels of CD19 and CD22 in bryostatin treated leukemia cell lines. Western blot analysis of **(A)** CD19 and **(B)** CD22 protein expression in Raji, NALM6 and REH cell lines with (+B) or without bryostatin treatment. **(C)** CD19 and **(D)** CD22 band intensity from three independent experiments was quantified and normalized to β-actin. For each line, treated and non-treated groups were compared. There was no significant difference (ns) between groups of at the protein level. RNA levels for **(E)** CD19 and **(F)** CD22 were quantified by ddRT-PCR. CD19 and CD22 copies per ng RNA were calculated and analyzed. Significant differences between treated and untreated groups were seen for CD22 in Raji cells ($p < 0.05$). *$p < 0.05$. ns, not significant.

This would extend the known effects of this agent to include modulation of global gene expression programs, and perhaps identify specific alterations. Cut&Tag analysis (Cleavage Under Targets and Tagmentation), developed by the Henikoff lab at the Fred Hutch, goes beyond ATACseq, in that specific epigenetic modifications of histones, as determined by specific antibody cleavage sites, are measured and characterized (22, 29). Increased H3K4me3 and H3K4me2 signal (trimethylation or demethylation of lysine 4 on the histone H3, associated with activation of transcription from nearby promoters) or the opposing H3K27me2 (dimethyl state of lysine 27 of histone H3, associated with inactivation of transcription) marks can be readily visualized by mapping resultant amplified segments. Global alignment of transcriptional start sites identified by Cut and Tag demonstrates that our analysis compares numerous bryostatin-induced changes in gene expression, and that bryostatin treatment in and of itself did not profoundly change the net transcriptional activity of the treated leukemia cell lines (**Supplementary Figure 1**). For CD22, small, but statistically significant, increases in reads for H3Kme2 in Raji cells, and for H3Kme3 for all three lines (Raji, NALM6, REH) were seen with bryostatin treatment (**Supplementary Figure 2**). The only significant change for CD19 was seen in Raji cells, and only for H3Kme3 (**Supplementary Figure 3**). Although there are slight increases in mRNA and total protein expression, and bryostatin does have measurable epigenetic effects, these are unlikely to account for the rapid increase in target antigen expression induced by bryostatin over 24 hours.

Coculture of Leukemia Cells With CD22 CAR-T Decreases On-Target and Off-Target Antigen Expression

The observation that relapsed disease is associated with a lower expression of CD22 antigen on the leukemia cell surface led us to explore the temporal interactions between CAR-T cells and leukemia cell line targets in the presence of bryostatin. Using a range of effector (CD22 CAR-T) to target (leukemia line) ratios (E:T) we found that the co-incubation of CAR-T with leukemia cell lines induces a profound decrease in the number of cell surface antigens expressed on the cell surface (**Figure 3**). The assay was carried out by culturing leukemia cells for 24 hours in the presence of 1 nM bryostatin for 24 hours, followed by the overnight addition of CD22-specific CAR-T for another 24 hour period, again in the presence of bryostatin. At the concentration used, bryostatin does not impact CAR-T activity (not shown). As expected, CD22 CAR-T induced profound and rapid down-regulation of CD22 antigen expression on the leukemia cell surface. Surprisingly, this effect was also seen when the levels of CD19 were analyzed on the leukemia cell surface, **Figure 3**. Thus, CD22 CAR-T cells rapidly down-modulate not only CD22 but also CD19. The effects were seen with or without bryostatin addition. However, including bryostatin did have an effect on the net amount of antigen down-modulation, in that moderately higher levels of antigen expression were noted for both targets during CAR-T co-culture. Thus, in a short-term assay, bryostatin impacts target antigen expression. Overall, co-incubation with CAR-T decreases CD22 and CD19 surface antigen expression on leukemia cells surviving CD22 CAR-T co-culture. Antigen expression was somewhat higher in Raji and NALM 6, and somewhat lower in REH treated with bryostatin. This informs us that inclusion of brysotatin, most clearly for NALM6, keeps target antigen expression at a higher level even while undergoing CAR-induced antigen down-modulation, and thus may aid in immune elimination.

Down-Regulation Occurs Rapidly, and Reverses Rapidly

To determine if CAR-T-mediated CD22 on-target and off-target antigen modulation was a lasting effect, CAR-T and leukemia cells were separated following overnight co-culture using anti-CD3 immunomagnetic beads, and leukemia cells cultured alone in fresh media. Following removal of CD22-specific CAR-T cells, cultured leukemia cell lines demonstrated differential re-expression of CAR target antigens, **Figure 4**. Raji cells co-cultured with CD22-CAR-T took more than 3 days to fully recover CD19 expression from CD22 CAR-T exposure, yet this recovery was complete. As expected bryostatin markedly upregulated CD22 on Raji cells, this increased level persisted to day 3, and returned to original levels by day 7. While CD22 CAR-T reduced CD22 levels for at least 3 days, this effect was markedly reversed by bryostatin. Thus, with Raji targets, bryostatin has a

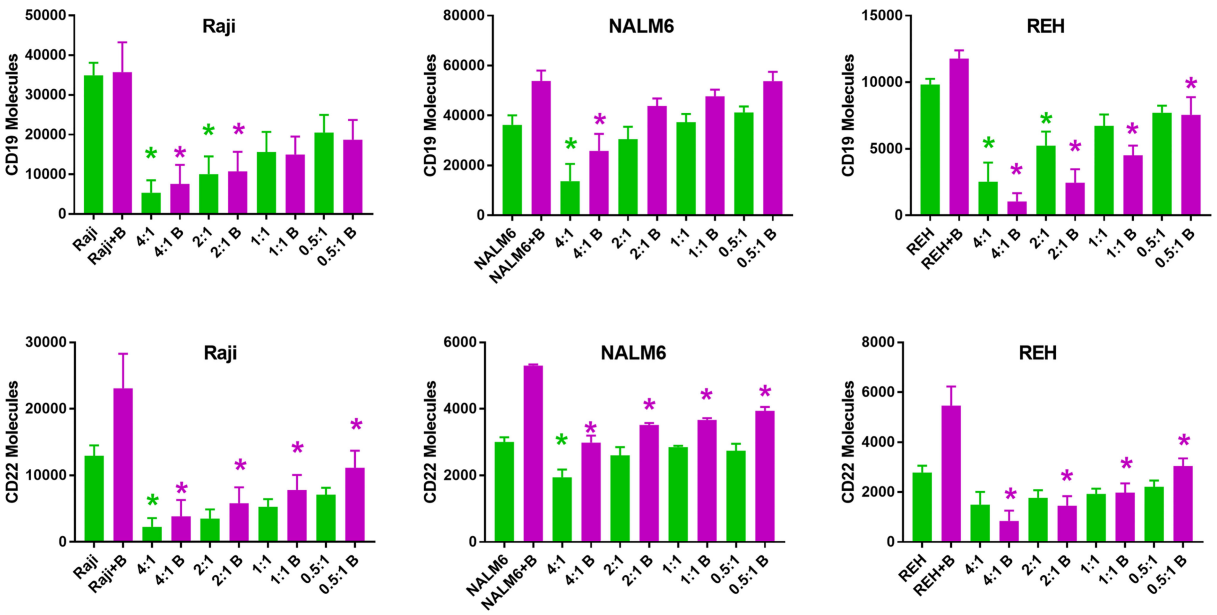

FIGURE 3 | Surface expression of CD19 and CD22 upon co-culture with anti-CD22 CAR-T. Using Quanti-Brite analysis, the number of CD19 and CD22 molecules (y-axis) on the surface of Raji, NALM6, and REH cell lines was quantified, following co-culture with CD22 CAR-T, at the indicated effector to target ratios, x-axis. The leftmost pair of columns quantifies surface expression on untreated cell lines. Significant differences from control are shown *p < 0.05. The x-axis lists the cell line tested, exposure to bryostatin (B, magenta bars) or CD22 CAR-T alone (green).

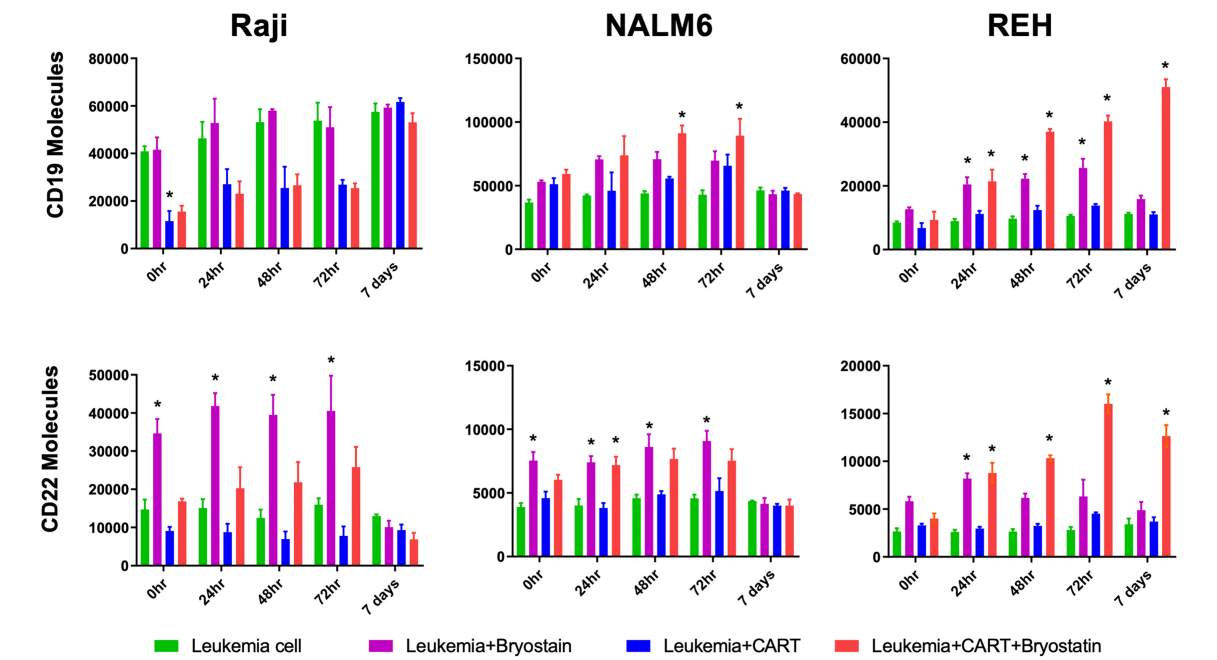

FIGURE 4 | Surface expression of CD19 and CD22 following bryostatin wash-out and CAR-T removal. After overnight culture with anti-CD22 CAR-T, the number of cell surface proteins was quantified using Quanti-Brite analysis, average of triplicate wells and standard deviations are shown. 0 hr, x-axis, is after the overnight culture, and each time point represents cell surface proteins on the surface of untreated Raji, NALM6, or REH (Leukemia cell, green bars), treated with bryostatin alone (Leukemia + Bryostatin, magenta bars), treated with CAR-T alone (Leukemia+CART, blue bars), or treated with both CAR-T and bryostatin (Leukemia+CART +Bryostatin, red bars), at the time points listed, x-axis. Significant differences from leukemia alone are shown *p < 0.05.

decidedly beneficial impact on the upregulation of CD22. NALM6 showed a similar preservation of both CD19 and CD22 upregulation following CD22 CAR-T co-culture in the presence of bryostatin. At the E:T ratio evaluated, no large down-regulation of CD22 expression was seen due to CD22 CAR pressure. This requires the higher E:T presented in **Figure 5**. The REH cell line displayed an unexpected result. Immediately following separation from CAR-T and at 24 hours, bryostatin alone and bryostatin and CD22 CAR-T had increased CD19 and CD22 expression. Either bryostatin treatment alone or CD22 CAR-T treatment had no long-term effect, as by day 7 expression levels returned to those of untreated REH. However, treatment or REH with bryostatin and CD22 CAR-T cells resulted in prolonged upregulation of both CD19 and CD22 expression. This result will be explored in future studies, and implies an interesting additive effect.

Trogocytosis Is Unlikely to Play a Major Role in Antigen Down-Regulation

One well-described mechanism for altering or sharing cell surface antigen expression is trogocytosis, defined as transposition of cell membrane or cell membrane proteins between cells during cell-cell interactions (30). We tested if CAR-T cells were able to acquire either on-target or off-target cell surface antigen upon co-culture with leukemia cells, **Figure 5**. When CAR-T cells specific for CD22 were analyzed for either CD22 or CD19 acquisition following co-culture with leukemia target cells, CD19 appeared to transfer more readily to the CAR-T cell surface than CD22. This likely reflects the relative increased abundance of CD19 on the membrane of the leukemia cell. Clearly, trogocytosis is not limited to the CAR target antigen, as both CD19 and CD22 were transferred to the T cell surface. Moreover, this supports the original definition of trogocytosis, the transposition of a membrane patch, as opposed to single protein transfer. Importantly, this effect was not uniform across the leukemia cell targets. While Raji cells appeared to readily transfer membrane (and thereby CD19 and CD22 expression on CAR-T), this effect was quite limited in NALM6 and REH cells and unlikely to drive the loss of target antigen expression at the cell surface we measured.

CD22 CAR-T and NK-92 Activity Against Leukemia Depends on the Leukemia Cell Type and the Effects of Bryostatin Treatment

Our motivation for studying the down-modulation of target antigens was to explore the effect of epigenetic modifiers on these changes, and to determine their overall effect on leukemia cell cytolysis. When cytolytic assays were caried out following pre-treatment of leukemia target cells with bryostatin, we found differential effects according to the cell line analyzed, **Figures 6A–D**. Without bryostatin, we found that increasing E:T ratios resulted in increased cytolysis for all cell lines, with the exception of K562, an antigen negative leukemia included as a control for NK cell-like activity. For the ALL lines NALM6 and REH, the killing of leukemia targets mediated by CD22 CAR-T

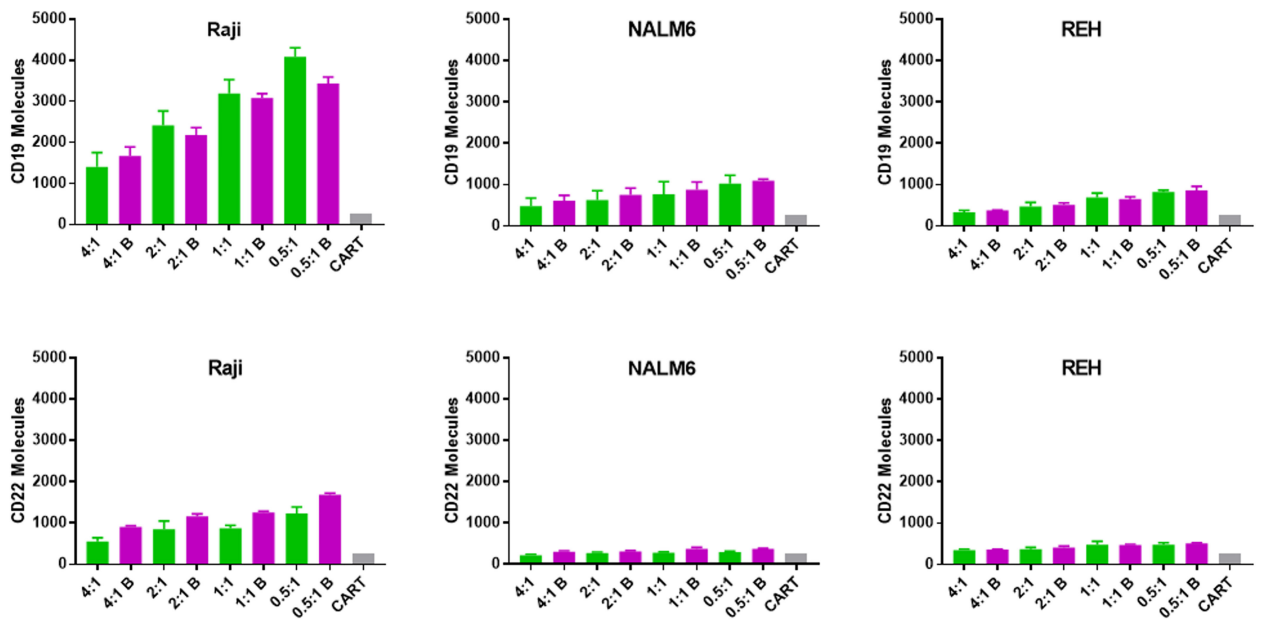

FIGURE 5 | Transfer of CD19 and CD22 to CD22 CAR-T following overnight culture with leukemia cell lines. The number of CD19 and CD22 molecules acquired by anti-CD22 CAR-T (trogocytosis) was quantified using Quanti-Brite analysis, as per **Figure 3**, however in this case the T cells were analyzed. Average antigen expression and standard deviation are shown. Background signal is shown as a gray bar for each subgroup (CART). The x-axis lists the anti-CD22 CAR-T to leukemia cell ratio (E:T) used for each condition and indicates if the leukemia line had been treated with bryostatin (B, magenta bars).

was greatly enhanced by bryostatin. However, we also saw an increase in the killing of these ALL lines mediated by untransduced/activated (UTD) T cells induced by bryostatin. Thus, treatment of leukemia targets (as indicated by +B in **Figure 6**) with bryostatin had a profound effect on cell-cell killing mediated by activated T cells in general, implying that non-CAR-T specific killing mechanisms were invoked. To the contrary, Raji cells showed the opposite effect. Although bryostatin does indeed increase the target antigen number on the cell surface (**Figure 1**), bryostatin treatment results in a marked inhibition of cellular cytotoxicity. A classic cellular immunology technique to block non-antigen dependent (usually NK-associated) killing is called "cold-target inhibition" (31–33). In this technique, used to differentiate between receptor-mediated ADCC, NK cell activity, and "natural" cytolysis by other immune cell subtypes, a 30:1 excess of unlabeled (in this case luciferase non-expressing) K562 cells are added into the cellular cytolysis assay, **Figures 6E–H**. Cold target inhibition had no effect in the Raji cytolysis assay. This indicates that CD22 CAR-T activity against Raji is strictly driven by the CAR, and not other target antigens initiating susceptibility to UTD-mediated killing. For the REH cell line, bryostatin treated cells upregulated ligands that were recognized by activated T cells, i.e. strong UTD-mediated killing was induced. When unlabeled K562 were added to the killing assay for cold-target inhibition, non-specific killing by non-CAR expressing activated T cells (UTD) was blocked, **Figure 6G**. This indicates that induction of a set of classical NK ligands on REH was responsible for the UTD-mediated killing. The same effect was seen when CD22 CAR-T and NALM6 were co-incubated, **Figure 6F**, although bryostatin appeared to have a more pronounced effect. Thus, bryostatin treatment induces B-ALL sensitivity to both CAR-T specific and non-specific killing mechanisms. The ability to block these effects with an excess of unlabeled K562 cells demonstrates that activation of T cells to produce CAR-T induces an NK-like activity. However, the ligands to detect this activity requires the ALL to first be activated by bryostatin. This could thus be classified as a bryostatin-induced off-target/on-tumor activity. We have termed this "CAR T-cell antigen-non-specific killing" or CTAK, to differentiate it from NK- or LAK-mediated killing. It requires both the induction of new targets on the leukemia and the ligands expressed on highly activated T cell populations, such as those induced by CAR-T production.

To further explore the activity of NK cells against bryostatin-treated B cell leukemia cell lines, we tested the NK92 cell line in direct cytolysis assays, **Figure 7**. Use of NK-92 cells avoids donor-to-donor variability and NK culture condition concerns. NK92 are currently being tested natively or modified with CARs in clinical trials, and may represent a complimentary treatment option to CAR-T (34, 35). Our data demonstrate that the Raji cell line is effectively lysed by NK92, and that this lysis is not impacted by the presence of K562 cold-target inhibition, **Figure 7E**. As the control experiment with K562 demonstrates (**Figures 7D, H**), cold-target inhibition completely abrogates cytolysis of the self-same target. The results with the pre-B ALL cell lines were unexpected, in that there was no lysis of ALL by NK-92 without bryostatin treatment. Moreover, once NK-92

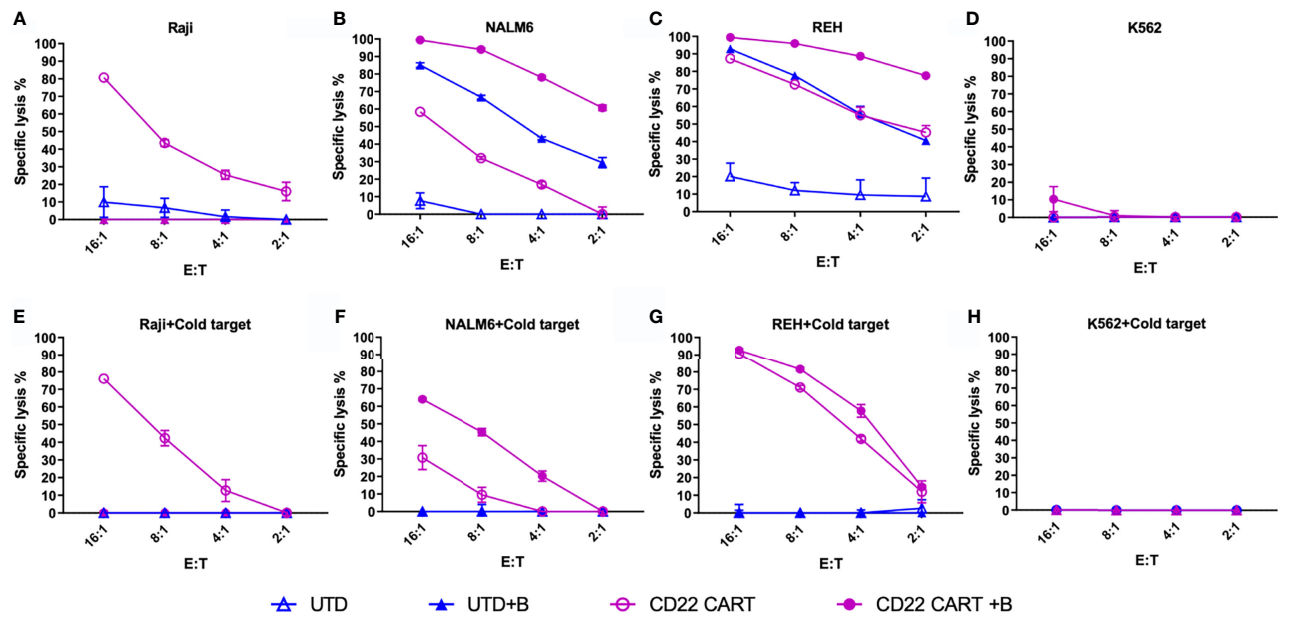

FIGURE 6 | Anti-CD22 CAR-T mediated cellular cytotoxicity (CTL) of bryostatin-treated leukemia. **(A–D)** Average lysis from triplicate wells for four cell lines (Raji, NALM6, REH, and K562) by anti-CD22 CART (CD22 CART, open circle) or un-transduced T cells from the same donor (UTD, open triangle), treated with bryostatin (+B, closed shape) or untreated (open shape), a the E:T ratios listed on the x-axis. **(E–H)** Assay tested in parallel including cold-target inhibition (addition of K562 at a 30:1 E:T ratio). Representative results for T cells from 3 donors are shown, each data point showing the average and standard deviation from three replicate wells.

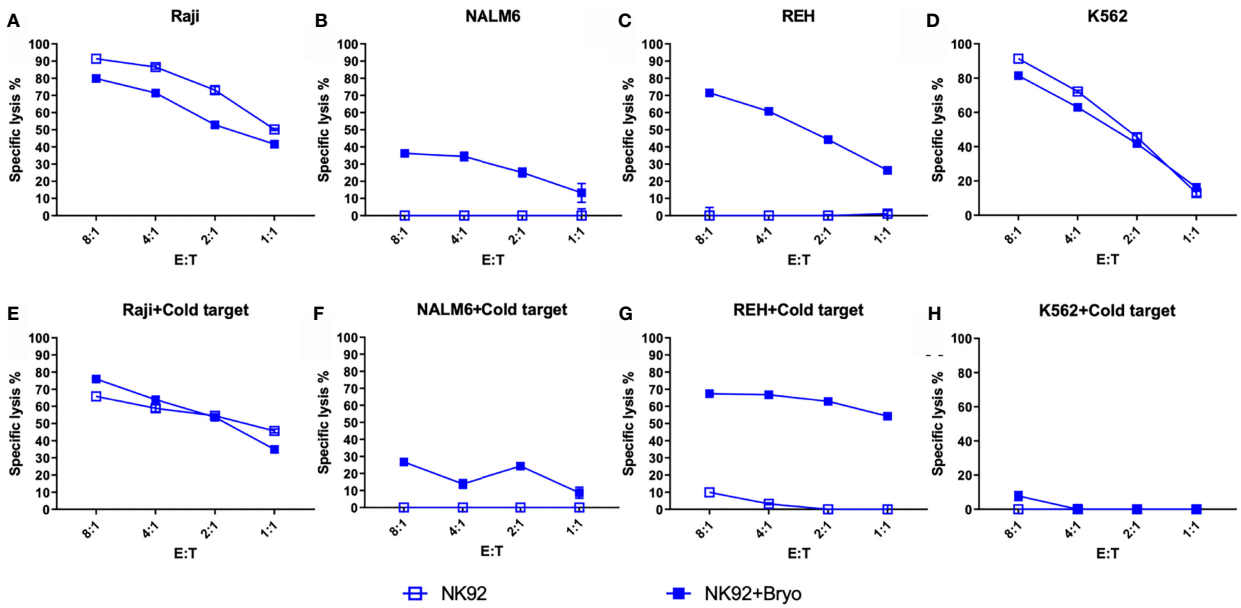

FIGURE 7 | NK92-mediated cellular cytotoxicity of bryostatin-treated leukemia. **(A–D)** Average lysis from triplicate wells for four cell lines (Raji, NALM6, REH, K562) mediated by NK92 cells using untreated (open square) or bryostatin-treated (closed square) targets at the E:T ratios listed on the x-axis. **(E–H)** Assay tested in parallel including cold-target inhibition. Representative results, average of triplicate wells and standard deviation, from 3 independent experiments are shown.

ligands were induced, these target antigens were not blocked by K562-based cold target inhibition. These results indicate that bryostatin induces two classes of targets for the innate immune system. Some are analogous to classic NK-targets (K562-like). Other leukemia expressed targets -while being recognized by NK-92- are not blocked by K562 cold-target inhibition, as illustrated in **Figure 8**. Our flow cytometric analysis of NK-92 is in agreement with previous studies, demonstrating strong CD56, as well as NKG2D, KIR2DL3, NKp30, NKp44, NKp46, and Fas staining; and low staining for NKG2C, KIR2DL1, FasL, DNAM, and KIR3DL1 (**Supplementary Table 2** and **Supplementary Figure S7**). Published analysis by others of potential NK targets expressed on K562 demonstrated very high expression for ICAM1, ICAM2, NKp30, HLA-F, MIC-A, ULBP2, ULBP3, CD48, CD80, CD112 (PVRL2/NECTIN2), CD155 (PVR) (36), thus providing multiple candidates whose expression, either singly or in combination, may be responsible for cold-target inhibition.

EBV Latency Reactivation in Not the Major Driver of CD22 CAR-T Resistance to Cytolysis in Bryostatin-Treated Raji Cells

Raji is an EBV-positive Burkitt lymphoma cell line. Principal Component Analysis (PCA) of bulk RNAseq data demonstrated that Raji clusters closer to normal B cells in comparison to either REH or NALM6, reflecting its well-established more differentiated B cell status as a Burkitt lymphoma (not shown). We tested the impact of inhibiting EBV replication or activation by culturing Raji cells in ganciclovir for two weeks. Previous work demonstrated the requirement for this extended time of treatment to insure complete viral quiescence for B-LCLs (37). CD22 CAR-T lysed Raji cells efficiently while control UTD did not, **Figure 9**. Furthermore, treatment with bryostatin renders Raji cells resistant to CD22 CAR-T-mediated cytolysis. The addition of ganciclovir reversed bryostatin-mediated resistance to a small degree, and some restoration of killing by CD22 CAR-T was demonstrated. Thus, bryostatin-mediated modulation of latent EBV gene expression may in some part explain the induced resistance to CAR-T mediated killing. We examined epigenetic alterations in EBV latency-associated genes to see if these were altered by the addition of bryostatin. No changes in histone methylation were seen for the Epstein-Barr virus associated latency antigens EBNA1, EBNA2/EBNA-LP, LMP1 or LMP2, although a slight decrease in mRNA expression was noted for the latency membrane proteins (**Supplementary Figures 4**, **5**). Changes in canonical markers of EBV reactivation, Zta and Rta, or for LF1,2 or 3 were not seen (**Supplementary Figure 6**). Interestingly, when we examined the regulation of EBNA3 promoter regions by methylation we did not find any changes for EBNA 3A, 3B, or 3C, but did increased marks for H3K4me3 (indicating increased transcriptional activity) for BLLF1, **Figure 10**. Unlike the EBNA proteins which serves as transcriptional regulators, BLLF1 encodes the major viral surface glycoprotein gp350. The gp350 receptor is CR2/CD21. CD21 is expressed on both T and NK cells, and interacts in concert with other receptors to mediate either cellular activation or viral infection (38, 39). Thus, in searching for a potential explanation as to why bryostatin induces Raji resistance to CD22 CAR-T, we found small changes in latent EBV viral genome regulation, and a

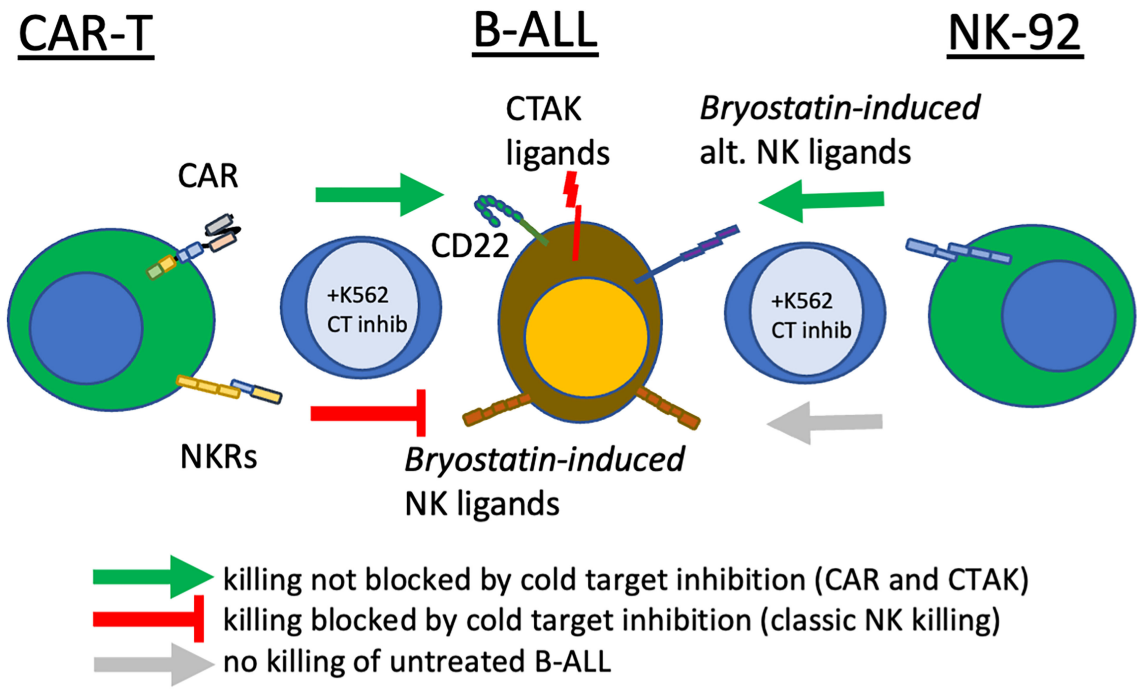

FIGURE 8 | Bryostatin treatment reveals multiple pathways that CAR-T cells use to eliminate leukemia. In the center of the diagram, pre-B ALL cells are illustrated, displaying the CAR-T target antigen, CD22, innate immune receptor ligands induced by bryostatin that are recognized by activated T cells (Bryostatin-induced NK ligands) and ligands recognized by T cells that have been: a) sensitized by CAR-T production, b) bryostatin-induced, and c) not blocked by cold-target inhibition (CTAK, CAR-T cell non-antigen-specific killing). Also shown are a non-overlapping set of alternative innate immune receptor ligands that are recognized by NK92 upon bryostatin-treatment (right-most effector cell). Cold-target inhibition does not affect NK92 or CD22-specific CAR-T killing. Cold-target does decrease killing evidenced by activated T cells (UTD), but incompletely for CTAK-mediated killing. Green arrows indicate successful cytolysis and blunt red arrow indicates killing impacted by K562-mediated cold target inhibition (classic NK killing).

minor but detectable reversal of the bryostatin effect by ganciclovir. Taken together this indicates that while the latent EBV genome in Raji does play a role in immuno-evasion, and bryostatin partially reverses this effect, the majority of bryostatin-mediated immune-evasion is attributable to factors inherent in the Burkitt lymphoma genome itself.

Increased Expression of Both Adhesion Molecules and NK Ligands Contributes to CAR T-Cell Antigen-Non-Specific Killing (CTAK), and CAR-T NK-Like Killing

To assess the contribution of known NK ligands on bryostatin-induced cytolysis of leukemia targets, we used both antibody and soluble protein-based inhibition assays. When NALM6 cells with or without bryostatin treatment were used as CD22 CAR-T or UTD targets, we again saw significant induction of UTD-mediated leukemia cell cytolysis induced by bryostatin treatment, **Figures 11A–D**. The addition of soluble DNAM-1 did not have an effect. NKG2D and ICAM did have some effect on bryostatin-induced killing by CD22 CAR-T. UTD was most affected by NKp30 and ICAM-1 blocking. Likewise, NKG2D and ICAM1 blocking impacts killing of bryostatin-treated REH by CD22 CAR-T and UTD, **Figures 11F, H**. NKp30 effects were limited to UTD for REH, just as for NALM6, **Figure 11G**. REH differed to a degree in that DNAM1-blocking now was shown to have an effect, and to a greater degree for UTD upon bryostatin treatment, **Figure 11E**.

Because Raji cells are universally sensitive to NK92 mediated killing, we restricted our analysis of NK92-mediated killing to the pre-B ALL lines. NALM6 killing was not impacted to a great degree by any of the 4 blocking agents tested. Although NKG2D blocking gave a statistically significant effect, the overall effect was small, **Figure 12B**. ICAM1-blocking did inhibit REH killing in the presence of bryostatin. NKG2D blockade had an effect on non-treated REH, but this difference was lost when bryostatin was added, as the overall killing was increased, **Figure 12F**. Taken together, we can assert that the decrease of ICAM1-mediated cell adhesion impacted bryostatin-induced killing by all three effectors tested, but impacted NK92-mediated killing less. NKG2D blockade impacted T cell mediated killing (both CAR-T and UTD), while NKp30 had activity in UTD but not CAR-T cell-mediated killing. Our findings indicate that well-characterized mediators of NK-like killing did have an effect in our system. However, the killing mechanisms are complex, and likely additive as no single blocking agents inhibited all killing activity.

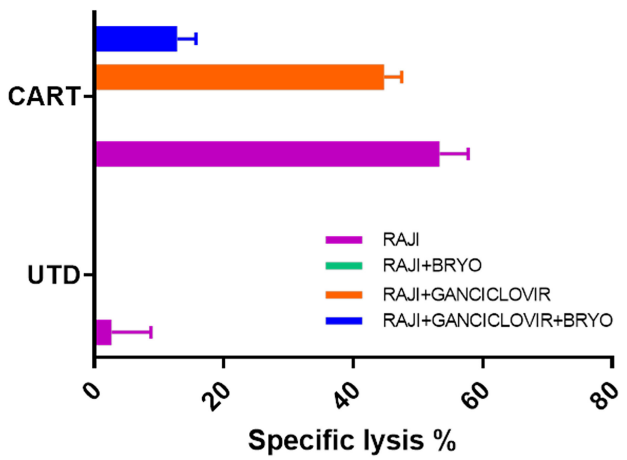

FIGURE 9 | Impact of EBV lytic cycle inhibition on CD22 CAR-T mediated killing of bryostatin-treated Raji leukemia cells. Raji cells were cultured for 2 weeks in the presence or absence of 15 uM ganciclovir (+Ganciclovir in legend), and for the final day of culture bryostatin was added where indicated (+Bryo in legend). Treated cells were then used as targets in CTL assays using anti-CD22 CAR-T (CART) or untransduced T cells (UTD) as effector cells. Average cytolysis of 3 replicate wells is plotted for each condition. Results are representative of three independent experiments.

We can also conclude that the multifactorial nature of innate immune cell-mediated cytotoxicity is activated in a novel way by the addition of bryostatin, as demonstrated herein.

Potential Bryostatin Epigenetic Changes Impacting Leukemia Cell Target Expression

We also carried out a comprehensive read analysis of Cut&Tag data, comparing bryostatin-treated and untreated REH, NALM6 and Raji cells (**Supplementary Figure S8**). Raji changes were the most dynamic, and NALM6 showed very few significant changes (The file comprising **Supplementary Table 4** contains the complete data set). We also specifically inspected the ligands for innate immune receptors that were expressed or induced in effector cells as detected by flow cytometry, **Supplementary Table 3A**. Among those ligands, HLA-ABC which would interact with iKIRs, changed the most, **Supplementary Table 3B**. We did very little to explore the Fas system in functional assays due in part due to the unchanging expression of FasL on effector cells, and low expression of Fas on NALM6 and REH. Although expressed on Raji cells, bryostatin did not alter Fas expression.

Promoter regions for ligands known to be important in NK cell activity were also compared by Cut&Tag analysis. Activating ligands (MICA, MICB, ULBP1, ULBP2, ULBP3, Nectin-2 (CD112), PVR (CD155); and inhibitory ligands (HLA-E, Nectin-1/CD111), showed no large alterations. NKp30 and NKp80 ligands (B7H6, BAG6, and CLEC2B) also were unchanged. Fas, TRAILR1 (DR4) and TRAILR2 (DR5) were also unchanged. Analysis of the SLAM family (FLAMF1, SLAMF3/LY9, SLAMF4/CD244, SLAMF4/CD84. SLAMF6, SLAMF7, SLAMF4LG/CD48) also showed no bryostatin effects. Analysis of KIR ligands HLA-A,-B, and -C showed no changes.

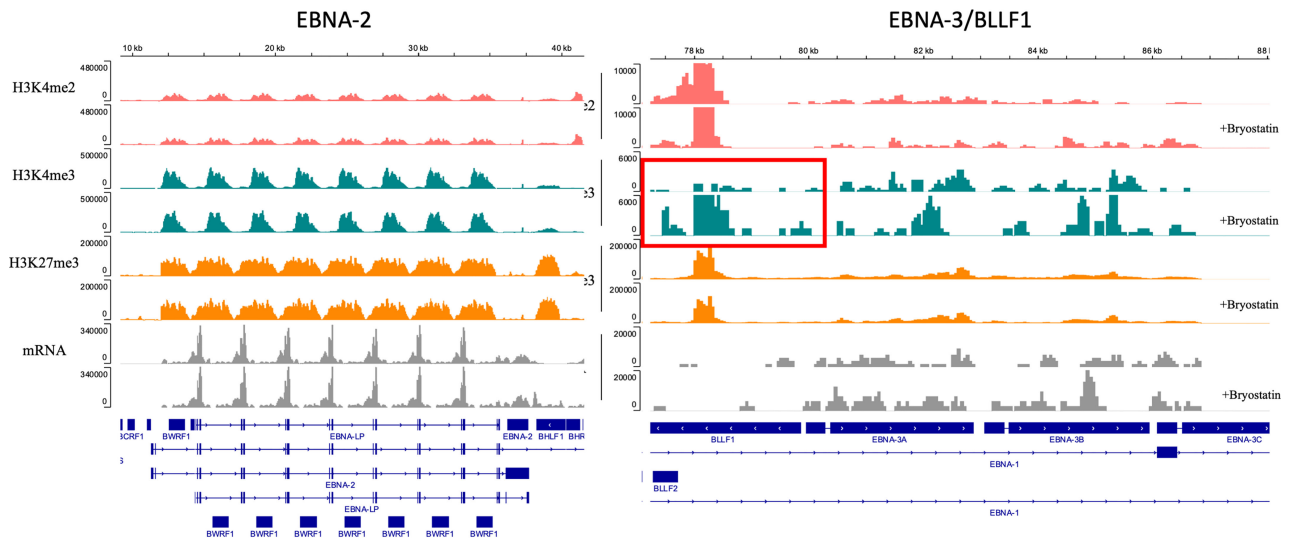

FIGURE 10 | Epigenetic modulation of the EBV genome in Raji leukemia cells mediated by bryostatin. Sequenced reads for transcriptional activators of EBV latency, left panel) EBNA-2 and EBNA-LP, and right panel) EBNA-3A,-3B,-3C and BLLF1; were analyzed by Cut&Tag analysis and mapped on the EBV genome for the presence of epigenetic modification of H3K4me2, H3K4me2, and H3K27me3 (y-axis). No reads were detected for the IgG control. Reads are presented as parallel samples for bryostatin-treated (+bryostatin) or untreated Raji. Shown below the immunoprecipitated Cut&Tag reads are total mRNA reads (in gray) displayed over the relevant portion of the EBV viral genome, shown at the bottom portion of the plot. Changes demonstrated for H4K4me3 reads for BLLF1 are indicated by the red square.

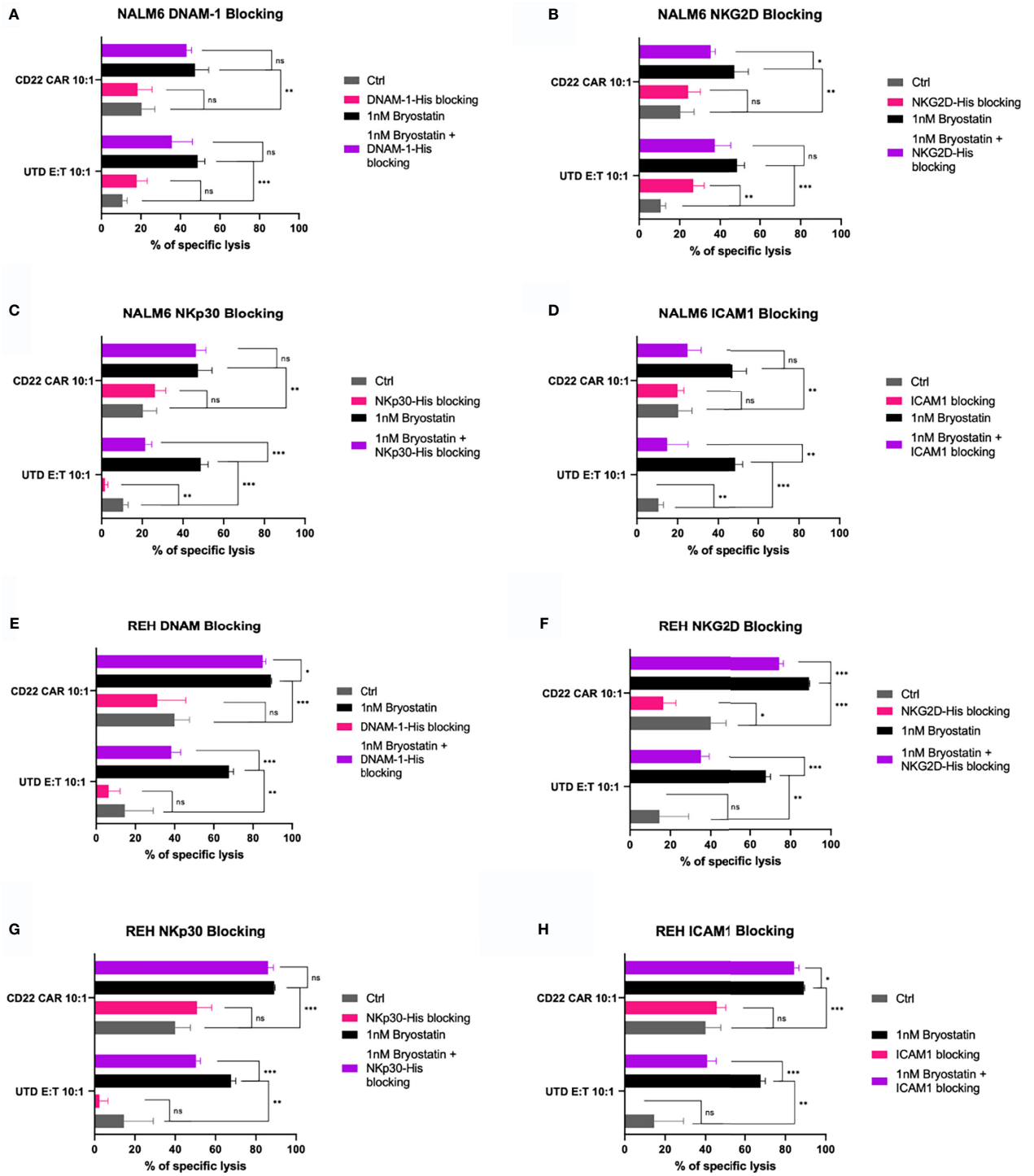

FIGURE 11 | Blocking of innate immunoreceptor ligands during CAR-T mediated cytolysis. The NALM6 pre-B ALL cell line was exposed to anti-CD22 CAR-T or control UTD cells at an E:T ratio of 10:1, y-axis. Results are grouped in each panel by cytolysis seen with untreated target (gray, control), bryostatin treatment (black), or treated with blocking agent (pink) or blocking agent and bryostatin (purple), using **(A)** recombinant DNAM-1, **(B)** NKG2D, **(C)** NKp30, or **(D)** anti-ICAM1 antibody, for 30 minutes prior to addition of effector cells. Average of 3 replicate wells are shown, with statistical difference between groups plotted, ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(E–H)** REH leukemia cells were similarly analyzed.

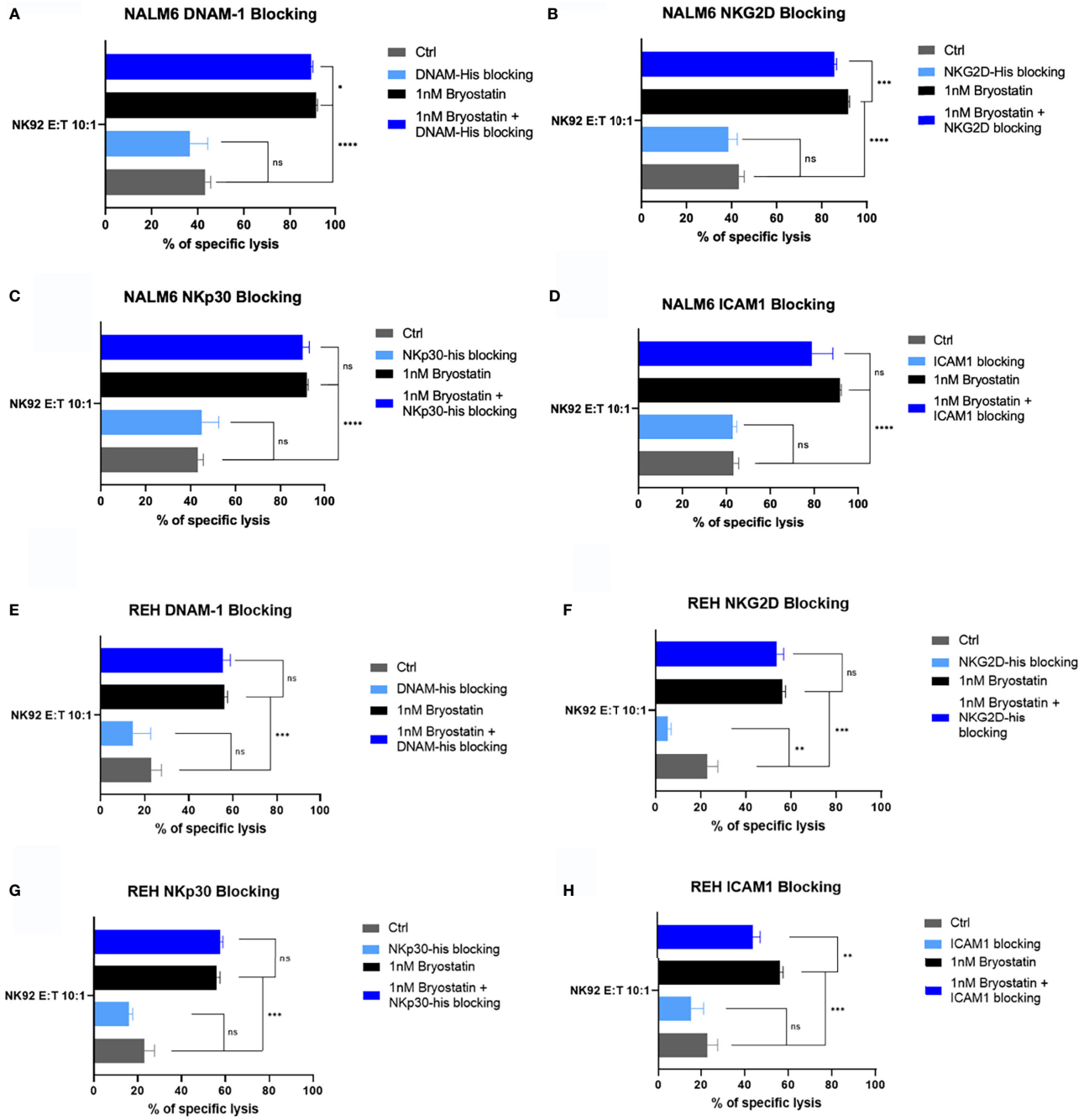

FIGURE 12 | Blocking of innate immunoreceptor ligands during NK92-mediated cytolysis. The NALM6 pre-B ALL cell line, with or without bryostatin-treatment, was cultured with NK92 cells at an E:T ratio of 10:1. Treatment groups are arranged according to the blocking agent tested: **(A)** DNAM-1, **(B)** NKG2D, **(C)** NKp30, or **(D)** anti-ICAM1 antibody as in **Figure 11**. Average of 3 replicate wells are shown, with statistical difference between groups plotted, ns $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. **(E–H)** REH leukemia cells were similarly analyzed.

HLA-G also was not changed, showing only H3K27me3 (inhibitory mark) reads present. Thus, we documented the presence and activity of well described innate immune receptors active in our system. Any single change in expression, as detected by flow cytometry, or attempts in direct protein blockade in functional assays reveal that these signals are integrated from multiple inputs. Further work remains to explain the specific signals operative in any one effector cell or cell line. The effects of bryostatin are layered on to the biology of CD19 and CD22 as expressed by B-ALL cell lines. The upregulation of these molecules

does sensitize ALL to cytolysis, but one must include in the analysis of CAR-T activity the strong induction of NK-like and CTAK killing activity, above and beyond CD22 antigen upregulation. Furthermore, bryostatin cannot be assumed to be universally applicable to B cell malignancies as Raji cells are rendered insensitive to cytolysis upon treatment.

DISCUSSION

B cell activation is a carefully regulated event. In addition to antigen- or developmentally-initiated positive signals, regulatory or inhibitory signals, like those mediated by CD22, are required to prevent hyperactivation (40). In keeping with the diversity of activity of the Siglec (sialic acid binding Ig-type lectin) family of receptors, CD22 has both negative regulatory activity, mediated through intracellular ITIM motifs that recruit SHP-1 and Grb2, as well as endocytic activity for ligands bearing specific glycoform structures, notably alpha2,6-linked sialic acid (41, 42). Recent studies with the B cell line DT40 have demonstrated that CD22 internalizes into early endosomes *via* clathrin-mediated endocytosis following B cell receptor (BCR) stimulation (43). Upon internalization, CD22 can either be marked for degradation by the E3 ubiquitin ligase cullin 3, or circulate back to the cell surface membrane, revealing a complex network amenable to multiple regulatory inputs. Thus, a number of clinically-relevant epigenetic modifiers or differentiation agents were explored, with bryostatin showing the broadest impact across the 3 lines tested on CD22 surface expression.

The average site density of CD22 on clinical pediatric ALL samples is 3,470 with a broad range (349-19,653) that is dependent in part on disease subtype (44). In an effort to overcome the evasion of B-ALL from CAR-T therapy, Ramakrishna et al., demonstrated that bryostatin is able to upregulate CD22, and to improve outcomes in a NSG animal model system (13). Laboratory and clinical studies have revealed that very little CD22 is shed, and although a possibility in our system, the evasion of immune effector cells by increased antigen target shedding is unlikely (45). The internalization of CD19 and CD22 was carefully described in studies evaluating anti-CD22 and anti-CD19 immunotoxins. In these studies, CD19 was expressed at 3-4 fold higher with respect to site density, but was far less effective as an anti-leukemic target for antibody-linked toxins due to its lower rate of internalization (46). Thus, there is documented differential internalization rate, even though the number of CD19 on the surface of B cell lines always exceeds that of CD22 (46). Immunofluoresence studies revealed that antibody-mediated ligation drives these receptors into the same intracellular compartment. This indicates both a differential mechanism with regard to ligation-dependent internalization, and some commonality as the initial endosomal compartment is the same. A global coregulation of CD19 and CD22 is also suggested by the lower levels of CD22 on ALL relapse post CAR-19 therapy (47). We explored the activity of a number of epigenetic modifiers and differentiation agents, **Figure 1**, to determine if other clinically relevant agents modulate CD22 target number on the cell surface, apart from overt cytotoxic activity.

Although panobinostat and vorinostat may stabilize antigen expression, only bryostatin appeared to consistently upregulate target antigen expression, and thus we continued our studies by focusing on bryostatin. Bryostatins are a family of cyclic polyketides, with most research focused on bryostatin 1 (48). The activity of bryostatin 1 is attributed to its interaction with the diacylglycerol biding site of the C-1 regulatory domain of protein kinase C. Upregulation of CD22 was noted alongside an increase in cell size and membrane projections in bryostatin-treated CLL (chronic lymphocytic leukemia) cells. Importantly, the effects of bryostatin on PKCbII change from activating to inhibitory with increased dosage or time in culture (14). At the lower concentration of 1 ng/mL, bryostatin induces CLL differentiation activating both PKCbII and Erk (49). Thus, it has a dual concentration-dependent effect. To examine the epigenetic effects of bryostatin we employed Cut&Tag analysis of two histone modifications associated with promoting transcription, H3K4me2 and H4K4me3, and one modification associated with repressing transcription, H3K27me3. CD19 and CD22 were not overtly altered, **Supplementary Figures S2, S3**, in keeping with the relatively unaltered overall transcript and protein levels, **Figure 2**. Responsiveness to bryostatin was clearly an attribute of transformed B cells, as normal B cell surface expression of both CD22 and CD19 was unaltered by bryostatin, **Figure 1**. The modulation of both CD19 and CD22 may be key attributes of successful CAR-T therapy, and will be explored in future studies. The ability of a CAR-T cells to release from a specific target and engage in serial killing would be inhibited if surface expression of the target molecule remained unchanged.

In addition to bryostatin treatment, we sought to determine the effect of CAR-T cells on cell surface CD22 expression. Much to our surprise, anti-CD22 CAR-T down-regulated both CD22 and the off-target antigen CD19, **Figure 3**. Increasing E:T ratios resulted in a greater decrease in CD22 and CD19 surface expression. This data suggests that the addition of bryostatin may keep target antigen surface expression higher and allow for a greater degree of CAR-mediated leukemia cell killing. This raises a key question, are we are selecting for a low antigen-expressing leukemia sub-clones, or observing antigen recycling and internalization at the cellular level? When the CAR-T + bryostatin challenged leukemia cells were isolated and re-cultured separately, interesting long-term changes were observed, that resolved in a week for 2 of the 3 lines, **Figure 4**, indicating that clonal selection was not the operative mechanism for detecting an antigen low population. For the Raji cell line, on-target CD22 expression decreased due to the addition of CAR-T. This effect lasted throughout 72 hours of post CAR-T co-culture, but normalized by day 7. For CD19 modulation in Raji, CD22 CAR-T induced CD19 down-modulation irrespective of bryostatin treatment. Effects on NALM6 CD19 and CD22 surface expression were not as dramatic, and returned to original levels by day 7. CD22 down-modulation by CAR-T was essentially reversed by bryostatin within 24 hours, indicating a long-term dominant effect that resolved within a week. The results seen with REH were unexpected in that there was a strong rebound effect for cultures treated with anti-CD22 CAR-T and bryostatin.

While CAR-T only and bryostatin-only cultures normalized CD22 expression levels by day 7, the combined treatment invoked a more permanent change in that even on day 7, CD22 and CD19 surface expression levels remained high. The genetic or epigenetic basis for this change will be explored in future studies. Our data illustrates that bryostatin has a profound effect on target antigen expression, even days after it is removed from the culture media.

Another potential mechanism for the loss of antigen expression on the target cell is trogocytosis mediated by the CAR-T cell. Antigen acquisition by CAR-T cells was detectable, and mirrored the relative antigen expression on each leukemia target cell, **Figure 5**. Importantly, this was an antigen non-specific process. The level of trogocytosis was also partially reflective of the degree of leukemia cell killing. Less transfer was seen was seen with higher E:T ratios. This may be due either to the greater number of T cells that can receive membrane associated surface antigens (signal dilution), or that cells being actively lysed do not "donate" membrane and membrane-associated proteins.

Investigating the cytolysis of bryostatin-treated leukemia cell lines gave unanticipated findings, **Figure 6**. Untreated Raji cells were readily killed by CD22 CAR-T. However, when bryostatin was added, killing was completely abrogated. Untransduced T cells (UTD) are activated T cells treated exactly like anti-CD22 CAR-T, with the exception that no LV vector transduction takes place. Both REH and NALM6 were efficiently killed by CAR-T, while UTD showed a very low killing activity, as expected. However, when bryostatin was added, UTD now mediated strong REH and NALM6 killing. This may indicate that the increased killing of REH and NALM6 is not due to the increased number of CD22 molecules on the leukemia cell surface, but due to bryostatin-induced innate immune ligands that make the cells susceptible to CAR-T and UTD antigen non-specific killing. Neither CAR-T nor UTD lysed K562 cells, indicating that the increased B cell leukemia killing was not mediated by standard NK cell interactions. Cold-target inhibition demonstrated that the bryostatin-induced killing of ALL lines could be blocked by the innate immune ligands expressed by K562, while preserving CAR-T mediated killing.

To specifically explore leukemia cell line sensitivity to NK cell killing, we used the NK92 cell line, **Figure 7**. Unlike CAR-T or UTD, NK92 had strong cytolytic activity against K562 cells. And, as expected this was abrogated with K562-mediated cold target inhibition. Raji cells were very sensitive to NK92 killing, with or without bryostatin addition, and this killing was completely unaffected by cold-target inhibition. NALM6 and REH were not killed by NK92 unless they were first treated with bryostatin. This killing also was not abrogated by K562-mediated cold target inhibition. These results indicate that CAR-T cells mediate killing through a number of mechanisms that include the CAR itself, NK-like killing that can be blocked by K562-mediated cold target inhibition, and killing induced by ligands induced by bryostatin. This led us to propose a new model for CAR-T mediated killing of bryostatin-treated cells, **Figure 8**. We now use the term CTAK (CAR-T activated killing) to refer to off-target cytotoxicity against B-ALL cell lines mediated by CAR-T cells. Moreover, CTAK activity is optimized by bryostatin treatment of target ALL cell lines.

The striking evasion of bryostatin-treated Raji cells to CAR-T cell-mediated cytotoxicity, but not NK92, led us to hypothesize that activation of EBV latency may be responsible for immune evasion. While the EBV genome present in Raji cells is not replication competent, and thus gangiclovir effects may not be directly EBV-related, the latent EBV genome present in Raji cells remains a focus of study on the immune evasion mechanisms utilized by EBV (50, 51). Preliminary RNASeq studies of bryostatin-treated Raji highlighted EBV reactivation pathways (not shown). When we treated Raji with ganciclovir, some sensitivity to CAR-T-mediated cytolysis was recovered, **Figure 9**. This did not correlate with epigenetic changes in control regions for EBNA-1 or EBNA-2/LP expression, nor were changes seen in the promoter regions associated with EBV reactivation from latency, Zta, Rta, and LF1,2,3 (**Supplementary Figure S6**). Upon examining other EBV latency promoters we noticed a marked increase in reads for BLLF1. BLLF1 encodes the major viral envelope glycoprotein gp350. Although gp350 does interact with B cell surface proteins, notably CR2/CD21, we did not explore this finding further in this report. Due to the minor role EBV latency gene expression plays in bryostatin-treated Raji immune evasion, and the examination of only one EBV-positive line, we cannot make a causal link to immunoevasion and EBV. An alternate hypothesis would be the effect bryostatin has on α2,6 sialic acid-bearing targets, which if increased would impact CD22 expression.

In our final set of studies we explored the contribution of ligands known to be involved in innate immune recognition of cancer targets. For NALM6, ICAM-1 blockade diminished CAR-T mediated cytolysis, **Figure 11D**. There was a decrease when NKG2D was blocked as well, but this difference did not reach statistical significance. DNAM1 blockade did very little in any of our assays, in opposition to previous reports showing DNAM-1 activation of NK cells *via* interaction with CD112 (Nectin-2) and CD155 (PVR) on myeloid leukemias (52). The REH cell line showed decreased CAR-T and UTD cytolysis when either ICAM-1 or NKG2D were blocked, **Figure 11F, H**. NK92 cell-mediated killing of NALM6 and REH was impacted by NKG2D or ICAM1 blockade, **Figure 12**. The only evidence of NKp30 activity in our assays was the partial blockade of UTD-mediated killing of bryostatin-treated REH or NALM6, **Figures 11C, G**. Due to the low expression of the NKp30 ligands B7H6 and BAG6 on leukemia target cell lines, we hesitate to ascribe this activity as being a key point of differentiation between the killing activities we described, but it indicates that transduction with a CAR may give rise to a different innate immune effector activity than that seen in UTD. In sum, the receptors tested as a single agents had a moderate effect. This implies that the killing activities observed are the result of additive signals that are integrated by the effector cell type being tested.

Bryostatin profoundly modulates cell surface antigen expression of targeted leukemia cells. For NK92-mediated killing, bryostatin induced a set of ligands on the B-ALL cell

lines REH and NALM6 that allowed them to be recognized and eliminated. Moreover, these signals were not those normally associated with NK cell activity, as cold target inhibition had no effect, **Figure 13**. For the more developmentally mature B cell line, Raji Burkitt lymphoma, bryostatin had no effect. However, Raji cells are universally sensitive to NK92. Effects on T cell-mediated killing of Raji were very different from NALM6 and REH. T cell killing of Raji was completely abrogated by bryostatin. This surprising result indicates that bryostatin cannot be assumed to be universally beneficial in CD22 CAR-T mediated killing. This also indicates that the increased killing cannot be solely attributed to an increase in the number of CD22 molecules on the cell surface. A portion of the bryostatin-amplified susceptibility to cytolysis is blocked by cold-target inhibition, indicating that canonical NK receptor interactions play a role for ALL. We have termed the non-canonical activity that could not be blocked by cold-target inhibition CTAK (CAR-T cell antigen non-specific killing) in order to differentiate it from NK cell and LAK cell-mediated killing.

Analysis of cell surface antigen dynamics revealed that trogocytosis occurs to some degree, but is unlikely to be a major source of antigenic modulation seen during CAR-T cell mediated killing. The dual activity of CAR-T and bryostatin induced changes in surface antigen expression for days, even when these agents were removed, **Figure 4**. Notably, the REH cell line maintained changes in both CD22 and CD19 antigen expression levels for 7 days when co-cultured with both CAR-T and bryostatin. Epigenetic analysis at 24 hours revealed changes in EBV antigen expression control regions in Raji cells, and alterations for other proteins as well, but not in the control regions of CD19 and CD22 (**Supplementary Table 2**). The complex dynamics of surface antigen expression in leukemia cells will be the focus of future studies. A recent analysis of CD22 CAR-T treated patients revealed that in addition to T cell exhaustion and a lack of stimulation due to antigen down-modulation, significant splice variations in CD22 have also been noted that may account for escape from immunotherapeutic control (53).

We have demonstrated that bryostatin induces innate immune receptor ligands on ALL that increase CAR-T cell killing, which can be blocked only in part by cold-target inhibition with K562. We have also demonstrated that Raji cells are rendered resistant to T cell mediated, but not NK92-mediated killing, by bryostatin. Furthermore, NK92 targets are induced on B-ALL when treated with bryostatin, and these also are not influenced by cold-target inhibition. We have described the mechanisms behind these effects only in part. Anti-ICAM1 antibody seems to partially block these effects for both T and NK effector cell types, and other innate immune receptors clearly play a role as well. We propose that for clinical studies where CAR-T cells are combined with bryostatin, that the leukemia cell type targeted should first be documented to have increased biological sensitivity to cytolysis. A simple increase in CD22 target cell number is not sufficient. Secondly, the addition of NK

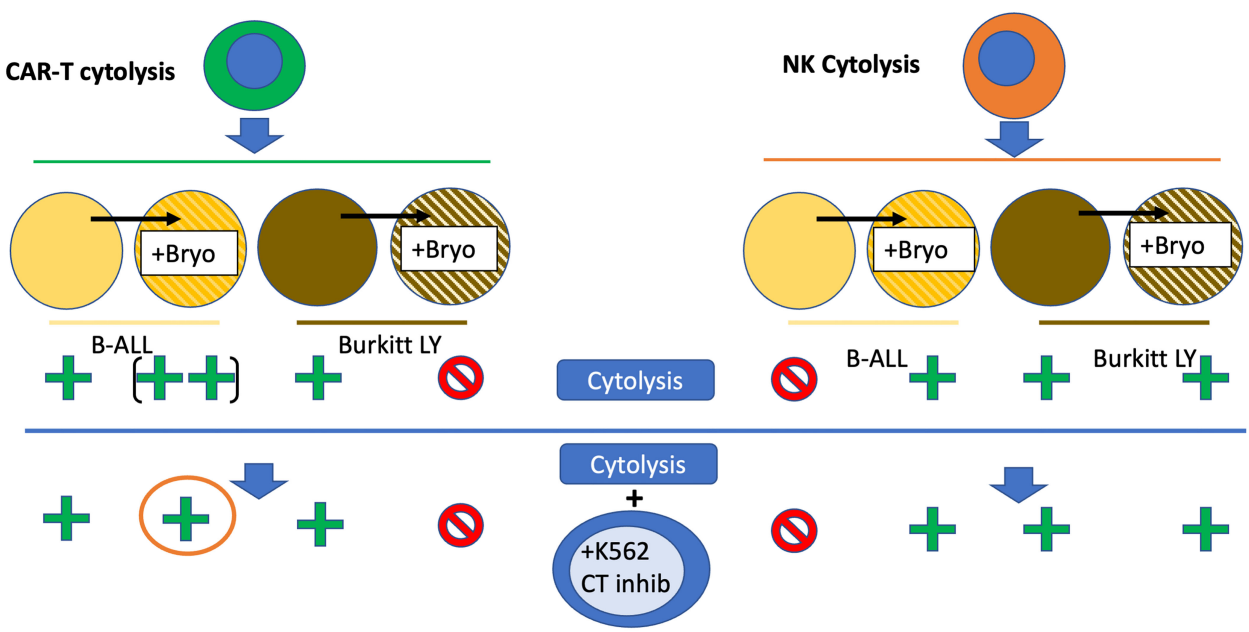

FIGURE 13 | Bryostatin modulation of CAR-T and NK92-based leukemia cell line cytolysis. Bryostatin treatment of pre-B ALL (yellow to striped yellow) and Burkitt (brown to striped brown) cell lines alters sensitivity to effector cell cytolysis. CAR-T cytolysis (left half, green cell) is amplified by bryostatin treatment (+ to ++) for B-ALL, and blocked for Burkitt's (+ to -). Progressing to the lower portion of the figure illustrates the effect of K562 cell mediated cold-target inhibition (+K562 CT inhib). CAR-T mediated killing of Burkitt's is unaffected, while some of the bryostatin facilitated killing of B-ALL is lost (orange circle). For NK cytolysis (orange cell, right half), the induction of a new set of innate immune ligands that now allow for killing of B-ALL is illustrated (- to +). Burkitt's remains unaffected, and universally sensitive. The induced ligands on B-ALL are unaffected by cold-target inhibition and remain sensitive to NK cytolysis.

cells to CAR-T cell therapeutic approaches may overcome escape mechanisms that more mature leukemia subtypes display, and should be considered on their own or in combination with bryostatin.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192965 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE192837.

AUTHOR CONTRIBUTIONS

The studies were conceived and designed by AL and RO. Experiments were carried out by LW, EA, and YZ. Bioinformatics analysis was carried out by YZ. Studies were supervised by RO. All authors contributed to the article and approved the submitted version.

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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.2022.825364/full#supplementary-material

Supplementary Figure 1 | (S1) Heatmap of histone enrichment anchored at TSS (Transcription Start Sites). Raji, NALM6, and REH cell lines were treated with bryostatin for 24 hours (Bryo), and then analyzed by Cut&Tag, or left untreated, Ctrl. Total reads were normalized by RPKM, aligned for transcriptional start sites, and signals for H3K4me2, K3K4me3, and H3K27me3 compared to untreated cells cultured in parallel. Both read length from the transcriptional start site (bottom scale), and frequency (vertical scale) are indicated.

Supplementary Figures 2 and 3 | (S2 and S3). Cut&Tag analysis of CD22 and CD19 transcriptional control regions. Cut&Tag reads mapped to genome regions encoding S2) CD22 and S3) CD19, are displayed for Raji, NALM6, and REH cell lines as listed on the y-axis. Next to each cell line the three immunoprecipitating antibody specificities are listed along with the IgG control antibody (H3K4me2, H3K4me3, H3K27me3, IgG). For each condition, two read tracks are presented for cell lines that have been treated (+Bryostatin) or untreated control samples. Thus, changes due to bryostatin treatment appear directly below the untreated cell line sample. Below each data set the corresponding genome map is illustrated. To the right of each panel, average of normalized total reads within the gene region from 3 experiments are presented for H3K4me2 and K3K4me3. For CD22: Raji showed increased H3K4me2 and H3K4me3 ($p<0.001$), REH ($p<0.001$) and NALM6 ($p<0.05$) showed increased H3K4me3 reads. n.s., not significant. For CD19 only H3K4me3 ($p<0.05$) was increased by bryostatin treatement.

Supplementary Figures 4 and 5 | (S4 and S5). Modulation of the EBV genome in Raji leukemia cells mediated by bryostatin. S4) Sequence reads for two of the major transcriptional activators of EBV latency, EBNA-1 and EBNA-2/LP analyzed by Cut&Tag analysis and mapped on the EBV genome for the presence of epigenetic modification of H3K4me2, H3K4me2, and H3K27me3 (y-axis). No reads were detected for the IgG control. Reads are presented as parallel samples for bryostatin-treated (+bryostatin) or untreated Raji. Shown below the immunoprecipitated Cut&Tag reads are total mRNA reads (in gray) displayed over the relevant portion of the EBV viral genome, shown at the bottom portion of the plot. S5) Analysis of the viral genome encoding two of the latency antigens that mediate B cell activation, C) LMP1/2 and D) LMP2A/B, demonstrated no overt changes in Cut&Tag signal, and a decrease in total mRNA reads for LMP-1 and LMP-2.

Supplementary Figure 6 | (S6) Cut&Tag analysis of EBV latency reactivation promoters in Raji. Cut&Tag Profiles for **(A)** Zta/Rta, **(B)** BLLF1, and **(C)** LF1,2,3 are presented for reads amplified from H3K4me3, H3K4me3, K3K27me3 immunoprecipitations, as in S2. Reads are presented in parallel with or without (+bryostatin) bryostatin treatment. Red square indicates difference in H3K4me3 reads for BLLF1.

Supplementary Figure 7 | (S7) Flow cytometry for innate immune ligands: effector cell and leukemia cell line characterization. **(A–N)** the three immune effector lines (NK92, UTD, and CD22 CAR-T) were characterized for expression of NKG2D, DNAM-1, NKp30, NKp44, NKp46, TRAIL, FasL, KIR2DL1/DS1, KIR3DL1/DS3, NKG2A, ICAM1, ICAM2, LFA1, and CD56. All antibodies used are listed in Methods. **(O-BB)** Four leukemia lines (NALM6, REH, Raji, K562) were analyzed for expression of MIC-A/B, ULBP1, ULBBP-2/5/6, ULBP3, Nectin-2, B7H6, BAG6, DR4, DR5, Fas, HLA-A,B,C, HLA-E, ICAM1, ICAM2. In all flow panels isotype control (gray), untreated cells (blue), and bryostatin-treated cells (red, 1 nM overnight) are compared.

Supplementary Figure 8 | (S8) Volcano plot of differentially enriched peak by Cut&Tag analysis. Enriched peak reads were normalized by read depth and compared between samples (control versus bryostatin treatment) by DESeq2. Shown is H3K4me2 (top row) and H3K4me3 signal (bottom row) for REH **(A, D)** NALM6 **(B, E)**, and Raji **(C, F)**. Green color indicates gene expression differential of >4-fold change and $p<0.001$ in H3K4me2, gene expression differential of >4-fold change and $p<0.005$ in H3K4me3. Gene identities can be found in ST2.

Supplementary Table 1 | (ST1) Epigenetic modifiers and differentiation agents modulate CD19 and CD22 expression at non-cytotoxic concentrations. Three cell lines (Raji, NALM-6, REH) were tested across a broad range of concentration to ensure a noncytotoxic concentration of epigenetic modifiers and differentiation agents were tested for the ability to increase the expression of the number of CD22 and CD19 molecules per cell, **Figure 1**. Agents that changed the expression of each target antigen are indicated by the greatest percent increase of the number of CD22 or CD19 molecules per cell are listed.

Supplementary Table 2 | (ST2) Expression of innate immune receptors on effector cells. MFI signal, (summarized from Supplementary Figure 7) for NK92, untransduced T cells (UTD T) and CD22 CAR-T cells, for expression of surface innate immune effector molecules. NK92 expression patterns are consistent with primary NK cells, while UTD and CAR-T cells lack CD56, NKp30, and NKp44 expression. Bryostatin has negligible effects on NK-92, and some induced (ind) expression of TRAIL and ICAM-1 on UTD and CAR-T cells.

Supplementary Table 3 | A, 3B Summary of gene expression by MFI, based on flow cytometry profiles, S3. **Table 3A**) Effector cells (NK92, UTD, CD22 CAR-T) with or without bryostatin treatment (+Bryo) were analyzed for expression of Activating Ligands (orange blocks, column 1): MFI for NKG2D, DNAM1, NKp30, NKp44, NKp46, TRAIL, FasL; and for Inhibitory Ligands (blue blocks, column 1) KIR2DL1/DS1, KIR3DL1/DS1, NKG2A; and for adhesion receptors (gray blocks, column 1) ICAM1, ICAM2, LFA1, CD56 are shown. **Table 3B)** NK ligands (column 3) interacting with Activating Receptors (column 2, orange blocks), Inhibitory Receptors (column 2, blue blocks), and adhesion receptor (gray block, column 1) are listed according to expression on leukemia cell line targets (NALM6, REH, Raji, K562) that have been untreated or treated (+bryo) with bryostatin. MFI for MIC-A/B, ULBP1, ULBP-2/5/6, ULNP3, NECTIN2, B7H6, BAG6, DR4, DR5, Fas, HLA-A,B, C, HLA-E, ICAM1, and ICAM2 are shown. MFI was calculated by (geometric mean of ligand fluorescence – geometric mean of isotype fluorescence).

Supplementary Table 4 | (ST4) Total reads and reads exceeding threshold. Table (ST2_CutNTag_differential) describes methylation patterns (me2 and me3) of histone 3 (H3) modifications at lysine 4 (K4). The first of each pair of tabs reports all signal from Cut&Tag analysis and the second tab lists differential expression between bryostatin-treated and control (non-treated) leukemia cells. The global tab contains the peak ID (column A), chromosome (column B), start and end sequence number (C,D), annotation (E), distance to transcription start site (TSS) (F), gene name (G), gene description and type (H,I). Signal for the cell lines, in triplicate, analyzed without bryostatin (J-L) with bryostatin (M-O), normalized signal by DESeq2 without bryostatin (P-R), normalized signal by DESeq2 with bryostatin (S-U); column V is not utilized, but gives signal intensity mean across all samples, column W, log2 fold-change (without bryostatin versus with bryostatin, and thus negative in value); column X, standard error of log2 fold-change; column Y, standard error of the log2 fold-change, Wald statistic; column Z, Wald test p-value; column AA, Benjamini-Hochberg adjusted p-value. All stats were calculated within the DSeq2 package. Thus, ST2 contains the following tabs: K4me2NALM6 K4me3NALM6_|FC|>4_P<0.001_green (fold change greater than 4, indicated p value) K4me3NALM6 K4me3NALM6_|FC|>4_P<0.005_green K4me2REH K4me2REH_|FC|>4_P<0.001_green K4me3REH K4me3REH_|FC| >4_P<0.005_green K4me2Raji K4me2Raji_|FC|>4_P<0.001_green K4me3Raji K4me3Raji_|FC|>4_P<0.005_green.

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SP1-Mediated Upregulation of circFAM126A Promotes Proliferation and Epithelial-Mesenchymal Transition of Oral Squamous Cell Carcinoma *via* Regulation of RAB41

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Background: Accumulating evidence indicates that circular RNAs have major roles in the progression of human cancers. Nevertheless, the molecular mechanism and effects of circFAM126A in oral squamous cell carcinoma (OSCC) remain unclear.

Methods: Quantitative real-time PCR (qRT-PCR) was used to detect expression levels of circFAM126A in OSCC tumor tissues and cell lines; the effects of circFAM126A small hairpin RNA (shRNA) on the proliferation, migration, and invasion of OSCC cells were detected by MTT, colony formation, and transwell assays; xenograft mouse models were used to determine the effects of circFAM126A shRNA on the growth of OSCC tumors *in vivo*; the expression of miR-186 and RAB41 in OSCC tissues and cells was examined by qRT-PCR; the targeting relationship between circFAM126A and miR-186 was verified by dual-luciferase reporter and RNA pull-down assays; and the relationship between miR-186 and RAB41 was explored.

Results: The expression of circFAM126A was significantly upregulated in OSCC tissues and cells. The transcription factor SP1 transcriptionally activated circFAM126A. However, knockdown of circFAM126A markedly suppressed the proliferation, migration, and invasion of OSCC cells *in vitro* and inhibited tumor growth and distant metastasis *in vivo*. Moreover, circFAM126A increased the expression of RAB41 and promoted its mRNA stability *via* binding to miR-186 and RNA-binding protein FUS. Overexpression of RAB41 antagonized the effects of circFAM126A knockdown and induced an aggressive phenotype of OSCC cells.

Conclusion: SP1 transcriptionally activated circFAM126A modulated the growth, epithelial-mesenchymal transition (EMT) of OSCC cells *via* targeting the miR-186/FUS/RAB41 axis, suggesting that circFAM126A is a potential biomarker for the treatment of OSCC.

Keywords: oral squamous cell carcinoma, circFAM126A, miR-186, RAB41, proliferation, epithelial-mesenchymal transition

INTRODUCTION

Oral squamous cell carcinoma (OSCC) is the most common oral malignant tumor worldwide (1). Although strategies for the diagnosis and treatment of OSCC have improved significantly in recent years, the 5-year overall survival rate for patients with advanced OSCC remains poor (2, 3). Therefore, further exploration of the pathogenesis of OSCC and identification of novel therapeutic targets, as well as potential diagnostic and prognostic biomarkers, may provide new opportunities for early diagnosis and treatment of OSCC.

Accumulating evidence has unveiled the roles of circular RNAs (circRNAs; a type of non-coding RNA) in cancers including bladder cancer (4), colorectal cancer (5), and cervical cancer (6). Abnormally expressed circRNAs can be used as diagnostic markers and targets for therapeutic intervention for various malignant tumors (7, 8). In OSCC, circRNAs can function as tumor suppressors or oncogenes (9). For example, hsa-circ-0008035, hsa-circ-0000670, and hsa-circ-0003159 have been found to be associated with OSCC tumorigenesis (10–12). However, the roles of circ_0001682 (circFAM126A), which is located on chromosome 7 with a spliced length of 181 base pairs, have not been elucidated.

Functionally, circRNAs function as competing endogenous RNAs (ceRNAs) and sponge microRNAs (miRNAs) to regulate gene expression and numerous biological processes, including proliferation, apoptosis, migration, and invasion of cancer cells (13–15). miRNAs pair with mRNA bases of target genes to induce silencing complex RISC, which further degrades the mRNA or inhibits its translation. The circRNA/miRNA/mRNA axis has been verified to be a regulator of multiple tumor-related pathways and to modulate tumorigenesis. For example, aberrant expressed circAKT1 induces malignant behaviors of cervical cancer cells *via* regulating the miR-942-5p/AKT1 axis (6); the circ-0067934/miR1324/FZD5 axis promotes the progression of hepatocellular carcinoma (16); and has-circ-0000670 promotes the proliferation, migration, and invasion of OSCC *via* regulating miR384/SIX4 axis (11).

Epithelial-mesenchymal transition (EMT) is considered as a classical theory for tumor metastasis (17). The processes of EMT is accompanied by the loss of epithelial function and acquisition of mesenchymal characteristics, which loses cell adhesion and enhances migration and invasion ability (18). Presently, increasing evidence has revealed the potentials of circRNAs in the EMT processes (19). For instance, circ_0008305 suppresses the EMT and metastasis of non-small cell lung cancer *via* miR-429/miR-200b-3p/TIF1γ axis (20). circPRRC2A-induced upregulation of TRPM3 promotes the EMT, angiogenesis and metastasis of renal cell carcinoma (21). circIGHG enhances the EMT of OSCC *via* regulating miR-142-5p/IGF2BP3 axis (22). However, the study on the roles of circRNAs in OSCC is still limited.

In this research, we investigated the roles of circFAM126A in OSCC tissues and cell lines using circRNA microarrays, bioinformatics, and functional studies. We found that circFAM126A could function as an oncogene in OSCC, and that its knockdown suppressed the proliferation and EMT of OSCC cells *via* regulation of the miR-186/RAB41 axis.

MATERIALS AND METHODS

Tissue Samples and Cell Lines

A total of 30 OSCC patients who underwent surgeries at the Affiliated Stomatological Hospital of Nanchang University were involved in this research. Adjacent normal tissues were taken 5 cm away from the edge of the tumor. The diagnosis of OSCC was confirmed by histological examination. Patients with OSCC who had received prior treatment for their tumor or had a history of other solid tumors were excluded. This study was approved by the Human Research Ethics Committee of the Affiliated Stomatological Hospital of Nanchang University. Informed consent was obtained from all patients.

OSCC cell lines CAL27, SCC25, SCC15, TSCCA, UM1 and UM2, normal human oral epithelial cells (NHOK), and HEK-293 T cells were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cell lines were cultured in 90% RPMI-1640 medium (Gibco, USA) with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin solution (Invitrogen) at 37°C in a moist atmosphere with 5% CO_2.

Microarray Analysis

The microarray data set GSE131182 analysis was performed with Limma R Human CBC circRNA under the following the standard: $|logFC| > 2$ and $P<0.05$. The number of differentially expressed circRNAs was 417, among which 383 circRNAs were upregulated and 34 downregulated.

RNase R Treatment

To prove that circFAM126A is a circRNA, total RNA from CAL27 and UM1 was treated with RNase R (Sigma) at 37°C for 15 min and then purified with phenol-chloroform (Sigma). The expression of circular or linear FAM126A was determined by quantitative real-time polymerase chain reaction (qRT-PCR).

Actinomycin D

The circFAM126A plasmids were transiently transfected into OSCC cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h, actinomycin D (5μg/mL) was added to the culture medium, followed by incubation for 0 h, 4 h, or 8 h, 16 h, and 24 h. mRNA stability was analyzed by PCR.

qRT-PCR

TRIzol™ reagent (Invitrogen, CA, USA) was used to extract total RNA according to the manufacturer's instructions. To ensure the purity of circRNAs, RNase R (Geneseed, Guangzhou, China) was used to digested the RNAs for 20 min. The RNA concentration was measured using a Nanodrop 2000 (Thermo, USA), and qRT-PCR was performed on a 7500 Fast Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific) using a SYBR Premix Ex Taq II kit (Takara Bio, Beijing, China) to examine the

relative expression of circRNAs. GAPDH was used to normalize circRNA expression levels.

First, total RNA was reverse transcribed into cDNAs by TaqMan Reverse-transcription. The cDNAs were synthesized using a PrimeScript RT reagent kit (Takara, Tokyo, Japan) transcriptase, random 6mers, RNase inhibitor, Oligo dT primer, dNTP mixture, and reaction buffer. The cycle conditions were 95°C for 30 s (initial denaturation), followed by 95°C for 5 s and 60°C for 34 s, for 40 cycles. We used the $2^{-\Delta\Delta Ct}$ method to analyze the data (23).

Cell Transfection

The circRNA small hairpin RNA (shRNA), miR-186 mimics and inhibitor, and specific negative control were synthesized and purchased from GenePharm (Shanghai, China). The circFAM126A vectors were constructed with amplified DNA fragments, including the sequence of exons 15 and 16 of the PTK2a gene with flanking introns containing complementary Alu elements (GeneChem, Shanghai, China). Cells were transfected using Lipofectamine 3000 (Invitrogen, CA, USA) for 48 h.

Cell Viability (MTT) Assay

Logarithmic-phase cells were digested with trypsin, collected, and used to prepare a cell suspension after centrifugation. A mixture of the cell suspension containing 100 µl cells was added to each well of a 96-well-plate. The cells were cultured in an incubator at 37°C with 5% CO_2. Cells were supplemented with 10 µl MTT solution (5 mg/ml, 0.5% MTT) and further cultured for 4 h. Then, 150 µl dimethyl sulfoxide was added to each well, followed by shaking at low speed for 10 min to fully dissolve the crystals. The absorbance values of each well were measured using a microplate reader at an optical density of 490 nm.

Colony Formation Assay

After 48 h transfection, cells were digested with trypsin. A cell suspension was prepared in complete medium. Then cells were washed with phosphate-buffered saline. Trypsin was added and the cells were centrifuged. The cells were seeded into six-well plates (500–1000 cells/well), shaken gently, and cultured for 14 days. Then, 1000 µl impurity-free crystal violet dye was added to the cells. Cells were visualized under a microscope.

Transwell Cell Migration and Invasion Assays

Transwell assays were used to determine the invasion and migration ability of the OSCC cells. After transfection for 48 h, cell culture transwell inserts (8-mm pore size; Falcon; BD Biosciences) were placed in 48-well plates in the upper chamber with or without precoated Matrigel (BD Biosciences, San Jose, CA, USA). The membrane was hydrated with FBS 2 h prior. Cells in the lower chamber were cultured with RPMI-1640 (600 µl) containing 10% FBS. After 24 h, the migrated or invaded cells were fixed with 100% methanol and cultured with crystal violet. The numbers of migrated and invaded cells were counted under a microscope.

RNA Probe Pull-Down Assay

Biotinylated probes binding to the junction region of circFAM126A or miR-186 were designed by GenePharm. The oligonucleotide probe was used as a negative control. Approximately 1×10^7 cells were lysed in lysis buffer and incubated with 3 µg biotinylated probe for 2 h. Cell lysates were incubated with streptavidin magnetic beads (Life Technologies, Gaithersburg, MD, USA) for 4 h to pull down the biotin-conjugated RNA complex. Cells were washed with lysis buffer five times. Subsequently, the bound miRNA in the pull-down complex was extracted using TRIzol reagent and analyzed by qRT-PCR.

Wound Healing Assay

Cells were seeded into 6-well plates. Then cells, cells at 80% confluence, were scratched using 20 µl pipette tip. Afterwards, cells were cultured with DMEM medium for 0 h and 24 h and captured using an inverted microscope.

Dual-Luciferase Reporter Assay

Target analyses of circFAM126A and miR-186 (https://starbase.sysu.edu.cn/index.php), and of miR-186 and RAB41 (http://www.targetscan.org/vert_72/), were performed on the biological prediction, respectively. The sequences of the circFAM126A and RAB1 3′ untranslated regions (UTRs) containing the miR-186 binding sites were cloned into luciferase reporter vectors (Promega, Madison, WI, USA) to form the wild-type vectors wt-circFAM126A and wt-RAB41. The mutant luciferase reporter vector constructs mut-circFAM126A 3'UTR and mut-RAB41 were created by mutating the binding sites of miR-186. For the dual-luciferase reporter assay, HEK-293 T cells were co-transfected with these vectors and an miR-186 mimic or negative control. After 48 h, luciferase activity was measured by using a dual-luciferase reporter assay system (Promega).

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed using a ChIP Kit (Millipore, USA). Briefly, cells were fixed in 1% formaldehyde. Afterwards, crosslinked chromatin was ultra-sonicated, and immunopreciated with anti-SP1 or control anti-IgG bound-protein G beads. The enrichments of DNA fragments were determined using qRT-PCR.

Mouse Xenograft Tumor Models

Mouse xenograft tumor models were established for *in vivo* assays. OSCC cells transfected with circFAM126A shRNA were subcutaneously injected into nude mice. The volumes of xenograft tumors were measured every week for 6 weeks. At the end of week 6, mice were sacrificed, and weights of tumors were measured. Immunohistochemical staining was performed to determine the expression of Ki67. This animal study was approved by the Animal Care Board of the Affiliated Stomatological Hospital of Nanchang University.

Statistical Analysis

Each experiment was conducted three times. Statistical analysis was performed with SPSS v.22.0 (IBM, SPSS, Chicago, IL, USA). Data were presented as mean ± standard deviation. Comparisons between two groups were performed using student's t-test. Differences among multiple groups were evaluated with one-way analysis of variance. A *P*-value less than 0.05 was considered to indicate statistical significance.

RESULTS

circFAM126A Is Overexpressed in OSCC Tissues and Cells

Figures 1A, B showed the differentially expressed circRNAs in OSCC patients (S) and healthy control (N), among which 383 circRNAs were upregulated and 34 downregulated. The expression of circFAM126A was more remarkable. We further determined the expression of circFAM126A in OSCC tissues and cells using qRT-PCR. As shown in **Figure 1C**, the expression of circFAM126A was significantly higher in OSCC tissues compared with healthy control ($P<0.01$). Moreover, high expression of circFAM126A was significantly associated with gender, tumor stage, and lymph node metastasis, but not with age (**Table 1**); additionally, high level of circFAM126A was associated with poor overall survival (**Figure 1D**, $P<0.05$). The expression of circFAM126A was significantly increased in OSCC cells, such as CAL27, SCC15, SCC25, TSCCA, UM1 and UM2 (**Figure 1E**, $P<0.01$, $P<0.001$), which was more remarkable in CAL27 and UM1 cells. Therefore, CAL27 and UM1 cells were used in the following experiments. To further verify the circRNA characteristics of circFAM126A, RNAse and PCR assays were performed. As shown in **Figures 1F, G**, circFAM126A was stable and could resist RNase R digestion in CAL27 and UM1 cells ($P<0.05$, $P<0.01$). Moreover, circFAM126A could be amplified in cDNA, but not gDNA (**Figure 1H**). Furthermore, fluorescence imaging showed that circFAM126A was located in the nucleus as well as in the cytoplasm (**Figure 1I**).

SP1 Transcriptionally Activates circFAM126A in OSCC

SP1 is a crucial transcription factor and functions as an oncogene in OSCC. As shown in **Figure 2A**, the expression of SP1 was significantly higher in patients OSCC tissues compared with healthy control ($P<0.01$). The expression of SP1 in OSCC samples was positively correlated with circFAM126A (**Figure 2B**, $P<0.01$). Moreover, knockdown of SP1 significantly decreased expression levels of circFAM126A (**Figure 2C**, $P<0.01$). To further verify the interaction between SP1 and circFAM126A, serial truncations of the circFAM126A promoter were inserted into the pGL3 vector in HEK-293 T cells. The luciferase activity was significantly increased when the 1419–1477 and 1778–2000 truncations were used (**Figures 2D, E**, $P<0.01$). Furthermore, knockdown of SP1 significantly suppressed luciferase activity in the 1419–1477 group (**Figure 2F**, $P<0.01$). The ChIP further verified that SP1 bound to the promoter of circFAM126A (**Figure 2G**, $P<0.01$).

Knockdown of circFAM126A Inhibits the Proliferation and EMT of OSCC Cells *In Vitro*

Next, to further explore the roles of circFAM126A in OSCC, we treated CAL27 and UM1 cells with circFAM126A shRNA. This knockdown of circFAM126A effectively reduced the expression levels of circFAM126A, with a particularly marked effect in the sh-circFAM126A 2# group (**Figure 3A**, $P<0.01$). Therefore, sh-circFAM126A 2# was used in the following experiment. Knockdown of circFAM126A also significantly inhibited the viability and proliferation of OSCC cells (**Figures 3B, C**, $P<0.01$), and markedly inhibited the migration and invasion of CAL27 and UM1 cells *in vitro* (**Figures 3D–G**, $P<0.01$). Moreover,

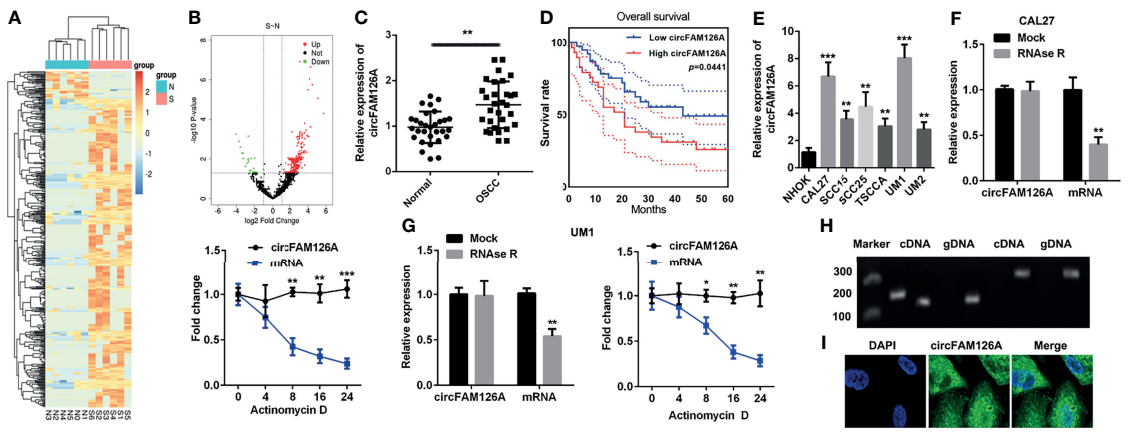

FIGURE 1 | circFAM126A is upregulated in OSCC. **(A, B)** Microarray analysis the differentially expressed circRNAs in OSCC. **(C)** Expression of circFAM126A in clinical samples. **(D)** The overall survival of OSCC patients. **(E)** Expression of circFAM126A in OSCC cells. **(F)** Relative RNA levels and RNA stability in CAL27 detected by qRT-PCR. **(G)** Relative RNA levels and RNA stability in UM1 detected by qRT-PCR. **(H)** Primers amplified in cDNA or gDNA determined by qRT-PCR. **(I)** Locations of circFAM126A detected by fluorescence assay. S: OSCC patients; N: normal group. **P*<0.05, ***P*<0.01, ****P*<0.001.

TABLE 1 | Clinical features of OSCC patients.

Parameters	Total	circFAM126A Expression		P value
		High	Low	
Tissues				<0.01
OSCC	30	21	9	
Health	30	7	23	
Age				0.0157
≥60	31	19	12	
<60	29	15	14	
Gender				0.0363
Male	33	23	10	
Female	27	11	16	
Stage				0.6452
I-II	6	2	4	
III-IV	24	21	3	
Lymph node metastasis				<0.01
	30	22	8	

circFAM126A knockdown significantly suppressed the protein expression of Snail, Vimentin, and N-cadherin, but increased E-cadherin (**Figures 3H, I**, $P<0.01$).

Knockdown of circFAM126A Inhibits Growth of OSCC Tumor *In Vivo*

Xenograft mouse models were established to determine the effects of circFAM126A shRNA on the growth of OSCC tumors *in vivo*. As shown in **Figures 4A–D**, knockdown of circFAM126A significantly decreased tumor size, weight, and volume, and liver metastasis; circFAM126A knockdown markedly decreased expression levels of circFAM126A and Ki67 (**Figures 4E–G**).

miR-186 Is a Direct Target of circFAM126A

miR-186 was predicted to have binding sites for circFAM126A (**Figure 5A**). To confirm the targeting relationship between circFAM126A and miR-186, we performed luciferase activity reporter and RNA pull-down assays. As shown in **Figure 5B** ($P<0.01$), miR-186 mimics markedly decreased luciferase activity in wt-circFAM126A-transfected cells but had no significant effects on mut-circFAM126A-transfected cells. Furthermore, a circFAM126A probe and biotin miR-186-probe were used to perform the RNA pull-down assay. The results showed that the miR-186 probe could enrich circFAM126A (**Figure 5C**, $P<0.01$). Moreover, the expression of miR-186 in OSCC cells was significantly increased by knockdown of circFAM126A but significantly decreased by overexpression of circFAM126A (**Figure 5D**). As shown in **Figure 5E**, the expression of miR-186 was significantly decreased in OSCC tissues in comparison with adjacent normal tissues ($P<0.01$).

RAB41 Is a Target of miR-186

miRNAs regulate biological progresses *via* binding to their targets. Online database TargetScan 7.2 was used to predict the

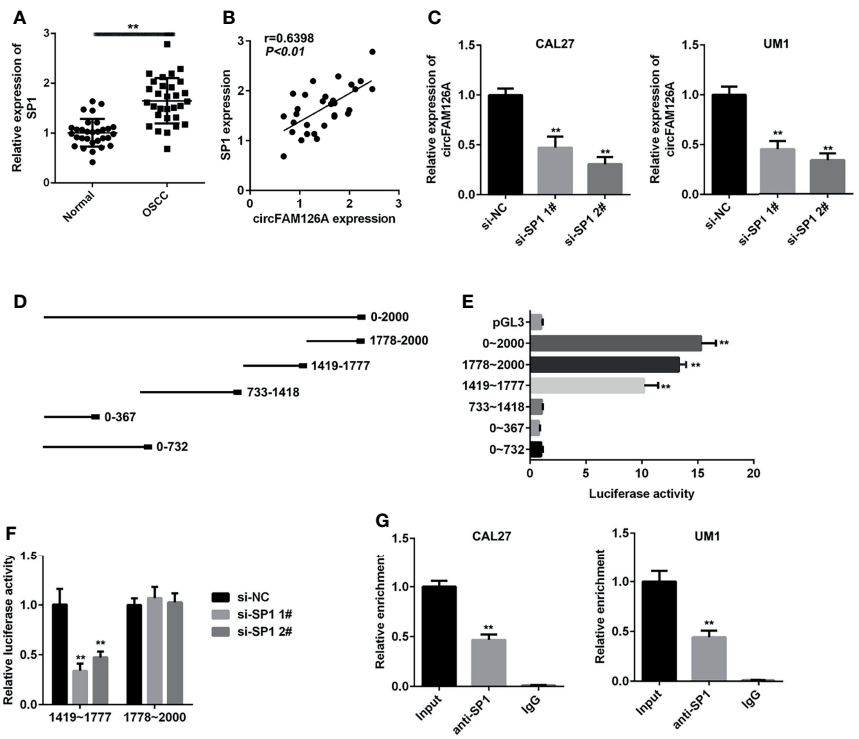

FIGURE 2 | SP1 transcriptionally activates circFAM126A in OSCC. **(A)** mRNA expression of SP1 in OSCC patients measured using qRT-PCR. **(B)** Correlation analysis of expression levels of SP1 and circFAM126A in OSCC patients. **(C)** Expression of circFAM126A detected by qRT-PCR. **(D)** Serial truncations of circFAM126A promoter inserted into pGL3 vector. **(E, F)** Interaction between SP1 and circFAM126A verified by luciferase assay. **(G)** Interaction between SP1 and circFAM126A verified by ChIP assay. $^{**}P<0.01$.

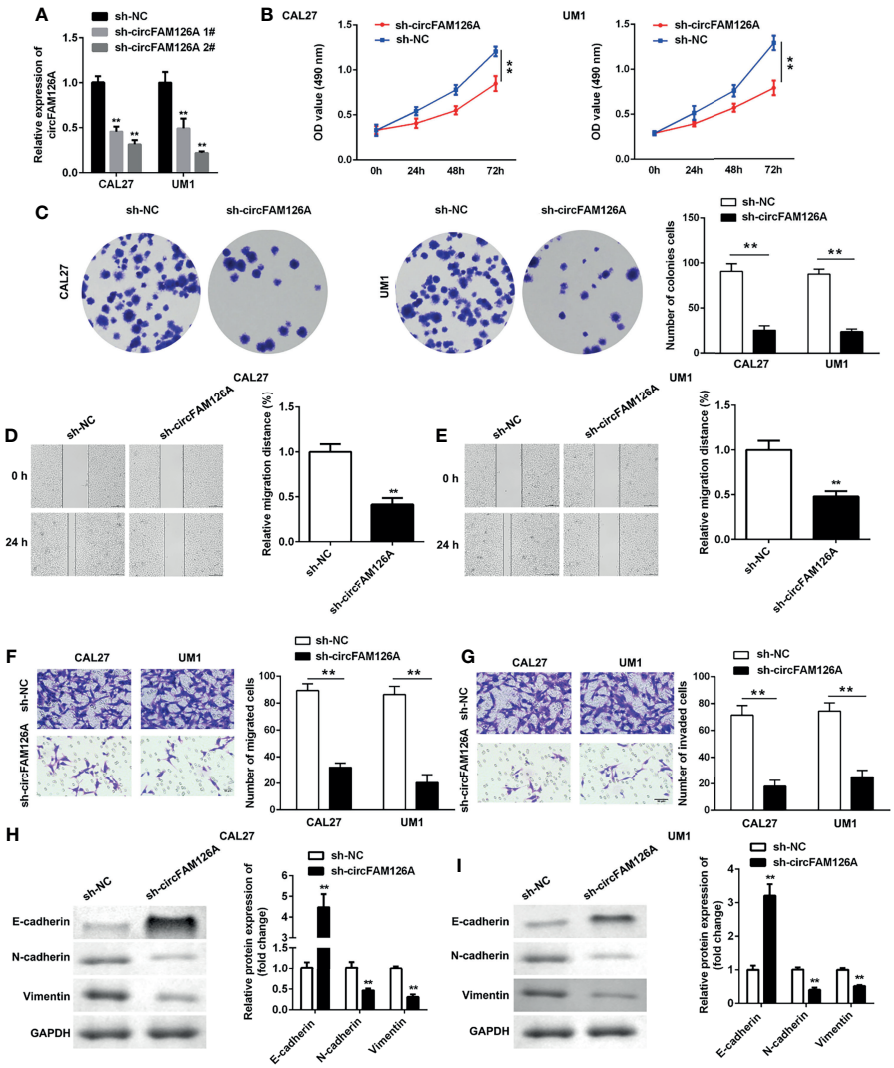

FIGURE 3 | circFAM126A shRNA inhibits proliferation, migration, and invasion of OSCC cells. **(A)** Expression of circFAM126A in OSCC cells detected by qRT-PCR. **(B)** Viability of CAL27 and UM1 cells determined by MTT assay. **(C)** Proliferation of OSCC cells detected by colony formation assay. **(D, E)** Migration detected using wound healing assay. **(F, G)** Migration and invasion of OSCC analyzed by transwell assay. **(H, I)** Protein expression determined using western blot. $^{**}P<0.01$.

targets of miR-186. **Figure 6A** showed the binding sites between miR-186 and RAB41 (**Figure 6A**). The luciferase assay showed that miR-186 mimics markedly decreased luciferase activity in wt-RAB41-transfected cells, whereas they had no significant effects on mut-RAB41-transfected cells (**Figure 6B**, $P<0.01$). The RNA pull-down assay further verified the interaction between miR-186 and RAB41 (**Figure 6C**, $P<0.01$). Overexpression of miR-186 significantly decreased the expression of RAB41 at both the mRNA and protein levels, whereas RAB41 expression was significantly upregulated by miR-186 inhibitors (**Figures 6D, E**, $P<0.01$). As shown in **Figure 6F**, the expression of RAB41 was significantly increased in OSCC tissues compared with adjacent normal tissues ($P<0.01$). The expression of RAB41 was positively correlated with circFAM126A expression and negatively correlated with miR-186 expression (**Figures 6G, H**, $P<0.01$).

circFAM126A Interacts With FUS to Promote mRNA Stability of RAB41

circRNAs regulate gene expression *via* binding to miRNAs or RNA-binding proteins (RBPs). RNA pull-down and mass spectrometry analyses showed that circFAM126A could bind to FUS (**Figure 7A**, $P<0.01$). The RIP assay further verified the interaction between FUS and circFAM126A and RAB41 (**Figure 7B**, $P<0.001$). The expression of FUS was significantly increased after transfection with FUS, indicating that cells had been successfully transfected (**Figure 7C**, $P<0.01$, $P<0.001$). Moreover, overexpression of FUS significantly increased its mRNA stability (**Figure 7D**, $P<0.05$, $P<0.01$), and circFAM126A

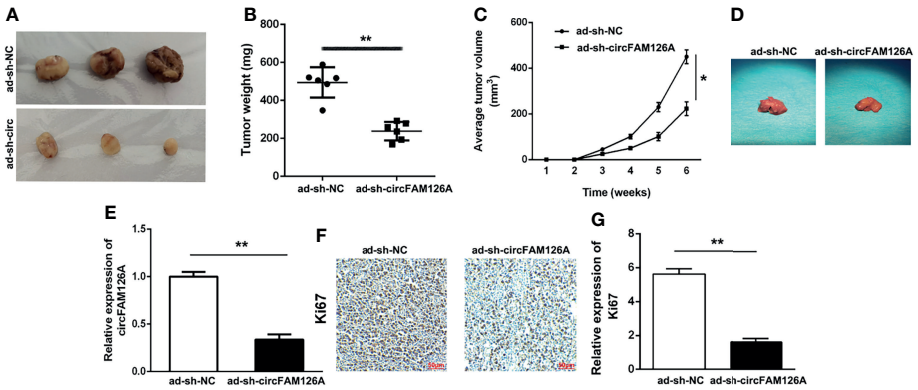

FIGURE 4 | circFAM126A shRNA inhibits the growth of OSCC tumor *in vivo*. **(A)** Xenograft analysis of tumor growth *in vivo*. circFAM126A knockdown suppressed OSCC tumor growth. **(B, C)** Tumor weight and volume after transfection with circFAM126 knockdown. **(D)** The suppression of liver metastasis induced circFAM126 knockdown. **(E)** Expression of circFAM126 *in vivo* detected using qRT-PCR. **(F, G)** Expression of Ki67 in OSCC determined by immunohistochemistry. **P*<0.05, ***P*<0.01.

modulated the interaction between FUS and RAB41 (**Figure 7E**, *P*<0.01). The decrease in mRNA stability induced by circFAM126A knockdown was reversed by overexpression of FUS (**Figure 7F**, *P*<0.01).

circFAM126A Regulates the Proliferation and EMT of OSCC Cells *via* Targeting the miR-186/RAB41 Axis

As shown in **Figure 8A**, RAB41 overexpression plasmids significantly increased the expression of RAB41 compared with sh-circFAM126A (*P*<0.01). Moreover, compared with circFAM126A knockdown, overexpression of RAB41 significantly promoted the proliferation (**Figures 8B, C**, *P*<0.01), migration (**Figures 8D, E**, *P*<0.01), and invasion (**Figure 8F**, *P*<0.01) of OSCC cells *in vitro*. Additionally, upregulated RAB41 antagonized the effects of circFAM126A knockdown on the protein expression of Snail, Vimentin, E-cadherin, and N-cadherin (**Figures 8G, H**, *P*<0.01).

DISCUSSION

Dysregulated circRNAs play a crucial role in the development of OSCC. circRNAs may function as anti-tumor genes or oncogenes in OSCC. In this study, we found that circFAM126A was upregulated in OSCC. Moreover, SP1-induced upregulation

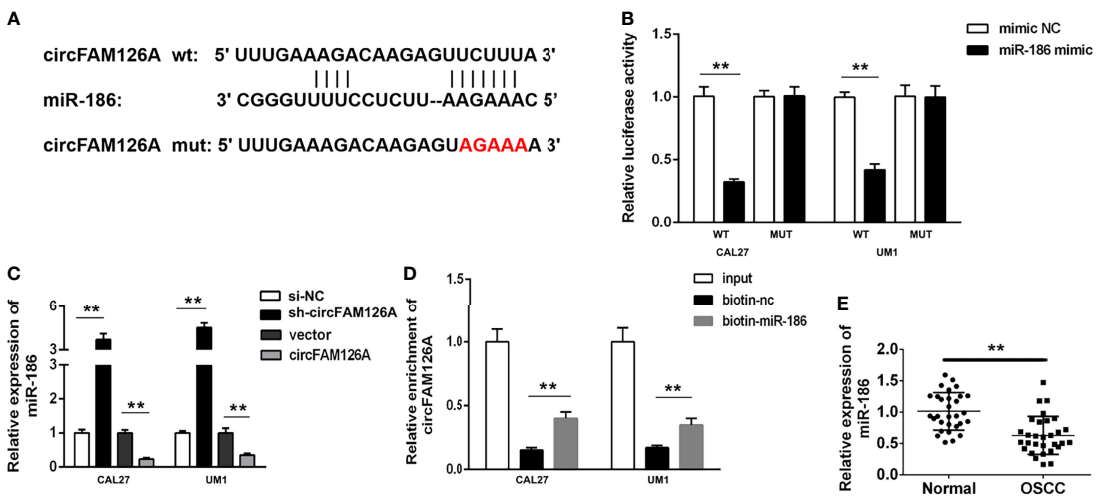

FIGURE 5 | circFAM126A acts as a sponge of miR-186 in OSCC cells. **(A)** The binding sites predicted by Starbase3.0. **(B)** The binding sites verified by dual-luciferase reporter assay. **(C)** The expression of miR-186 detected using qRT-PCR. **(D)** The interaction between circFAM126A and miR-186 determined by RNA pull-down assay. **(E)** Expression of miR-186 in clinical samples determined by qRT-PCR. ***P*<0.01.

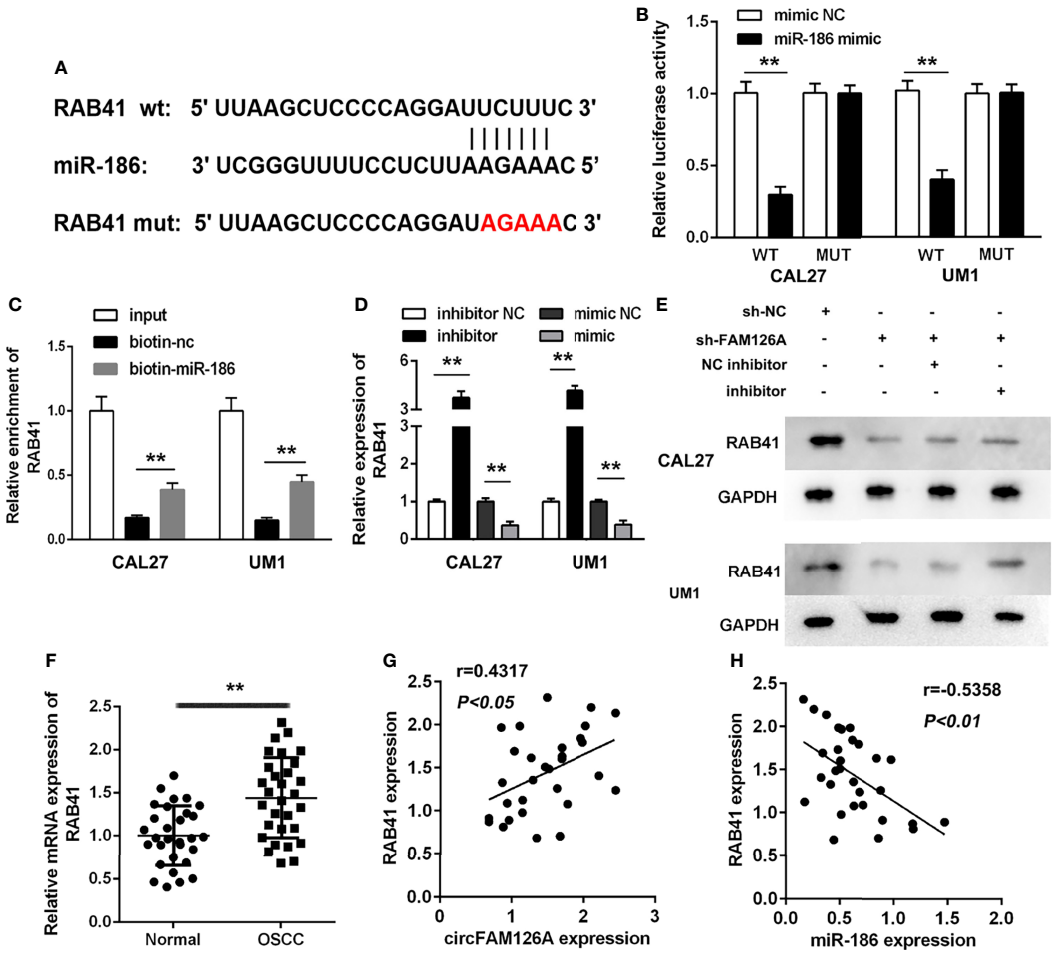

FIGURE 6 | RAB41 is a target of miR-186 in OSCC cells. **(A)** The binding sites predicted by TargetScan7.2. **(B)** The binding sites verified by dual-luciferase reporter assay. **(C)** The interaction between RAB41 and miR-186 determined by RNA pull-down assay. **(D)** mRNA expression of RAB41 in OSCC cells detected using qRT-PCR. **(E)** Protein expression of RAB41 in OSCC cells measured using western blot. **(F)** Expression of RAB41 in OSCC clinical samples determined by qRT-PCR. **(G)** Correlation analysis of RAB41 and circFAM126A in clinical samples. **(H)** Correlation analysis of RAB41 and miR-186 in clinical samples. $^{**}P<0.01$, $^{\#\#}P<0.01$.

of circFAM126A promoted the proliferation and EMT of OSCC cells by regulating the miR-186/FUS/RAB41 axis *in vitro*. However, knockdown of circFAM126A suppressed the aggressiveness and metastasis of OSCC *in vitro* and *in vivo*. This is the first study to investigate the mechanism, expression, regulation, and clinical implications of circFAM126A in OSCC.

circRNAs are a type of non-coding RNA. Owing to their stability, diversity, high expression, and high sequence conservation, and other biological characteristics, circRNAs have potential as diagnostic markers and therapeutic targets in tumorigenesis and progression. Many studies have reported abnormal expression of circRNAs in different types of tumors. For example, Zhang et al. (24) found evidence that circ-100876 is downregulated in colorectal cancer tissues, and that low expression of circ-100876 predicts poor prognosis and increases the risk of relapse of colorectal cancer patients. Yu et al. (25) found that the expression of circRNA SMARCA5 is significantly downregulated in liver cancer tissues and is associated with early tumor stage and poor prognosis of liver cancer patients. Moreover, circRNAs play vital roles in many physiological and pathological processes, including cell cycle progression, autophagy, proliferation, invasion, metastasis, and carcinogenesis (26, 27). In the present study, circFAM126A was found to be upregulated in OSCC. Moreover, knockdown of circFAM126A markedly inhibited OSCC cell proliferation and EMT of OSCC cells, manifested by the upregulation of epithelial marker (E-cadherin) and downregulation of mesenchymal markers (N-cadherin and Vimentin) (17–21). Additionally, circFAM126A knockdown inhibited tumor growth and metastasis of OSCC *in vivo*. Therefore, the above data suggest that circFAM126A may have an oncogenic role in the progression of OSCC.

Previous reports demonstrate that transcription factors participate in the progression of cancer (28–30). Dysregulated transcription factors promote tumorigenesis *via* transcriptionally

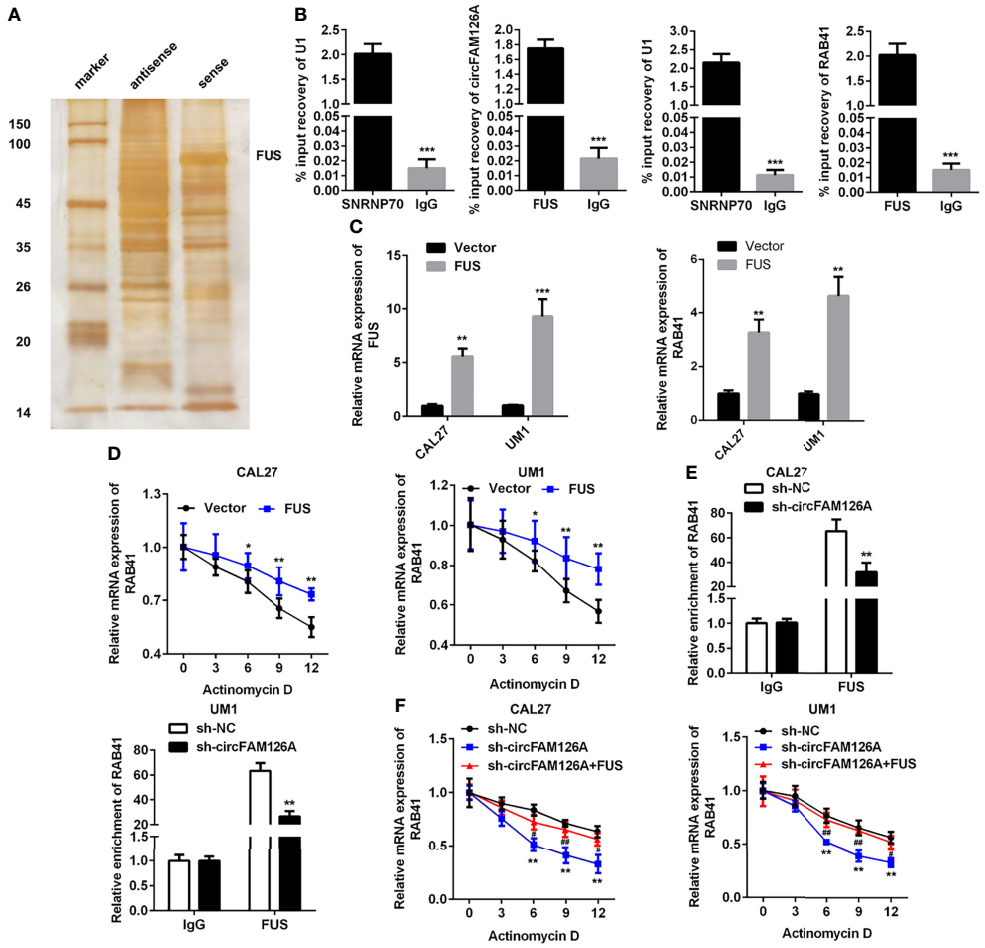

FIGURE 7 | circFAM126A binds to FUS to promote the mRNA stability of RAB41. **(A)** The potential proteins interacting with circFAM126A detected using RNA pull-down. **(B)** Interaction between FUS and circFAM126A or RAB41 confirmed by RIP assay. **(C)** mRNA expression of FUS and RAB41 detected by qRT-PCR. **(D)** mRNA stability of RAB41 determined using qRT-PCR. **(E)** Interaction between FUS and circFAM126A or RAB41 verified by RIP assay. **(F)** mRNA stability of RAB41 determined using qRT-PCR. *P<0.05, **P<0.01 and ***P<0.001.

regulating non-coding RNAs, such as long coding RNAs and circRNAs (29). We further investigate the upstream of circFAM126A. SP1 functions as an oncogene in various cancer, including OSCC (31–33). In this study, SP1 was overexpressed in OSCC tissues and cells. Moreover, SP1 transcriptionally upregulated circFAM126A, which further contributed the tumor growth and metastasis of OSCC.

Accumulating evidence demonstrates that miRNAs have essential roles in the regulation of tumor progression (34–36), and circRNAs might exert their functions through targeting miRNAs. miR-186 has been suggested to function as a tumor suppressor in the progression of OSCC (37). In this study, miR-186 was predicted and proved to be a target of circFAM126A. In addition, circFAM126A interacted with miR-186 to regulate the proliferation and EMT of OSCC cells. Therefore, circFAM126A may exert its carcinogenic function *via* targeting miR-186.

circRNAs, which lack the ability to encode proteins, function as ceRNAs to regulate gene expression *via* binding to miRNAs or RBPs (38). The circRNA/(miRNA/RBP)/mRNA axis may intensively participate in the progression of cancers including OSCC (39, 40). In this study, circFAM126A modulated the expression of RAB41 *via* sponging miR-186 and promoted its mRNA stability *via* interacting with RNA binding protein FUS. RNA pull-down assay and mass spectrometry analysis showed that circFAM126A could bind to FUS to increase the mRNA stability of RAB41. circFAM126A increased the expression of RAB41 *via* sponging miR-186. These results further elucidated the underlying mechanism, in which circFAM126A increased the mRNA expression of RAB41. RAB41 is a member of RAB family, which frequently acts as oncogenes in various cancer (41). RAB41 plays an essential role in membrane trafficking, high expression of which is associated with poor clinical results of lung adenocarcinoma (42). However, the roles of RAB41 in OSCC have not been fully elucidated. In this study, RAB41 was found to be overexpressed in OSCC. The expression of circFAM126A was positively correlated with RAB41. Overexpression of RAB41

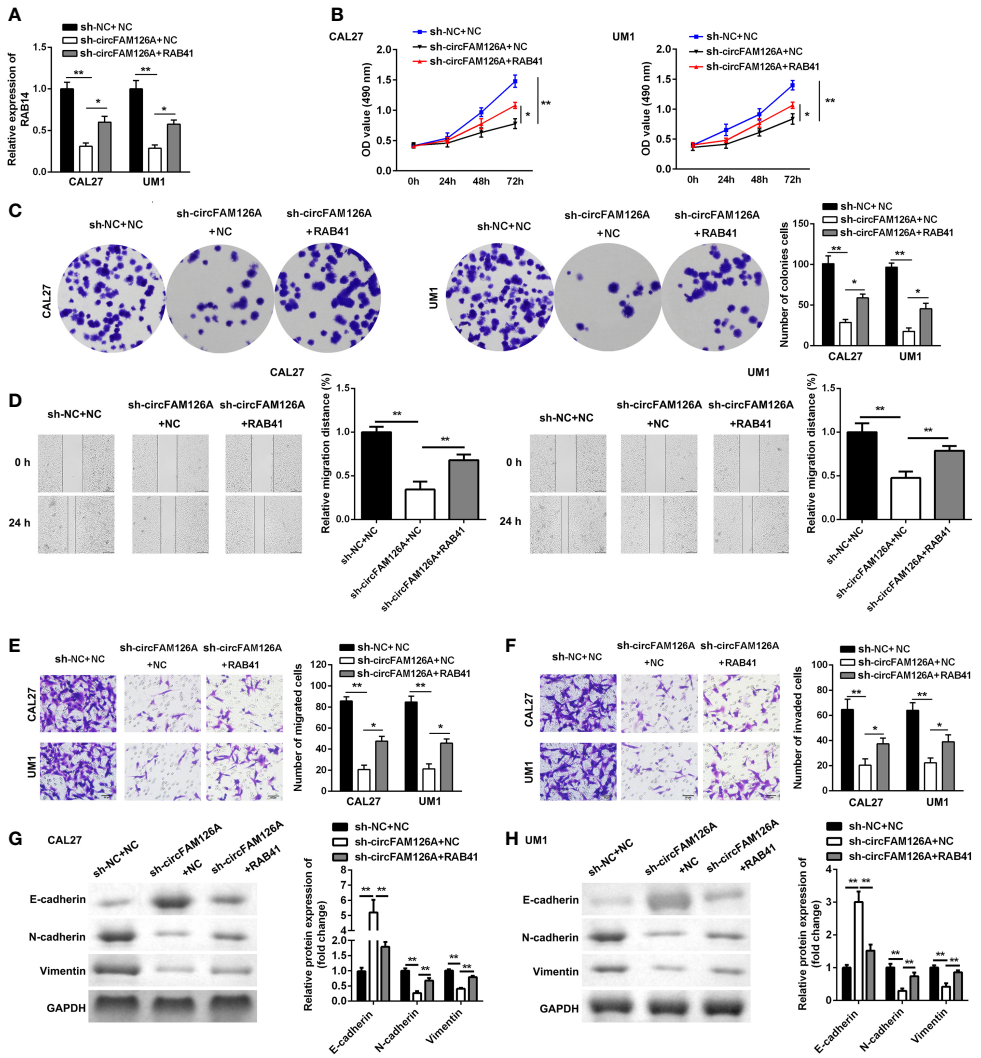

FIGURE 8 | Overexpression of RAB41 reverses the anti-tumor effects of sh-circFAM126A. **(A)** Expression of RAB41 in OSCC cells detected using qRT-PCR. **(B, C)** Viability and proliferation ability of OSCC cells detected by MTT and colony formation assay. **(D)**. Migration determined using wound healing assay. **(E, F)** Migration and invasion ability of OSCC cells measured using transwell assay. **(G, H)** Protein expressed determined using western blot. $^{*}P < 0.05$, $^{**}P < 0.01$.

alleviated the effects of circFAM126A knockdown and promoted an aggressive phenotype of OSCC cells. Taken together, these results suggest that circFAM126A regulates the growth and metastasis of OSCC cells *via* modulation of the miR-186/FUS/RAB41 axis.

CONCLUSION

Taken together, circFAM126A played vital roles in the progression of OSCC. SP1-mediated upregulation of circFAM126A promoted the growth and metastasis of OSCC cells *via* the miR-186/FUS/RAB41 axis. These results could indicate a new target for the treatment of OSCC.

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 human participants were reviewed and approved by Affiliated Stomatological Hospital of Nanchang University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JW was responsible for the organization and coordination of the trial. SO was the chief investigator and responsible for the data analysis. SZ, XZ, MC, XF, YC, and LL developed the trial design. All authors contributed to the writing of the final manuscript.

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CD19-Targeted Immunotherapies for Diffuse Large B-Cell Lymphoma

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Surgical resection, chemotherapy and radiotherapy were, for many years, the only available cancer treatments. Recently, the use of immune checkpoint inhibitors and adoptive cell therapies has emerged as promising alternative. These cancer immunotherapies are aimed to support or harness the patient's immune system to recognize and destroy cancer cells. Preclinical and clinical studies, based on the use of T cells and more recently NK cells genetically modified with chimeric antigen receptors retargeting the adoptive cell therapy towards tumor cells, have already shown remarkable results. In this review, we outline the latest highlights and progress in immunotherapies for the treatment of Diffuse Large B-cell Lymphoma (DLBCL) patients, focusing on CD19-targeted immunotherapies. We also discuss current clinical trials and opportunities of using immunotherapies to treat DLBCL patients.

Keywords: monoclonal antibodies, antibody-drug conjugates, bispecific T cell engagers, genetic modification, engineered T cells, CAR-T cells, CAR-NK cells

INTRODUCTION

The 2016 World Health Organization Classification of Tumors defines Diffuse Large B-Cell Lymphoma (DLBCL) as a disease originating from mature B-cells, for a large proportion of which there are no clear and accepted classification criteria. Despite the DLBCL heterogeneity, the neoplastic cells typically express pan-B-cell markers CD19, CD20, CD22, CD79a, PAX5 (1), paving the way for the introduction of targeted therapies. Among these, the use of the anti-CD20 monoclonal antibody rituximab represented the cornerstone. Rituximab is a chimeric monoclonal antibody, whose murine variable regions bind to CD20 on B-cells, while the human constant regions mediate effector mechanisms (2, 3), such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Large randomized trials comparing standard chemotherapy alone to the addition of rituximab showed a clear survival advantage for the combined immunochemotherapy approach (4–6), leading to the association of chemotherapy to rituximab as the current standard of care for DLBCL patients (7). Despite the successful history of anti-CD20 immunotherapy in DLBCL, approximately 40-50% of patients ultimately do not respond to frontline treatment (8). Several mechanisms of resistance have been hypothesized, including CD20 loss, expression of CD20 variants lacking the determinants recognized by rituximab, and polymorphisms of FcγRIIIA negatively affecting effector cell functions (3), making the identification

of alternative targets for immunotherapy a definitive need. Among pan-B-cell markers, CD19 is an attractive target due to both its broad presence through B-cell ontogeny and its functional role. CD19 is a 95 kDa, type I transmembrane glycoprotein whose expression starts early in B-cell maturation, concurrently with immunoglobulin gene D-J regions rearrangement in Pro-B cells, and ends with terminally differentiated plasma cells (9). The almost ubiquitous expression among B-cell lymphopoiesis of CD19 underlines its fundamental role in B-lineage functionality and commitment (9). In a murine model of B cell lymphoma, Chung and coworkers demonstrated a correlation between CD19 mRNA levels and the oncogene MYC expression, suggesting a role of CD19 in lymphomagenesis and arguing that CD19 ligation through targeted agents could represent a strategy to disrupt MYC signaling and interfere with oncogenesis (10). At present, four classes of drugs have been designed to target CD19: unconjugated monoclonal antibodies (mAb), antibody-drug conjugates (ADC) and molecules that specifically recruit T-cells, including bispecific T cell engagers (BiTE) and chimeric-antigen receptors (CAR).

MONOCLONAL ANTIBODIES

Inebilizumab (MEDI-551) is a humanized, a-fucosylated anti-CD19 antibody developed from the murine HB12b mAb through a two-step process: 1) HB12b humanization and Fab rearrangement, respectively to reduce immunogenicity and optimize CD19-affinity and 2) fucose removal to increase affinity for human CD16/FcγRIIIA, optimizing antibody-dependent cell cytotoxicity (ADCC) performed by Natural Killer (NK) cells and macrophages (11). Interestingly, subsequent observations underlined the role of F/V 158 FcγRIIIA polymorphisms in NK cell-mediated killing: in an *in-vitro* assay, heterozygosity for the high-affinity FcγRIIIA (namely, 158V allotype) was sufficient for efficient B-cell leukemia cells killing, while homozygosity for the weak-binding allotype FcγRIIIA (i.e. 158F allotype) was associated with the absence of activity (12). Despite a phase 1 trial showing both a good safety profile and some evidence of activity of single-agent inebilizumab treatment for B-cell malignancies, including DLBCL (NCT01957579), subsequent combination studies failed their endpoints, included a phase 1b/2 trial performed using inebilizumab in combination with an anti-PD1 mAb (NCT02271945).

Tafasitamab (MOR208) is an engineered antibody characterized by two amino acid substitutions, S239D/I332E enhancing FcγR and C1q binding and, therefore, effector cells recruitment (ADCC) and complement cascade activation (CDC) (13). In contrast to a-fucosylated antibodies, the S239D/I332E modification increases the affinity to all activating FcγR receptors (i.e. FcγRI, FcγRIIA, and FcγRIIIA) (14) irrespective of the FcγRIIIA-V/F allotype (15). A phase 2a trial with the single-agent tafasitamab showed promising activity in 35 patients affected by DLBCL (NCT01685008). Refractoriness to rituximab or FcγRIII-158F allotype did not impact tafasitamab treatment efficacy (16). Several combination trials, mainly with lenalidomide or bendamustine, are testing tafasitamab both in the relapsed and refractory (R/R) patients and first-line setting. The phase 2 L-MIND trial evaluated MOR208 plus lenalidomide for R/R DLBCL (NCT02399085), focusing on the synergic NK cell-mediated-ADCC observed when MOR208 is combined to lenalidomide (17), a potent NK cell activator (18). In this trial, 60% of the patients achieved a response and the median progression-free survival was 12.1 months (19). Moreover, an updated analysis showed activity even in high-risk categories (i.e. previously refractory) and beyond the second line (20). Based on L-MIND results, the tafasitamab-lenalidomide combination achieved the FDA approval for the R/R DLBCL. Currently, the phase 3 randomized, frontMIND trial (NCT04824092) aims to test tafasitamab plus lenalidomide in combination with the first-line chemotherapy regimen R-CHOP.

ANTIBODY-DRUG CONJUGATES

Denintuzumab mafodotin (SGN-CD19A) is a humanized anti-CD19 monoclonal antibody conjugated with monomethyl auristatin F (MMAF), a synthetic analogue of the natural antimitotic agent dolastatin 10. As a tubulin-binding molecule, dolastatin exerts its cytotoxic effect through the inhibition of microtubule assembly and tubulin-dependent GTP hydrolysis, leading to cell cycle arrest and apoptosis (21). MMAF differs from another auristatin derivative, monomethyl auristatin E (MMAE), for a C-terminal modification which is aimed to limit membrane permeability and reduce bystander and off-target toxicity (22). A phase 1 study (NCT01786135) demonstrated the safety of SGN-CD19A in the clinical setting of R/R B-cell NHL, with 30% of evaluable patients achieving a complete response (23). Two subsequent studies with denintuzumab mafodotin in combination with chemotherapy were interrupted with no further development (NCT02592876; NCT02855359).

Loncastuximab tesirine (ADCT-402): upon ligation, CD19 is rapidly internalized, making it an ideal target for immune-conjugates, which carry highly cytotoxic molecules directly within the cell. ADCT-402 is composed of the humanized anti-CD19 antibody RB4v1.2 linked with tesirine (SG3249), a drug-linker which delivers, through lysosomal degradation, the pyrrolobenzodiazepine (PBD) dimer warhead SG3199. SG3199 forms a covalent bond with the minor groove of DNA (24) through a minimal distortion of the DNA helix, hence slowing DNA repair and promoting intracellular persistence (25). Moreover, once released by damaged $CD19^+$ cells within the medium, its high permeability allows bystander cytotoxicity, even among $CD19^-$ cells (26). Interestingly, loncastuximab might not preclude a subsequent CD19-targeted therapy: a small series of 14 patients who failed loncastuximab conserved CD19 expression and responded to anti-CD19 CAR-T cells (27). A phase 1 study (NCT02669017) in patients affected by R/R B-cell NHLs has shown a good safety profile and encouraging activity (28), confirmed by the phase-2 LOTIS-2 trial (NCT03589469), where single-agent loncastuximab achieved an overall response rate (ORR) of 48%, half of which in

complete remission (CR) (29). Loncastuximab is being tested even in combination with targeted molecules such as ibrutinib, venetoclax or durvalumab (NCT03684694; NCT05053659; NCT03685344) in the R/R setting, as well as a first-line agent, together with chemotherapy (NCT04974996).

BISPECIFIC T CELL ENGAGERS

Blinatumomab (MT103): Bispecific T cell engagers (BiTE) represent the attempt to engage T cells in a polyclonal fashion, thus overcoming limits of clonal-specific response. Blinatumomab is a bispecific antibody composed of four variable domains, oriented to form two single-chain antibodies (scFvs), respectively directed against CD19 and CD3 (30). A short amino acidic linker keeps the two scFvs together and allows sufficient flexibility for the crosslink (31). Pre-clinical data with blinatumomab highlighted that T cells, once recruited, are much more potent effectors than NK cells and monocytes/macrophages; moreover, both $CD8^+$ and $CD4^+$ T cells can exert cytotoxic functions, independently of CD28 co-ligation or IL-2 exposition/exposure (31). The phase 2 study in R/R DLBCL (NCT01741792) showed remarkable activity and suggested a refinement in its administration to avoid neurotoxicity (32), a complication already emerged during phase 1 (NCT00274742). The phase 2/3 trial (NCT02910063) evaluated blinatumomab as a second-salvage strategy through a dose-escalating approach, to avoid toxicities. Despite efficacy (ORR: 36%) in a highly unfavorable cohort, only 46% of patients completed the first cycle, mainly due to concomitant disease progression (33); the dose-escalating approach might have hampered efficacy in patients with a rapidly progressive disease. As a consolidation strategy after the rituximab-chemotherapy-based first-line, blinatumomab was remarkably able to convert positive minimal residual disease (MRD) to negativity (NCT03023878) (33).

TNB-486 CD19/CD3: cytokine releasing syndrome (CRS) and neurotoxicity can represent life-threatening complications of CAR-T cells and BiTE therapies, limiting their use especially in frail patients. TNB-486 is a fully human, CD19/CD3 bi-specific antibody specifically designed to reduce the cytokine release from activated $CD3^+$ cells upon engagement. The molecule is constituted of a high-affinity anti-CD19 heavy chain and a low-affinity anti-CD3 light chain, the latter with low-activating potential. *In vitro* models have demonstrated that the cytokine secretion (i.e., IL-2, IFN-γ, IL-6, IL-10, and TNF) by $CD3^+$ cells is minimal even at saturating doses for tumor lysis (34, 35). A phase 1 study (NCT04594642) is currently testing TNB-486 for R/R B-cell non-Hodgkin lymphoma in patients who have received 2 or more prior lines of therapy.

CAR-CD19 ENGINEERED T CELLS

Chimeric Antigen Receptor – T Cells

Several aspects impact CAR-T cells biology, generating differences in expansion, persistence, and toxicity. Current evidence about relevant biological variables will be analyzed, together with a final update on the commercially approved products for DLBCL.

Chimeric Construct

The extremities of an anti-CD19 CAR construct, *in extenso* the extracellular scFv CD19 binding-region FMC63 and the intracellular CD3ζ signaling tail, are "fixed components" in the majority of products. Differences involve the hinge and the costimulatory domain (CD) which, respectively, optimize antigen-reach and prevent early exhaustion upon antigen-ligation. The combination of CD8α-derived hinge & transmembrane (TM) region with the 41BB CD (8-8-41BB CAR, adopted for tisagenlecleucel) is common. Alternatives are a full CD28 sequence (28-28-28 CAR, adopted for axicabtagene) or a combination of IgG4, CD28, and 41BB (IgG4-28-41BB, adopted for lisocabtagene). The incorporation of CD28 drives to a pronounced expansion, a favorable effector:target ratio and a faster tumoricidal activity, counterbalanced by a prolonged persistence for 41BB (36). In this view, CAR-T dynamics might be driven by downstream metabolic pathways: CD28 signaling leads to anaerobic glycolysis, typical of effector T-cells, 41BB to mitochondrial fatty-acid oxidation, and central-memory differentiation (37). A higher pro-inflammatory cytokines release might increase complications in CD28-based products (38). Interestingly, a clinical trial testing a 28-28-41BB product showed rates of inflammatory and neurological complications superimposable to 28-28-28 CAR-Ts, suggesting that the hinge-TM region, rather than the costimulatory domain, might be involved in mediating CAR-T-associated toxicity (38).

Manufacturing Process

It is composed of mononuclear cells apheresis and manipulation into the final product. Despite apheresis cryopreservation allows major flexibility, concerns may rise about post-thaw viability. Panch et al. confirmed a reduction of viable T-cells 2 days after thawing; nevertheless, in the presence of a sufficient apheresis, anti-CD19 CAR-T generation was not hampered (39). With regards to the final product, measures can be taken to control the CAR-T subsets composition and ratios. Sommermeyer et al. demonstrated that naïve (TN) CD4 and central memory (TCM) CD8 CAR-T cells have, separately, high anti-CD19 activity. Thus, hypothesizing a synergism, with CD4 producing IL-2 that activate and expand CD8 cells, they demonstrated that a fixed 1:1 CAR-T ratio of $CD62L^+/CD45RO^-$ CD4 TN and $CD62L^+/CD45RO^+$ CD8 TCM has the strongest activity against CD19 tumors (40).

Lymphodepletion

The lymphodepleting therapy consists in a course of chemotherapy, administered shortly before the CAR-T infusion to create a favorable immunological environment. Indeed, lymphodepletion increases chemotactic factors (MCP-1) and homeostatic cytokines (IL-2, IL-7 and IL-15), promotes eradication of regulatory T-cells and myeloid-derived suppressor cells, and the induction of costimulatory molecules. A combination of fludarabine and cyclophosphamide is the most

employed regimen, relying on early trials where the addition of fludarabine to cyclophosphamide improved CAR-T expansion and persistence (41–43). Moreover Hirayama et al. demonstrated an association between higher doses of cyclophosphamide and a favorable cytokine profile (defined as day 0 MCP-1 and peak IL-7 concentrations) (44).

Commercially Available Anti-CD19 CAR-T Products

Tisagenlecleucel (CTL019)

Tisagenlecleucel represents the first-in-class, autologous anti-CD19 CAR-T against DLBCL. Its approval followed the results of the phase 2 trial JULIET (NCT02445248) in R/R DLBCL. The manufacturing process consists in the lentiviral transduction of unselected T-cells, cryopreserved after collection (45). The JULIET trial tested tisagenlecleucel in 93 patients affected by R/R DLBCL, ineligible for or progressed after hematopoietic stem-cell transplantation. Half of the infused patients achieved a response, 40% of which as a complete remission. Promisingly, 65% of treatment-sensitive patients conserve a response (46). A trial update (47) and real-life experiences (48) support original data. Several trials involve tisagenlecleucel, included primary CNS lymphoma (NCT04134117) and pediatric R/R B-cell non-Hodgkin lymphoma (NCT03610724). The randomized, phase 3 BELINDA trial (NCT03570892) failed its aim to test tisagenlecleucel earlier as a second-line strategy (49). A phase 3 trial (NCT04094311) is investigating out-of-specification tisagenlecleucel for commercial release.

Axicabtagene Ciloleucel (KTEX19)

Axicabtagene manufacturing relies on the manipulation of a fresh apheresis and a gamma-retroviral transduction (50). KTEX19 was approved following the phase 1/2 study ZUMA-1 (NCT02348216), which exhibited remarkable results in a cohort of heavily pre-treated patients: 82% achieved a response, 54% a complete remission. Interestingly, responses were not negatively impacted by high-risk variables such as high IPI score, bulky disease and refractoriness to the previous line. A recent update showed that one-third of patients still in response at 24 months no longer had circulating CAR-T cells, suggesting that responses are not dependent on CAR-T persistence over time. Two multicenter trials are testing axicabtagene for high-risk DLBCL in an earlier setting: the ZUMA-7 (NCT03391466) as a second line, and the ZUMA-12 (NCT03761056) as a frontline treatment, respectively. Recent data from the ZUMA-7 demonstrated axicabtagene superiority in terms of overall response and risk of progression/death, in a comparison with a standard second line treatment comprehensive of high-dose chemotherapy followed by autologous transplant (51).

Lisocabtagene Maraleucel (JCAR017)

JCAR017 is a fixed 1:1 ratio of CD4 and CD8 cells (40). The manufacturing process, through which CD4 and CD8 T cells are separately activated and transduced through a lentiviral vector, leads to an enrichment in less differentiated, predominantly memory T-cells (52). The phase 1 TRANSCEND trial (NCT02631044) demonstrated high clinical activity (Response Rate 73%, Complete Remission 53%) with a low incidence of moderate/severe CRS and neurological events. The trial allowed the recruitment of secondary CNS lymphoma: in this subgroup, lisocabtagene achieved a 50% remission rate without fatal neurological events (53). A pooled analysis from 3 clinical trials (NCT02631044; NCT03744676; NCT03483103) in the outpatient setting provided encouraging data, with 46% of patients not requiring hospitalization after infusion (54). The TRANSFORM trial, aimed to compare lisocabtagene with high-dose chemotherapy followed by autologous stem-cell transplantation in a second-line setting, demonstrated a significant improvement in the probability of remission and a prolongation in event-free survival, in patients with early relapse or refractory disease (NCT03575351). Despite the need for a longer follow-up, an improvement in overall survival seems to emerge (55).

TOWARDS CAR-NK CELLS

In order to overcome the hurdle of manufacturing timelines and the poor fitness of autologous T cells, two factors that can affect the CAR-T therapy efficacy, ongoing clinical trials (NCT03666000, NCT03939026 and NCT04416984) are testing allogeneic CAR-T products. In particular, treatment with PBCAR0191, an anti-CD19 CAR-T product in which endogenous TCR is disrupted by gene editing to prevent GvHD, together with an intensified lymphodepletion, has shown clinical benefit in the majority of NHL patients, yielding high rates of overall and complete response with promising activity in both CD19 CAR naïve subjects and those who progressed following auto-CD19 CAR therapy (56, 57). Other ongoing studies are testing ALLO-501/ALLO-501A, alternative allogeneic anti-CD19 CAR-T products modified by gene editing to disrupt the T-cell receptor alpha constant gene and the CD52 gene, respectively to reduce the risk of GvHD and allow the use of anti-CD52 mAb to delay host T cell reconstitution and graft rejection, have provided encouraging results (58, 59).

However, CAR-NK cells represent a more appealing alternative strategy to reduce the disadvantages related to the production and use of anti-CD19 CAR-T cells.

CAR-NK cells can be prepared in advance to be rapidly available on demand and, most likely, less capable of inducing CRS and neurotoxicity. Notably, CAR-NK cells can kill tumor cells even in a CAR-independent manner by their native receptors (including NCRs, NKG2D, DNAM-1, and activating KIRs), counteracting tumor escape mechanism due to lack of CAR-targeted antigen. Clinical-grade CAR-NK cells can be manufactured on a large scale starting from multiple sources, including NK92 cell line, peripheral blood mononuclear cells (PBMCs), umbilical cord blood (UCB), and induced pluripotent stem cells (iPSCs) (60–65).

The use of NK92 cell line can be advantageous for its unlimited ability to expand *in vitro*, even after repeated freeze/thaw cycles, but disadvantageous for their lack of some relevant

NK receptors (including CD16), its potential tumorigenicity risk, and its low *in vivo* proliferation due to the irradiation needed before the infusion in the patient (66).

Differently, PBMC-derived NK cells may represent a good source for CAR-NK cell production (63, 67). Indeed, upon CAR-transduction NK cells maintain the expression of the main native activating receptors (NCRs, NKG2D, DNAM-1, CD16), can be administered without irradiation and, in a large fraction, exhibit a mature phenotype with high cytotoxicity. Moreover, each CAR-NK product obtained from a single donor can be used for the treatment of more patients in HLA-mismatched conditions. Finally, the limited lifespan of CAR-NK cells in the circulation and the reduced risk for GvHD allow repeated CAR-NK cells administrations (68).

Similarly, CAR-NK cells can also be produced from UCB NK cells, but, the limited amount of NK cells derived from a single UCB unit and the lower anti-tumor cytotoxicity of UCB-NK cells, mainly related to their less mature phenotype, represent obstacles (69, 70).

Finally, iPSCs have recently become an attractive source of CAR-NK cells for their unlimited proliferative capacity (71, 72). Indeed, CAR-engineered iPSCs can be induced to differentiate *in vitro* into hematopoietic progenitor cells and then into CAR-NK cells (72). Notably, from a limited number of iPSCs it is possible to obtain a large number of CAR-modified NK cells, even characterized by a homogeneous phenotype (73). However, even in this case, iPSCs-derived NK cells are usually expressing an immature phenotype (i.e. low KIRs/CD16 and high NKG2A expression).

In recent years, there has been a rapid increase in clinical trials using CAR-NK cells and investigating their possible application as therapeutic approach against hematological malignancies, including DLBCL (**Table 1**). Phase 1 and 2 of the pioneering clinical trial NCT03056339 enrolling 11 patients with R/R $CD19^+$ malignancies, of which 2 DLBCL patients, showed promising results (74) and indicated the feasibility of adopting CAR-NK therapy for patients with high-risk B cell lymphoma and leukemia. Indeed, no patient infused with anti-CD19 CAR-NK cells, manufactured by transducing UCB derived NK cells (64), had shown neurotoxicity events, CRS, and GvHD. Moreover, 8 out of 11 patients (73%) had a clinical response, and 7 out of 11 (63%) achieved a CR. The maximum tolerated dose was not reached even with the higher infusion of CAR-NK cells (10^7 CAR-NK cells per kilogram of body weight) and CAR-NK cells were detectable at low level for up to 1 year after infusion.

Others active clinical trials (NCT04245722, NCT04555811, NCT04887012) are registered to investigate the use of CAR-NK targeting CD19 derived from manufacturing iPSCs, however detailed results are not yet available. First evidences on the use of an anti-CD19 iPSCs-derived CAR-NK product (FT596 by Fate Therapeutics) in preclinical studies and clinical trials (NCT04245722, NCT04555811) suggest safety and well tolerability of the product (75, 76). FT596 is a CAR-NK product derived from iPSCs engineered to express a non-cleavable CD16 and IL-15 receptor fusion to promote additional functional activation (71). Its safety in advanced lymphoma treatment is under investigation both as monotherapy and as combined therapy with obinutuzumab or rituximab. A case of a heavily pre-treated DLBCL patient was enrolled in the first dose cohort of the study (lower infusion of CAR-NK cells - $30x10^7$cells) (77). A partial response has been observed upon infusion of one dose of FT596 that got better after a second infusion as proved by further decrease of tumor size and metabolism. The positive response to treatment wasn't compromised by dose related toxicities and severe adverse effects, events of any grade of CRS, immune effector cell-associated neurotoxicity syndrome (ICANS), or GvHD (77) (https://ir.fatetherapeutics.com/news-releases/news-release-details/fate-therapeutics-reports-fourth-quarter-2020-financial-results).

Only a few months ago, the first clinical trial targeting $CD19^+$ R/R B cell malignancies using CAR-NK cells obtained by engineering peripheral blood NK cells from healthy donors has been approved (NCT05020678). The purpose of this phase 1 study is to identify the optimal treatment dose with NKX019 product of Nkarta Therapeutics (https://ash.confex.com/ash/2021/webprogram/Paper146602.html). NKX019 expresses a CD19-targeted CAR, OX40 costimulatory domain, CD3ζ signaling moiety, and a membrane-bound form of IL-15 (mbIL-15) (78). Equipping CAR-NK cells with on-board cytokines, such as IL-15, lays the foundations for new therapeutic options aimed at improving clinical efficacy by enhancing both persistence and cytotoxicity against tumor cells (79).

DISCUSSION

The optimization of mAbs production and cell therapies development have shown remarkable results and changed the

TABLE 1 | Anti-CD19 CAR-NK mediated active clinical trials including DLBCL patients.

Identifier	NK cell origin	Construct	Location	First Posted	Status
NCT03056339	CB-NK cells	CAR.CD19-CD28-zeta-2A-iCasp9-IL15	USA	2017	Active, not recruiting
NCT04245722	iPSC (FT596)	CAR.19-NKG2D-2B4-CD3ζ-IL15RFhnCD16	USA	2020	Recruiting
NCT04555811	iPSC (FT596)	CAR.19-NKG2D-2B4-CD3ζ-IL15RFhnCD16	USA	2020	Recruiting
NCT04887012	iPSC (CAR-NK019)	Full construct undeclared (CAR.CD19, IL15 and modified CD16)	China	2021	Recruiting
NCT04796675	CB-NK cells	Full construct undeclared (CAR.CD19 and IL15)	China	2021	Recruiting
NCT05020678	PB-NK cells (NKX019)	CAR.CD19-OX40-CD3ζ-mIL-15	USA/Australia	2021	Recruiting

clinical history of many tumor patients, even affected by DLBCL (**Figure 1**). The identification of stably expressed tumor-associated antigens to be targeted by immunotherapies and the improvement of the CAR structure are relevant issues to be explored in the next future. In this regard, simultaneous dual antigen targeting by tandem CARs could represent a way to overcome antigen loss by tumor cells and the subsequent, antigen escape-mediated relapse. The first clinical trial (NCT03097770) designed to evaluate the effect of an autologous, bispecific anti-CD19/anti-CD20 CAR-T in R/R B-cell lymphoma has shown its safety and ability to induce a durable antitumor response, possibly due to a superior immune-synapsis stability and the mitigation of antigen-negative escape by tumor cells (80).

Contemporarily with the improvement of anti-tumor efficacy, there is an urgent need to reduce the risk of significant, potentially life-threatening consequences of CRS and ICANS, which currently affect available CAR-T therapies. In this context, it has been demonstrated that activated monocytes and macrophages are the major source of IL-1 and IL-6 production during CRS and play a key role in the amplification of the inflammatory response (81). Currently, there is an effort to elaborate strategies aimed to target pro-inflammatory cytokines and their pathways contemporarily with CAR-T infusion with a prophylactic or pre-emptive purpose (NCT04432506, NCT04359784, NCT04148430) (82).

Furthermore, the choice of the adoptive immune cells to be modified with CAR is a critical field of investigation. In this context, NK cells represent an attractive source for genetically modified cellular immunotherapies (69, 83, 84). Unlike T cells, allogeneic NK cell infusions have reduced risks for GvHD and can be used to produce "off-the-shelf" products eliminating the need for a personalized product that is necessary for T cell-based therapies. Moreover, therapeutic approaches combining cell therapies with drugs, such as immune checkpoint inhibitors or ADCC triggering immunotherapies, could be exploited in order to target multiple tumor-associated antigens (85, 86) and further improve clinical outcomes.

In conclusion, we have many tools at our disposal, and others will certainly be developed in the coming years, that we can

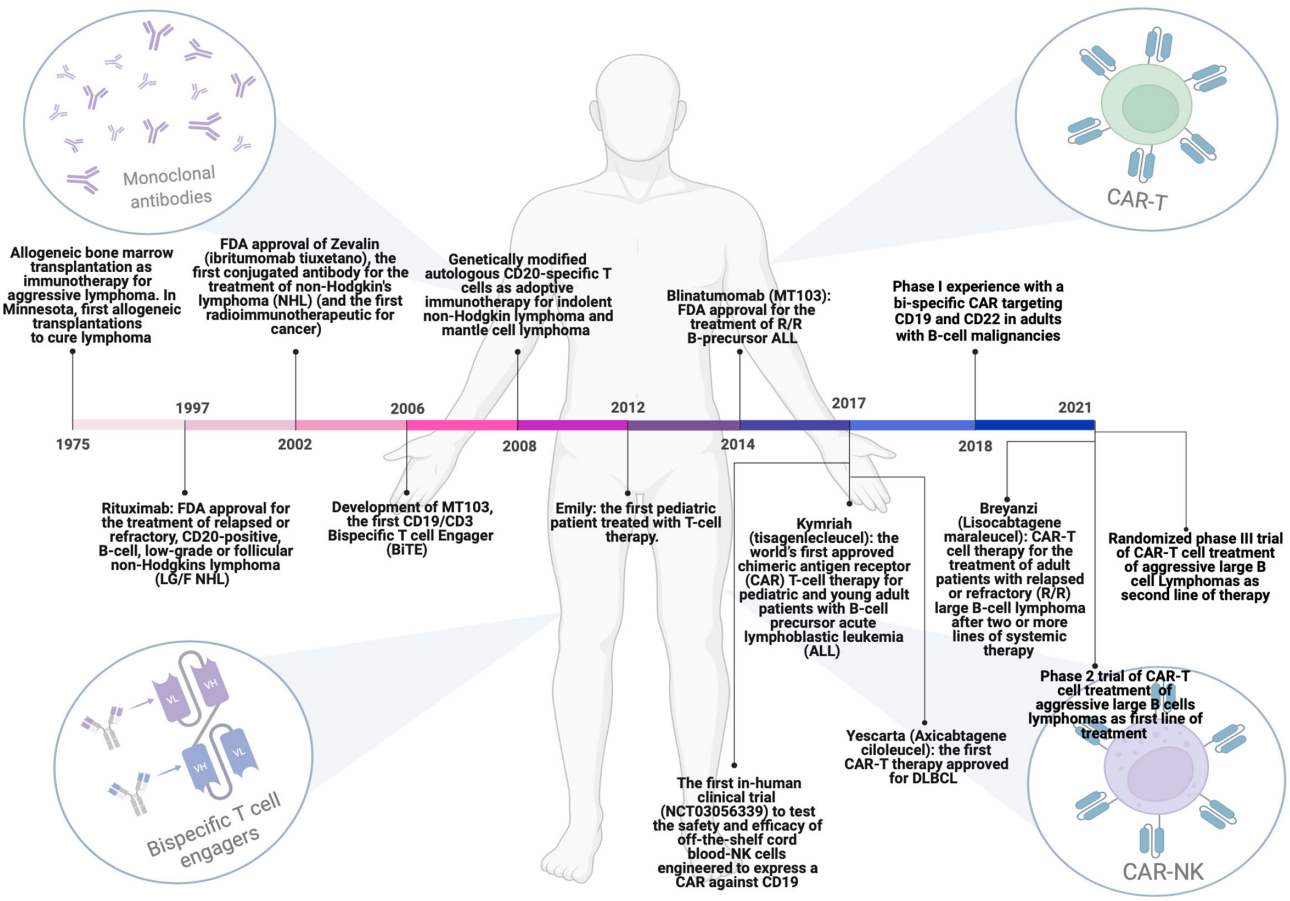

FIGURE 1 | Milestones achieved over the years regarding the evolution of immunotherapeutic strategies for the treatment of DLBCL patients. From allogenic bone marrow transplantation to the use of monoclonal antibodies, Bi-specific T-cell engagers (BiTEs) and T or NK cells engineered with chimeric antigen receptors (CARs). This figure has been created using BioRender.

combine to further improve the clinical outcomes of patients affected by aggressive and still lethal cancers.

AUTHOR CONTRIBUTIONS

MG, SC, EA and SS designed and wrote the manuscript. CS designed and prepared the figure. LG, CGh, CGi, SL, AS, AB were involved in the search of the literature. AMR, MF and MD were involved in the revision of the manuscript. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved the submitted version.

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REVIEW
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Targeting BCMA to Treat Multiple Myeloma: Updates From the 2021 ASH Annual Meeting

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With the gradual improvement of treatment regimens, the survival time of multiple myeloma (MM) patients has been significantly prolonged. Even so, MM is still a nightmare with an inferior prognosis. B-cell maturation antigen (BCMA) is highly expressed on the surface of malignant myeloma cells. For the past few years, significant progress has been made in various BCMA-targeted immunotherapies for treating patients with RRMM, including anti-BCMA mAbs, antibody-drug conjugates, bispecific T-cell engagers, and BCMA-targeted adoptive cell therapy like chimeric antigen receptor (CAR)-T cell. The 63rd annual meeting of the American Society of Hematology updated some information about the application of BCMA in MM. This review summarizes part of the related points presented at this conference.

Keywords: B-cell maturation antigen, CAR-T cell therapy, antibody-drug conjugates, bispecific T-cell engagers, immunotherapy, multiple myeloma

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1 INTRODUCTION

In recent years, the strategies for treating multiple myeloma (MM) have advanced across the board (1). In the second half of the previous century, Melphalan chemotherapy combined with steroids use such as prednisone or dexamethasone was the basic therapeutic regimen for treating MM (2). Later, with the widespread application of proteasome inhibitor (PI) and immunomodulatory drug (IMiD), the prognosis of MM patients has been dramatically improved. From the finding of targeted monoclonal antibodies (mAbs), which have a favorable curative effect in MM (3, 4), the treatment for MM has shifted to focus on multiple immunotherapies, and their most salient point was undoubtedly targeted immunotherapy. B-cell maturation antigen (BCMA/CD269), which belongs to TNF receptor superfamily member 17 (5), is highly selectively expressed on the surface of MM cells, as the ideal target of majority targeted agents studied currently for the patients with MM (6), such as anti-BCMA mAbs, antibody-drug conjugates (ADCs), bispecific T-cell engagers (BiTEs), and BCMA-targeted adoptive cell therapy like chimeric antigen receptor (CAR)-T cell (**Figure 1**). The data relating to the efficacy and safety of these targeted immunotherapy products have gotten more comprehensive based on a great number of preclinical and clinical trials. The 63rd annual conference of the American Society of Hematology (ASH) showed us the latest progress of multiple anti-BCMA immunotherapies. This review aims to summarize some of the main points in this meeting about the application of BCMA in MM, with a special focus on clinical achievements.

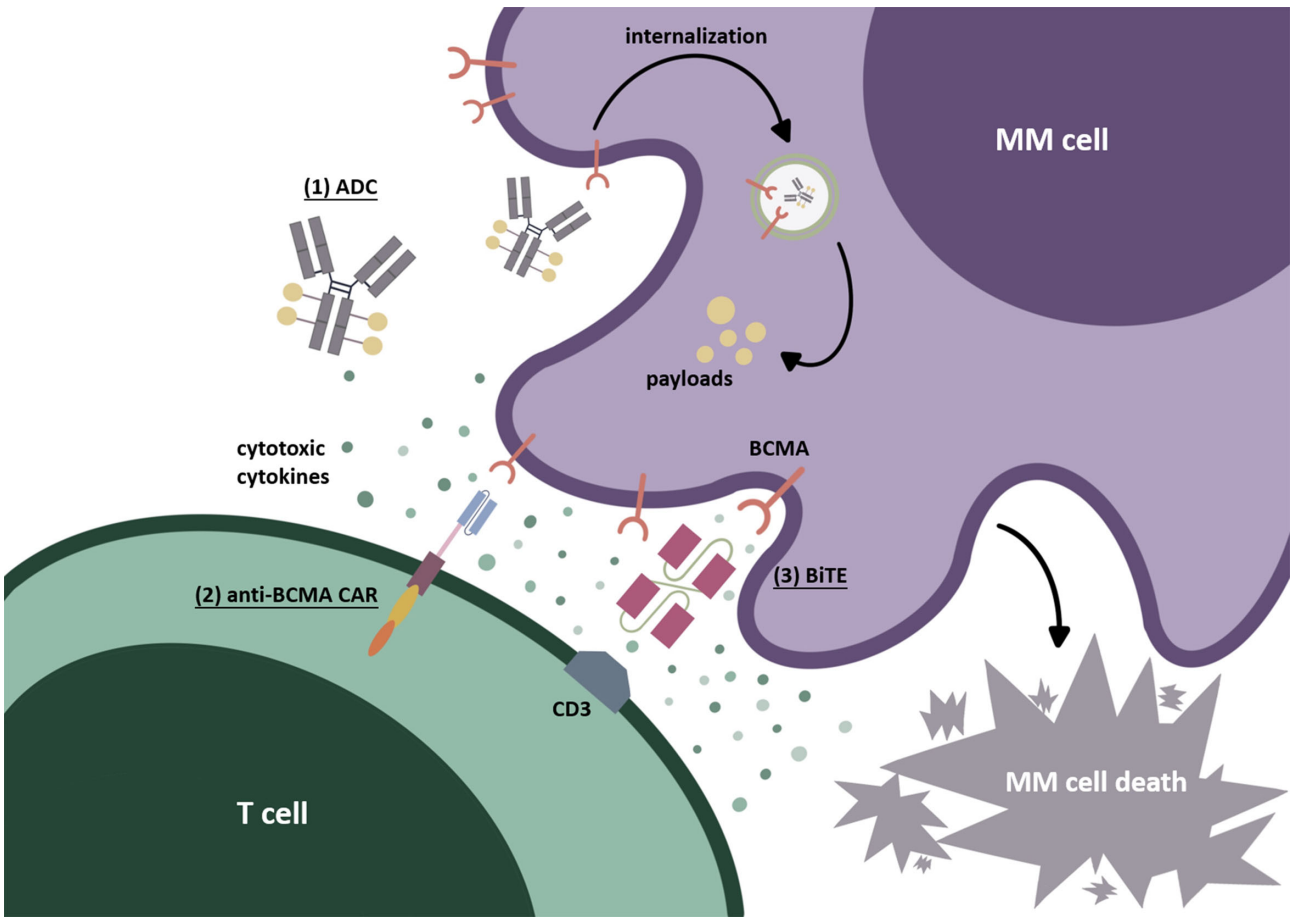

FIGURE 1 | BCMA-targeted immunotherapies. (1) Antibody-drug conjugate (ADC). After identifying BCMA on the cell surface, ADC internalizes into myeloma cells. Through the degradation by lysosomes or endosomes, the payloads are released, resulting in cytotoxicity. (2) Chimeric antigen receptor (CAR) T cell. The second-generation CAR commonly used today is mainly composed of an extracellular recognition domain (the most commonly used is scFv), a spacer, a transmembrane part, and intracellular structures (costimulatory domain such as CD28 or 4-1BB and an activating domain CD3-zeta). The recognition domain binds to BCMA on the myeloma cell surface as signal 1. The costimulatory domain (CD28 or 4-1BB) is then "aroused" to send signal 2, which is beneficial to CAR-T-cell activation and to prevent their disability. Finally, signals 1 and 2 are transmitted to the CD3-zeta domain to induce CAR-T-cells' final activation. (3) Bispecific T-cell engager (BiTE). BiTEs can target BCMA on MM tumor cells and CD3ε domain of TCR on T cells simultaneously. After causing the binding of T cells to myeloma cells, the cytotoxic T cells can be activated and secrete cytotoxic factors, thus producing the cytolethal effect.

2 PROGRESS OF THE MECHANISM RELATED TO BCMA

Under physiological conditions, BCMA is mainly expressed on plasmablasts (7) and terminally differentiated plasma cells (PCs) (8). In the pathological case, BCMA is expressed nearly on all MM tumor cell lines (80%–100%) (9), and the quantity of BCMA on the surface of malignant PCs is much higher than regular PCs (10). The ligands of BCMA include BAFF and a proliferation-inducing ligand (APRIL), which is a homolog of BAFF (11). APRIL has a higher affinity for BCMA than BAFF (12), and both of them can activate the downstream signals of BCMA like nuclear factor kappa-B (NF-κB) (13), rat sarcoma/mitogen-activated protein kinase (RAS/MAPK), and phosphoinositide-3-kinase–protein kinase B/Akt (PI3K-PKB/Akt) (14), thus promoting the expression of antiapoptotic proteins (e.g., Mcl-1, BCL-2, BCL-XL) and the activation of specific signaling pathways or factors (e.g., cell adhesion molecules, angiogenesis factors, immunosuppressive molecules) about cells' proliferation (14). One of these factors is c-Jun N-terminal kinase (JNK) (7), which can work together with NF-κB, JAK/STAT, and other related signaling molecules to synergistically promote tumor cell survival in the tumor microenvironment (TEM) (15). A study reported in ASH2021 (16) found that the expression of SETD2 can activate the BCMA-JNK pathway, thus facilitating the proliferation and maintenance of myeloma cells. Bridging this gap is the regulation of H3K36 trimethylation (H3K36me3) by SETD2, which provides us with a new perspective to explain the upstream activation of BCMA and the stimulation of its downstream signal pathways through epigenetic mechanisms.

It is worth mentioning that an increasing number of studies have confirmed the critical role of epigenetics in MM. For instance, the overexpression of histone methyltransferase MMSET can stimulate H3K36me2, which has been identified as one of the pathogenic mechanisms of t(4;14)$^{+}$ MM (17). The membrane-bound BCMA can break off from the cell membrane by the shear function of γ-secretase and turn into a soluble BCMA (sBCMA) (18), which is closely related to the development of MM and the prognosis of patients (19, 20). The formation of sBCMA reduces the distribution of BCMA on tumor cells' surface, thus relieving the effect conducted by BCMA activation. However, this mechanism may lower the efficacy of BCMA-targeted immunotherapies as well, resulting in MM cells' immune escape. With the gradual deepening of our awareness about the underlying mechanism related to BCMA, the modification to multiple existing anti-BCMA immunotherapies is also accelerating its pace.

3 PROGRESS OF BCMA-TARGETED IMMUNOTHERAPIES

3.1 BCMA-Targeted mAbs

The finding of BCMA-targeted mAbs can be regarded as an essential milestone in the field of targeted immunotherapy for MM. The first two approved mAbs agents target CD38 antigen (daratumumab) (3) and signaling lymphocytic activation molecule family member 7 (SLAMF7) (elotuzumab) (4), respectively. Although their effectiveness has been proved, there are still a large number of patients who relapse after receiving more than 3 prior lines of therapy (LOT), the mAbs, and further progress to relapsed or refractory multiple myeloma (RRMM) (21). At present, along with myriad novel immunotherapy agents being developed, researchers are also looking for more mAbs that can work better. SEA-BCMA, a novel humanized nonfucosylated IgG1 mAb, targets BCMA, which is expressed on the malignant PCs. The working mechanisms of SEA-BCMA may include blocking of BCMA activation with its downstream proliferative signaling pathways, regulating antibody-dependent cellular phagocytosis effect, and reinforcing the antibody-dependent cellular cytotoxicity. ASH2021 updated some findings regarding this agent (22, 23).

The preliminary results of its phase I clinical trial (SGNBCMA-001; NCT03582033) (23) are reported in this meeting. Part A of SGNBCMA-001 conducted a dose-escalation trial (from 100 to 1,600 mg, Q2W) of SEA-BCMA monotherapy for RRMM patients without any prior treatments. At the 800-mg Q2W regimen, 1 of 7 patients reported a grade 3 infusion-related reaction (IRR), which was the single dose-limiting toxicity (DLT) observed during dose escalation. At the maximum dose (1,600 mg Q2W, n = 22), the objective response rate (ORR) was 14% (n = 3). One patient got very good partial responses (VGPR), and two got partial responses (PR). The adverse events (AEs) were fatigue (32%), pyrexia (23%), IRR (23%), hypertension (23%) unrelated to hematological incidents, and anemia (14%) related to hematologic incidents from high to low. The other parts of this trial designed to verify whether using SEA-BCMA in higher doses (part B, Q1W induction dosing of SEA-BCMA for 8 weeks is followed by Q2W maintenance dosing) or combining it with dexamethasone (DEX) (part C) can produce better therapeutic results for the patients who have received ≥3 prior LOT for MM and were triple-class refractory. Surprisingly, DLTs did not occur in these two parts. Two of eight (2 PR) and two of twelve (1 VGPR, 1 PR) patients reported a certain OR in parts B and C, respectively. The pharmacokinetics (PK) analysis showed that the half-life of SEA-BCMA was approximately 10 days, and either ascending dose (from Q2W to Q1W) or combining DEX had no significant effect on its metabolism.

Moreover, Taft et al. reported the binding and saturation pharmacodynamics (PD) of SEA-BCMA in patients enrolled in part A of SGNBCMA-001. They suggested that the sBCMA in plasma may affect tumor cell clearance because of the formation of sBCMA : SEA-BCMA complex. Interestingly, an amplification dose of 1,600 mg seems to overcome this negative effect and support malignant plasma cell drug exposure. Nevertheless, the dose dependence of SEA-BCMA needs a more comprehensive evaluation. These latest results proved the safety of SEA-BCMA and the possibility to combine it with other medicines for patients with MM. Further studies will carry out in the subsequent part D, and it is expected to be used into clinical application as a promising anti-BCMA agent.

3.2 Bispecific Antibodies

Bispecific antibodies (BsAbs), which have affinities for two different epitopes on tumor cells and specific immune cells like T cells, as a bridge, induce the formation of immunological synapses between T cells and tumor cells, which can make granular enzymes and perforin released by T cells produce lethal effect to the targeted tumor cells (24). Up to now, BCMA, CD38, and SLAMF7 have been selected as the targets to prepare BsAbs for the treatment of MM (25). BiTE, a special BsAb, can physically bind BCMA and CD3ε on T-cell receptors (TCR) for redirecting T cells to myeloma cells to exert its cytotoxicity (25). Also, many new targets have been identified, like G-protein coupled receptor C family 5D (GPRC5D) (26), which are also expressed highly on the surface of PCs (27). ASH2021 provided us with the latest data from the early-stage clinical trials of multiple novel BsAbs for treating MM, which could certify the efficacy and safety of these new agents (**Table 1**).

Some information about **Table 1** should be added: firstly, the target CD16a of RO7297089 is expressed on the innate immune cells such as monocyte subsets, macrophages, and natural killer (NK) cells. Among the five dose cohorts in this study, ten patients had stable disease as their best response at dose levels of 60 mg (1/3 patients), 180 mg (2/5 patients), 360 mg (3/4 patients), and 1,080 mg (4/6 patients). Its PK parameter was nonlinear (a more than dose proportional increase) as the doses of RO7297089 increased from 60 to 1,080 mg, and then approached linear at doses higher than 1,080 mg. The disposition of this agent was mediated by its target. Secondly, the phase I study of teclistamab has obtained its recommended phase II dose (RP2D), which was applied in the phase II study. The data on the effectiveness of this drug in **Table 1** showed how

TABLE 1 | Updated clinical data for BsAbs.

	RO7297089-GO4158 (NCT04434469)	Teclistamab (JNJ-64007957)-MajesTEC-1 (NCT04557098)	REGN5458-(NCT03761108)	Tnb-383B-(NCT03933735)	Elranatamab (PF-06863135)–MagnetisMM-1 (NCT03269136)
Phase	1	1/2	1/2	1	1
Structure	BCMA×CD16a (BsAbs)	BCMA×CD3 (BsAbs)	BCMA×CD3 (BsAbs)	BCMA×CD3 (BsAbs)	BCMA×CD3 (BiTEs molecule)
Schedule	Dose escalation: 60, 180, 360, 1,080, 1,850 mg	1,500 μg/kg/w followed by step-up doses of 60 and 300 μg/kg	Dose escalation: full doses ranging from 3 to 400 mg	Dose escalation/ expansion: 0.025–120 mg	Part 1: 80, 130, 215, 360, 600, and 1,000 μg/kg/w (SC) Part 1.1/2A (RP2D): single priming dose (600 μg/kg or equivalent fixed dose of 44 mg), then the full dose (1,000 μg/kg or equivalent fixed dose of 76 mg) Q1W or Q2W followed (SC) Part 1C/1D: single priming dose (32 mg), then the full dose (44 mg) Q1W followed one week later in combination with either LEN (25 mg) or POM (4 mg) on days 1 to 21 of a 28-day cycle (SC)
Patients (*n*)	21	159 (phase 1: *n* = 40; phase 2: *n* = 119)	68	103 (dose escalation: *n* = 73; dose expansion: *n* = 30)	58 (part 1.1: *n* = 50; part 1C: *n* = 4; part 1D: *n* = 4)
Efficacy					
ORR (%)	NA	65 (phase 1 pts)	73.3 (96 and 200 mg dose levels)	79 (19/24) (≥40 mg dose-escalation cohort); 64 (28/44) (≥40 mg dose-escalation and dose-expansion cohorts)	70 (14/20) (part 1, at the efficacious dose range 215–1,000 μg/kg)
≥CR rate (%)	NA	40 (phase 1 pts)	19.1 (13/68) (across all dose levels)	29 (7/24) (≥40 mg dose-escalation cohort); 16 (7/44) (≥40 mg dose-escalation and dose-expansion cohorts)	30 (6/20) (part 1, at the efficacious dose range 215–1,000 μg/kg)
≥VGPR rate (%)	NA	60 (phase 1 pts)	36.8 (25/68) (across all dose levels)	63 (15/24) (≥40 mg dose-escalation cohort); 43 (19/44) (≥40 mg dose-escalation and dose-expansion cohorts)	35% (7/20) (part 1, at the efficacious dose range 215–1,000 μg/kg)
Safety					
Nonhematologic TRAEs	IRR (48%); back pain (24%); ALT rise (19%)	CRS (67%); injection site erythema (23%); fatigue (22%); ICNS (4 pts)	CRS (38.2%); fatigue (20.6%)	77% (Gr ≥3:32%, serious AEs:22%): CRS (52%); neutropenia (17%); fatigue (14%)	
Hematologic TRAEs	Anemia (52%); thrombocytopenia (19%)	Neutropenia (53%); anemia (41%); thrombocytopenia (33%)	Neutropenia (16.2%)		
TEAEs			97.1% (≥Gr3: 76.5%): fatigue (42.6%); CRS (38.2%); nausea (32.4%)	8%: infections (28%); pneumonia (5%)	CRS (83%); lymphopenia (64%); neutropenia (64%); anemia (55%); injection site reaction (53%); thrombocytopenia (52%)
Reference	(28)	(29)	(30)	(31)	(32)

BCMA, B-cell maturation antigen; BsAbs, bispecific antibodies; BiTEs, bispecific T-cell engagers; w, week; LEN, lenalidomide; POM, pomalidomide; ORR, overall response rate; NA, not applicable; CR, complete response; VGPR, very good partial response; DOR, duration of response; TRAEs, treatment-related AEs; IRR, infusion-related reaction; CRS, cytokine release syndrome; pts, patients; Gr, grade; AEs, adverse events; ICANS, immune effector cell-associated neurotoxicity syndrome; TEAEs, treatment-emergent AEs; DLT, dose-limiting toxicity; SC, subcutaneous.

the 40 patients who participated in phase I (median follow-up: 6.1 months) performed in phase II (median follow-up: 8.2 months), which was consistent with previously presented data (65% ORR and 58% VGPR rate) in phase I study. Thirdly, the median follow-up duration of the patients enrolled in REGN5458 clinical trial was 2.4 months. Although median DOR was not reached in this trial, the probability of DOR ≥8 months was 92.1%. Fourthly, the RP2D of Tnb-383B was 60 mg Q3W. The median follow-up time of the ≥40mg dose-escalation cohorts and the ≥40mg combined dose-escalation and dose-expansion cohorts were 6.1 and 3.1 months, respectively. Fifthly, elranatamab is a humanized bispecific molecule. Its subcutaneous (SC) cohorts from MagnetisMM-1 contained five parts: dose escalation (part 1), monotherapy with priming (part 1.1), lenalidomide (LEN) combination (part 1C), pomalidomide (POM) combination (part 1D), and monotherapy expansion with priming (part 2A). In part 1, the efficacious dose range was 215–1,000 μg/kg. ASH2021 updated the ORR and sCR/CR

rate under these doses, and the confirmed ORR at the RP2D was 83% (5/6) in this part. One last thing worth mentioning is that, although the patients enrolled in the REGN5458 clinical trial were penta-refractory after 5 or so prior LOT, the rest of the patients enrolled in the other trials relapsed after ≥3 prior LOT including a proteasome inhibitor, an immunomodulatory drug, and a CD38-targeted therapy.

3.3 Novel BCMA-Targeted Tri-Specific Agents

Frankly speaking, today's researchers are no longer satisfied with the dual-target immunotherapies for treating the patients with RRMM. The current studies have reached a level of developing triple or multiple specificity agents, which may have better efficacy. ASH2021 reported that HPN217, a half-life extended (median serum half-life: 74 h) (33) tri-specific T-cell activation construct (TriTAC) synchronously targeting BCMA, serum albumin to prolong the half-life period, and CD3ε to active and redirect T cells, could exert their cytotoxic effect to myeloma cells (34). The preclinical translational studies showed that HPN217 could eliminate 71% of tumor cells at a 0.45-T cell/MM cell ratio. The density of BCMA and the sBCMA in circulation affected the tumor killing effect of HPN217. Consistent with this result, GSI (e.g., LY-3039478), which increased the expression of BCMA on the surface of myeloma cells, could enhance the efficacy of this agent. Moreover, the negative effect of DEX on the HPN217-redirected T cells may be restricted (34). The phase I clinical trial is ongoing, whose preliminary results showed us that the maximum safe dose of HPN217 was 2,150 μg/week and its treatment-emergent AEs were transient and controllable (33). Another BCMA-targeted tri-specific agent who was undergoing preclinical evaluation has been reported in this meeting as well (35). CDR101, targeting CD3, BCMA, and PD-L1, could guide T cells to BCMA-expressed tumor cells and play a role in combating immunosuppression caused by the interaction of PD-L1 and PD-1 at the immune synapse site, which may reduce the possibility of "on-target off-tumor" effects. Compared with BCMA × CD3 bispecifics, CDR101 resulted in at least 10-fold increased T-cell-mediated tumor cells lysis and it performed better than the combination of the PD-L1 inhibitors and BCMA × CD3 bispecifics. Based on these findings, it is suggested that novel tri-specific immunotherapy agents argue for a high clinical potential and promising translation into the clinic.

3.4 Antibody-Drug Conjugates

Antibody-drug conjugates (ADCs), which connected mAbs with bioactive drugs through chemical linkers (36), can accurately identify tumor cells and exert high-efficiency cytotoxic effects on malignant cells without damaging healthy tissues (37). Belantamab mafodotin (GSK2857916), which is a microtubule-disrupt agent (38), consists of humanized BCMA-targeted IgG1 and monomethyl auristatin-F (MMAF). Blenrep was approved by the U.S. Food and Drug Administration (FDA) in 2020 for treating patients with RRMM. As the first licensed BCMA-targeted immunotherapy for marketing (39), belantamab has been tested in multiple clinical trials (38–40), which could confirm its safety and efficacy. The first-in-human DREAMM-1 study showed that the belantamab monotherapy (3.4 mg/kg, Q3W) induced deep (overall response: 60%, 21/35) and durable (median DOR: 14.3 months) responses (38). The results of DREAMM-2 (NCT03525678), a multicentric phase II clinical study of this ADC, have confirmed that the recommended regimen for its future studies was 2.5 mg/kg, Q3W instead of 3.4 mg/kg, Q3W, which was the RP2D after the phase I trial. Under this dose, the ORR was 31% (30/97) with manageable safety profile (41). Even so, belantamab also has a certain extent of boundedness. DREAMM-2 demonstrated that the toxicity of this agent was mainly reflected in thrombocytopenia and lesions about the cornea, which presented as microcyst-like epithelial changes or superficial punctate keratopathy (41). Moreover, adverse ocular signs like dry eye and diminution of best-corrected visual acuity (BCVA) have occurred during the administration of belantamab as well (41). Given that changing its administration regimens may reduce the incidence of corneal events without compromising the therapeutic effect, a new phase II, 5-arm, open-label and multicentric clinical trial DREAMM-14 is preparing to determine if there are better dosage choices than 2.5 mg/kg Q3W. This study will initiate in the springtime of 2022 (42). To relieve stress in the real world, a study in ASH2021 analyzed if those relatively simple clinical indicators or convenient judgment methods such as questionnaires, could replace the professional eye examinations for determining whether to change the in-use medication regiments (43). The conclusion of this study was unequivocally positive, and once these strategies are applied in clinical practice, the burden of either patients or physicians will greatly reduce. DREAMM-1 and DREAMM-2 have studied the efficacy of belantamab monotherapy. ASH2021 updated the results of belantamab/DEX and belantamab/DEX + POM for the patients with triple-class refractory disease. After the combination of belantamab (2.5 mg/kg Q3W) with DEX (20–40 mg Q1W, median 3 cycles), the ORR was 46%, the CR rate was 14%, and 18% of all patients achieved ≥VGPR with 7.4 months median follow-up duration. Median progression-free survival (PFS) was 4.9 months, with 7.4 months' median overall survival (OS). The incidence of AEs were anemia (83%), keratopathy (82%; Gr3/4: 56%), thrombocytopenia (70%), neutropenia (30%), and elevated liver function tests (53%) from high to low (44). On the other hand, after the combined application of belantamab (1.92, 2.5, or 3.4 mg/kg, Q4W, designed by 3 + 3 dose escalation strategy), POM (an IMiD) (4 mg day 21/28 days), and DEX (40/20 mg weekly), the ORR was 88.9% (48/54) and the sCR, ≥VGPR, and PR rates were 24.1% (13/54), 68.5% (37/54), and 20.4% (11/54), respectively. The median PFS was 24.2 months based on a median of 8.6 months follow-up. Keratopathy (96.9%) also was the most common AEs, and 56.7% of such patients have reached Gr 3/4 (45). These two studies demonstrated that POM and DEX may have positive impacts on the efficacy of belantamab. However, keratopathy remains a challenge in the treatment process. In addition to belantamab mafodotin, several other BCMA-targeted ADCs, such as AMG 224, MEDI2228, and HDP-101, are also undergoing multiple preclinical or clinical studies in different phases.

3.5 BCMA-Targeted CAR-T-Cell Therapy

Compared with the mAbs, BsAbs, and ADCs mentioned above, the therapeutic effect for BCMA-targeted CAR-T-cell therapy presented in the 63rd ASH seems to be more optimistic. Moreover, the preliminary results of many other related studies, such as the engineering improvement strategies to existing CAR-T-cell products or the effects induced by multiple factors inside and outside the body, were reported in this meeting. The relevant data about the safety and efficacy of those products, including ciltacabtagene autoleucel (cilta-cel) (46–48), CT053 (49, 50), CT103A (51), C-CAR088 (52), PHE885 (53), CART-ddBCMA (54), and bb21217 (55), are presented in **Table 2**, and the relevant supplementary explanations will be carried out later in this paper.

3.5.1 Ciltacabtagene Autoleucel

Cilta-cel, one of the BCMA-targeted CAR-T-cell products with two anti-BCMA single-domain antibodies to present avidity, a CD3-ζ signaling domain, and a 4-1BB costimulatory domain (56), has gotten favorable responses in its phase Ib/II open-label study CARTITUDE-1 from 97 patients with MM who had relapsed after more than three prior LOTs, such as PI, IMiD, or MoABs (56). According to the past report, after 5–7 days of single cilta-cel infusion (0.75×10^6 cells/kg) and median 12.4 months of follow-up, the ORR was 97%, with 67% of patients achieving sCR. Twelve months PFS rate and OS rate were 77% and 89%, respectively. There were two reports (46, 57) in ASH2021 presented the subsequent results of these patients and the performance of the subgroups in CARTITUDE-1. After 18 months median follow-up, the resulting ORR was 97.9%, which was well-matched to all the subgroups. 80.4% of all subjects got sCR, and 94.8% achieved VGPR or better. The median DOR was 21.8 months. The rate of 18 months' PFS and OS was 66.0% and 80.9%, respectively. These data also were consistent with most of the subcohorts. As for minimal residual disease (MRD), 91.8% of those who had been tested ($n = 61$) reported MRD negative at the 10^{-5} threshold. Across all the subgroups, the data were 80% to 100%. In terms of the safety, the mainly hematologic AEs graded 3 or 4 were neutropenia (94.8%), anemia (68.0%), leukopenia (60.8%), thrombocytopenia (59.8%), and lymphopenia (49.5%), without cytopenia-related fatalities. 94.8% of all the patients occurred CRS, and 98.9% of them obtained remission within 14 days. No neurotoxicity case related to CAR-T cells happened since the last report. The efficacy and safety of cilta-cel can be proved by the results from this phase Ib/II study, and the comparison between cilta-cel and other kinds of therapeutic methods for MM can extend its advantages to real-world clinical practice (RWCP). LocoMMotion (58), which can be seen as an external control cohort of CARTITUDE-1, is the first prospective study for cilta-cel's applicability in the real world (59). In LocoMMotion, 246 patients with RRMM who relapsed after more than triple class exposure to IMiDs, PIs, and MoABs were enrolled, they then received more than ninety other treatment regimens besides CAR-T-cell therapy. Based on the comparative analysis of many aspects between LocoMMotion and CARTITUDE-1, the prognosis of the patients treated with other therapeutic strategies was worse. Cilta-cel had a better outcome reflected by many indicators including ORR, CR, PFS, and OS.

CARTITUDE-2, a phase II multicohort clinical trial for cilta-cel, is currently ongoing. Two reports in ASH2021 provided us with the updates of cohort A (47) and cohort B (48) in CARTITUDE-2, respectively. In cohort A, 20 patients who were refractory after more than three prior LOTs especially lenalidomide were treated with cilta-cel (0.75×10^6 cells/kg, 5–7 days). The ORR was 95%; 85% of patients performed better than complete response (CR), and 95% of them were superior to VGPR. Median DOR has not been reached, but the 6-month PFS rate was 90%. In total, 13 patients were evaluated for MDR, and 92.3% of them got MRD negative based on the 10^{-5} criterion. The common hematologic AEs were neutropenia (95%), thrombocytopenia (80%), anemia (75%), lymphopenia (65%), and leukopenia (55%). Although the incidence rate of CRS was 95%, 90% of these cases were cured within 7 days. This, together with the neurotoxicity that happened in only 20% of all patients, demonstrated the manageable safety profile of cilta-cel. In cohort B, the cilta-cel infusion (0.75×10^6 cells/kg, 5–7 days) performed in 18 patients who relapsed within 12 months after receiving autologous stem cell transplantation or other anti-MM therapies. After an average of 4.7 months' follow-up, the ORR reached 100%. In total, 31.2% of them achieved better than CR, and 75% were superior to VGPR. All the evaluated patients ($n = 9$) performed MRD negative. With 4 days median time of duration (ranged 1–7), CRS (grades 1–4) occurred in 83.3% of patients, and ICANS (grade 1) occurred in only one patient. For cilta-cel, the latest results of CARTITUDE-1 and CARTITUDE-2 jointly highlight its potential as a promising method for heavily pretreated patients with RRMM. Further studies including CARTITUDE-4 (NCT04181827) have been carried out. However, this agent has not been approved yet for marketing.

3.5.2 Idecabtagene Vicleucel (ide-cel, bb2121)

Based on the positive results from the pivotal single-arm, open-label phase II clinical trial called KarMMa (60), Abecma (ide-cel), one of the BCMA-targeted CAR-T-cell products, which is used to treat the patients with RRMM after four or more prior LOTs including IMiD, PI, and MoABs (61), has been approved for listing by FDA as the first one around the world. ASH2021 updated the study results of health-related quality of life (HRQoL) in KarMMa (62). The results which have been reported previously have shown the significant clinical benefits of ide-cel on HRQoL during a 9-month follow-up (63), and the updated performance of the patients enrolled in this trial also proved that after 24 months follow-up, notable HRQoL improvements in multiple predefined domains were achieved. In those predefined prime HRQoL domains, 40%–70% of all 128 patients had clinically meaningful advances reflected by many indicators, such as QLQ-C30 fatigue, pain, physical functioning, and global health status/QoL scores at the later time points. In addition, 30%–40% of these patients got improvements in cognitive function, disease symptoms, and side effects, with 40%–60% of them remaining stable in these domains. Among those predefined secondary HRQoL domains, the improvement in role functioning, emotional functioning, social functioning,

TABLE 2 | BCMA-targeted CAR-T cells in clinical trials.

Name (manufacturer)	Clinical trial information	Inclusion/exclusion criteria	Pt characteristics	Dosage	Major response	Most common AE
Ciltacabtagene autoleucel (Janssen, Xi'an, China)	Phase 1b/2 (NCT03548207) (46)	RRMM who received or were refractory to ≥3 prior lines, including PI, IMiD, CD38 mAb	97 pts; median age 61; median prior lines 6	Single cilta-cel infusion (target dose 0.75 × 10^6 CAR^+ viable T cells/kg; range 0.5–1.0 × 10^6) 5–7 days after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 97.9%; sCR 80.4%; VGPR 14.4%; PR 3.1%; NR 2.1%	G3-4 neutropenia (94.8%), anemia (68.0%), leukopenia (60.8%), thrombocytopenia (59.8%), lymphopenia (49.5%); CRS (94.8%); neurotoxicity (0%)
	Phase 2 (NCT04133636) (47, 48)	Cohort A (47) RRMM who received or were refractory to ≥3 prior lines, including PI, IMiD, CD38 mAb, lenalidomide relapse; hx of BCMA-directed therapy were excluded	20 pts; median age 60; median prior lines 2	Single cilta-cel infusion (target dose 0.75 × 10^6 CAR^+ viable T cells/kg) 5–7 days after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 95%; CR 85%; VGPR 10%	G3-4 neutropenia (95%), thrombocytopenia (35%), anemia (45%), lymphopenia (60%), leukopenia (55%); CRS (95%), G3-4 CRS (10%); G1-2 neurotoxicity (20%)
		Cohort B (48) RRMM who received or were refractory to 1 prior line, including PI, IMiD, had disease progression either ≤12 months after ASCT or ≤12 months after start of antimyeloma therapy except ASCT, were tx-naïve to CAR-T or anti-BCMA therapies	18 pts; median age 57	Single cilta-cel infusion (target dose 0.75 × 10^6 CAR^+ viable T cells/kg) 5–7 days after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 100%; CR 31.2%; VGPR 43.8%; PR 25%	Neutropenia (88.9%), thrombocytopenia (61.1%), anemia (50.0%), leukopenia (27.8%), and lymphopenia (22.2%); G1-4 CRS (83.3%); G1 neurotoxicity (5.6%)
CT053 (CARsgen, Shanghai, China)	Phase 1 (NCT03975907) (NCT03380039, NCT03716856, NCT03302403) (49, 50)	RRMM who received or were refractory to ≥2 prior lines, including PI, IMiD, CD38 mAb	38 pts	0.5 (n = 1), 1.0 (n = 4), 1.5 (n = 32), 1.8 (n = 1) × 10^8 CAR^+ viable T-cell infusion after lymphodepletion	ORR 92.1%; CR 78.9%; VGPR 7.9%; PR 5.3%; NR 7.9%	G1-2 CRS (73.7%); G3 neurotoxicity (0%); DLT (0%)
CT103A (Sana, Seattle, USA) (IASO, Nanjing, China)	Phase 1/2 (NCT05066646) (51)	RRMM who received or were refractory to ≥3 prior lines, including PI, IMiD, CD38 mAb	71 pts; median age 58; median prior lines 4	1.0 × 10^6 CAR^+ viable T cells/kg single infusion 1 d after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 94.4%; CR 50.7%; VGPR 26.8%; PR 16.9%	CRS (93%), G3 CRS (2.8%); G2 neurotoxicity (1.4%)
C-CAR088 (CBMG, Delaware, USA)	Phase 1 (NCT04295018, NCT04322292, NCT03815383, NCT03751293) (52)	RRMM who received or were refractory to ≥2 prior lines, including PI, IMiD, CD38 mAb	31 pts; median age 61; median prior lines 4	1.0, 3.0, 4.5~6.0 × 10^6 CAR^+ viable T cells/kg infusion after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 96.4%; CR 57.2%; VGPR 32.1%; PR 7.1%	CRS (93.5%), G1 CRS (58.1%), G2 CRS (25.8%), G3 CRS (9.7%); neurotoxicity (3.2%)
PHE885 (Novartis, Basel, Switzerland)	Phase 1 (NCT04318327) (53)	RRMM who received or were refractory to ≥2 prior lines, including PI, IMiD, CD38 mAb	6 pts; median prior lines 5	5.0, 14.3 × 10^6 CAR^+ viable T cells/kg infusion after lymphodepletion	ORR 100%; CR 17%; VGPR 33%; PR 50%	≥G3 anemia (100%), neutropenia (100%), thrombocytopenia (67%), leukopenia (33%), ALT and AST increase (33%), decreased blood fibrinogen (33%); CRS (33%); G3 CRS (100%); G2 neurotoxicity (33.3%)
CART-ddBCMA (Arcellx, Maryland, USA)	Phase 1 (NCT04155749) (54)	RRMM who received or were refractory to ≥3 prior lines, including PI, IMiD, CD38 mAb	16 pts; median age 66; median prior lines 5	100, 300 × 106 (± 20%) CAR^+ viable T cells/kg infusion after lymphodepletion (300 mg/m^2 cyclophosphamide, 30	ORR 100%; sCR 43.8%; CR	CRS (100%); ≥G3 CRS (6%); G3 neurotoxicity (13%)

(Continued)

TABLE 2 | Continued

Name (manufacturer)	Clinical trial information	Inclusion/exclusion criteria	Pt characteristics	Dosage	Major response	Most common AE
				mg/m^2 fludarabine daily for 3 days)	12.5%; VGPR 18.7%; PR 25%	
bb21217 (bluebird bio, Massachusetts, USA)	Phase 1 (NCT03274219) (55)	RRMM who received or were refractory to ≥3 prior lines, including PI, IMiD, CD38 mAb	72 pts	150, 300, 450 × 10^6 CAR$^+$ viable T cells/kg infusion after lymphodepletion (300 mg/m^2 cyclophosphamide, 30 mg/m^2 fludarabine daily for 3 days)	ORR 69%; CR 28%; VGPR 30%; PR 11%	CRS (75%); G1-2 CRS (70.8%) G3 CRS (1.4%); neurotoxicity (15%)

Pt, patient; AE, adverse event; ORR, overall response rate; sCR, strict complete response; VGPR, very good partial response; PR, partial response; NR, no response; G, grade; CRS, cytokine release syndrome; hx, history; CR, complete response; mo, month; ASCT, autologous stem cell transplantation; tx, treatment; DLT, dose-limiting toxicity.

dyspnea, insomnia, constipation, diarrhea (QLQ-C30), future perspectives (QLQ-MY20), health utility index scores (EQ-5D-5L), and VAS scores (EQ-5D) had clinical significance. It is worth mentioning that there was also a study in ASH2021 (64), which was a qualitative analysis of the interviews with patients in KarMMa after 6–24 months ide-cel treatment, provided us a novel insight to evaluate the posttreatment life quality by analyzing the attitude of patients. Undoubtedly, 73% of all interviewed subjects (n = 33) had positive attitude towards ide-cel infusion.

Moreover, ASH2021 also touched on some of other studies derived from KarMMa. Because of the difference in overall OS and median PFS (34.2, 24.8 months and 8.8, 8.6 months, respectively) between the results of KarMMa and an earlier phase I study of ide-cel named CRB-401 (65), further research was conducted on the patients enrolled in KarMMa who relapsed after ide-cel treatment. A report (66) showed us the difference between those who received subsequent antimyeloma therapy (sAMT) (n = 68) and the anti-BCMA therapy (n = 11) after ide-cel infusion: the median PFS and OS of the patients with sAMT were 6.1 and 24.8 months, respectively. The duration of overall sAMT was 215 days, and the second disease progression (PFS2) was 13.6 months (inclusive of time on ide-cel therapy). The median PFS and OS of the patients who were applied anti-BCMA therapy was 12.1 and 31.0 months, and the median duration of the first sAMT was 48 days with 15.5 months' PFS2, which was more favorable. Therefore, patients who relapsed after the first ide-cel infusion may benefit from the follow-up anti-BCMA therapy, while the emergence of this phenomenon requires conditions referred to a past study (67). There was also a report (68) about the infectious complications after ide-cel treatment in patients with RRMM from CRB-401 and KarMMa. The overall incidence of infection matched the previous data of CD19 CAR-T-cell therapy. Generally, bacterial infections were the most common, and only one patient developed fungal infection despite none of the patients receiving antifungal prophylaxis. This study provided us with some other explicit information on specific infectious complications of the particular crowd, and it was pregnant to the clinical application of ide-cel.

3.5.3 Updated Information of Other Existing BCMA-Targeted CAR-T Products

In addition to cilta-cel and ide-cel, a variety of other BCMA-targeted CAR-T-cell products were mentioned at this ASH meeting. The relevant data are presented in **Table 2**, and there are some points that should be added: firstly, CT053, an all-human CAR-T-cell product, has shown promising efficacy and safety in its phase I clinical trial (LUMMICAR STUDY 1 and CG) (49, 50). Two things interesting were that, the ORR of CT053 to treat the RRMM patients relapsed after three or more LOT with the extramedullary disease (EMD) being 91.7%, the CR rate being 58.3%, and the median PFS being 9.3 months, better than the results of those past treatment strategies such as combination therapy with permadomide and dexamethasone (ORR: 30%; CR rate: 15.3%) (69) and carfilzomib-based combination therapy (ORR: 27%, CR rate: 0%, median PFS: 5 months) (70). For the patients with high-risk cytogenetic abnormalities [del(17p), t(4;14), t(14;16)/1q21], the ORR and CR rate of CT053 were 84.2% and 73.3%, respectively, with the 15.6 months median PFS, better than the results of ishatuximab, permadomide, and dexamethasone combination (ORR: 50%, CR rate: 0%, median PFS: 7.5 m) (71), carfilzomib monotherapy (ORR: 25.8%, CR rate: 0%, median PFS: 3.5 m) (72), and even the infusion of bb2121 (ORR: 73%, CR rate: 33%, median PFS: 8.2 m) (60). Secondly, the relationship between the dosage and curative effect of C-CAR088 has been studied (52). Among the selected doses of 1.0, 3.0, and 4.5~6.0 × 10^6 CAR$^+$ T cells/kg, the cohorts whose dosage ≥3.0 × 10^6 CAR-T cells/kg had deeper and more durable responses, which needed further research. Thirdly, PHE885 is a novel fully human CAR-T-cell product modified with T-Charge^{TM}. This platform can reduce the *in vitro* culture time of CAR-T cells to about 24-h, thus taking only less than 2 days to acquire the final products, which totally depends on the *in vivo* proliferation after infusing CAR-T cells (53). The application of this new platform also can retain the naïve-like and stem cell memory T cells (Tnaïve$^+$Tscm) (CD45RO$^-$/CCR7$^+$), which are beneficial to the persistence of CAR-T cells. By contrast, the CAR-T cells (TM_PHE885), having the same single-chain variable fragments (scFvs) of PHE885, which were prepared by the conventional methods, just keeps central-memory T cells (CD45RO$^+$/CCR7$^+$)

(73). Strong cell amplification was observed in all patients by qPCR technique (the maximum amplification of T cells in circulation was 283,000 copies/μg, the median maximum amplification time was 21.1 days) and flow cytometry (the maximum amplification of T cells in circulation was 69.3%, the median maximum amplification time was 16.4 days). PHE885 can be detected in the peripheral blood of each patient during follow-up (1–6 months). Fourthly, CART-ddBCMA is a special CAR-T-cell product with a synthetic BCMA binding domain. Differing from the classical scFvs with a 4-1BB costimulatory domain and a CD3ζ activation domain, it is a smaller stable protein containing only 73 amino acids, thus reducing the threats of immunogenicity (54). Finally, a multicenter phase I trial of bb21217 named CRB-402 (NCT03274219) is underway. The preliminary results of its preclinical study have been reported before, and the subsequent results presented in ASH2021 showed that adding PI3K inhibitor (BB007) during the *in vitro* culture stage to amplify memory-like T cells ($CD62L^+$ and $CD27^+$) (74, 75) could advance the persistence of CAR-T cells literally. This positive effect was reflected on a better DOR of bb21217 compared with bb2121 (65), which shared the same CAR structure with it (55). Moreover, after infusing bb21217, CAR-T cells could be detected in 30/37 (81%) patients and 9/15 (60%) patients at 6th and 12th months. Analysis of the peripheral blood samples showed that less differentiated, more proliferative CAR-T cells at peak expansion are associated with the prolonged response period (median DOR of higher $CD62L^+$ $CD27^+$ $CD8^+$ CAR-T cells vs. lower: 27.2 vs. 9.4 months) (**Figure 2**).

3.5.4 Allogeneic BCMA-Targeted CAR-T-Cell Products

Patients with relapsed or refractory hematological malignancies who are suitable to be treated by CAR-T-cell therapy usually have a high tumor load and many deficiencies in their T-cell population (76). These limitations increase the difficulty of obtaining sufficient qualified T cells as the materials for CAR-T cells' manufacture after apheresis (76). Up to now, allogeneic CAR-T-cell therapy has shown a certain extent efficacy for the patients who have appropriate donors (77, 78). However, allogeneic materials bring the risk of graft-versus-host disease (GvHD) undoubtedly (79). The most effective measure at present is using gene-editing technology such as zinc-finger nucleases (ZFN), transcription activator-like effector nuclease (TALEN) technology, and the CRISPR/Cas9 system to knockout T-cell receptors (TCR) at the DNA level to reduce the risk of GvHD (80). Moreover, many novel strategies such as RNA silencing (80) and membrane protein intracellular retention technology have been used to knockdown TCR at mRNA or other levels. ASH2021 updated two novel allogeneic BCMA-targeted CAR-T-cell products: ALLO-715 (81) and CYAD-211 (82). ALLO-715 used Collectis TALEN technology to disrupt the TCR alpha constant (TRAC) and CD52 gene, which required multiple operating steps and longer culture time to increase the exhaustion of T cells. By comparison, CYAD-211 converted to use short hairpin RNA (shRNA) to knockdown TCR expression at mRNA level, which could reduce the preparation duration. The shRNA in CYAD-211 was coexpressed with its CAR, so it required only one step to achieve the genetic modification. The phase I trials of these two, named UNIVERSAL and IMMUNICY-1 respectively, have already begun, and the former provided more details to us. A report in ASH2020 about UNIVERSAL suggested that a higher dose level of ALLO-715 could improve clinical efficacy. After the 320/480 × 10^6 CAR-T-cell infusion and 7.4 months median follow-up, the ORR (n = 26) was 61.5%, and the rate of VGPR was 38.5%. The incidence rate of CRS was 52.4%, and just one of them was rated level 3. Among ten patients who have been tested, eight obtained negative results of MRD, proving the efficacy of ALLO-715 to some extent. Regrettably, information about GvHD did not present here (81). As for IMMUNICY-1, none of the nine enrolled participants showed GvHD after CYAD-211 inputting. However, the grafts only lasted 3 to 4 weeks *in vivo*, which can be interpreted as the rejection of patients' healing immune system. Moreover, the effectiveness of CYAD-211 needs further assessment. There is also a clinical study showing that allogeneic CAR-T cells from the same donor were used as one of the preprocessed methods for subsequent allogeneic hematopoietic stem cell transplantation (Allo-HSCT) (83). The results showed that this strategy was effective in treating patients with MM who had relapsed after multiple LOTs. The conclusions of these studies, along with part of previous findings (84–87), demonstrated the prospect of allogeneic BCMA-targeted CAR-T-cell therapy and its clinical availability as an adjuvant treatment in combination with other traditional or neoteric therapeutic methods such as allo-HSCT. Rather than stop here, we need more in-depth studies in this area. We think allogeneic CAR-T-cell therapy can be seen as a "stepping stone", and the development and improvement of universal CAR-T cell will become mainstream one day.

3.5.5 Bispecific BCMA-Targeted CAR-T Cell

Although BCMA-targeted CAR-T-cell therapy has shown a favorable efficacy, the disease recurrence after this agents' treatment remains a critical concern (88). One of the reasons of palindromia is the tumor cells' immune escape, which is induced by the adaptive decrease of BCMA expression after long-term treatment, the amplification of a small number of BCMA-negative minimal residual lesions surviving from the lethal effect of CAR-T cells, or other mechanisms (89, 90). To solve this question, an increasing number of studies on dual-targeting or combined-targeting CAR-T cells have been carried out (91, 92). ASH2021 updated two novel bispecific BCMA-targeted CAR-T-cell products: the first targets two tumor-associated antigens (TAAs) (BCMA and CD24) (93), and another one constructs a synthetical CAR targeting the pan-TAAs, containing MHC class I polypeptide-related sequence A/B (MICA/MICB), as the companion target of the classic target BCMA (94). Previous studies showed us that tumor-initiating cells' (TICs) survival and amplification after CAR-T-cell therapy could seed relapse by acquiring the resistance. Part of these cells were the $CD24^+BCMA^-$ subgroups (95). As expected, these BCMA-CD24-targeted CAR-T cells, which target and kill TICs effectively, can be activated by exposing to the $CD24^+$ microenvironment. When $CD24^+$ MM cells (ARP-1 CD24OE or OCI CD24OE cells) were co-cultured with these CAR-T cells

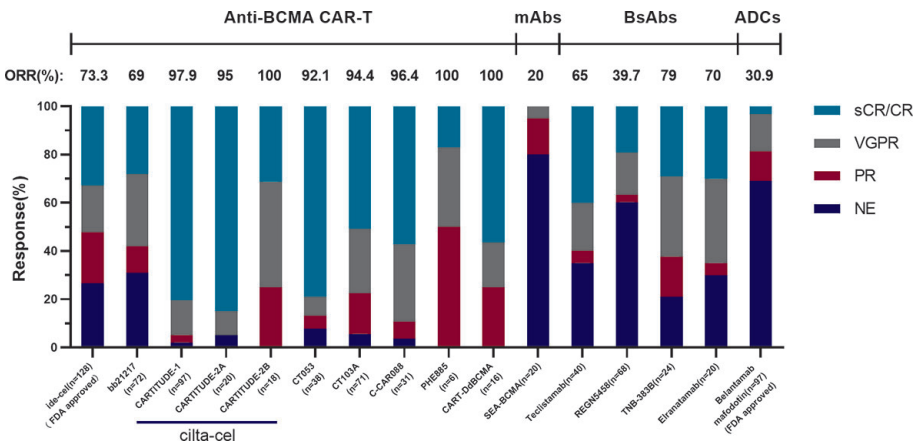

FIGURE 2 | The comparison of different anti-BCMA agents. These results come from phase II clinical trial KarMMa of Idecabtagene Vicleucel (*n* = 128, 13.3 months median follow-up), phase I clinical trial CRB-402 of bb21217 (*n* = 72, 9 months median follow-up), phase Ib/II clinical trial CARTITUDE-1 of cilta-cel (*n* = 97, 18 months median follow-up), cohort A in phase II clinical trial CARTITUDE-2 of cilta-cel (*n* = 20, 9.7 months median follow-up), cohort B in phase II clinical trial CARTITUDE-2 of cilta-cel (*n* = 18, 4.7 months median follow-up), phase I/II clinical trial LUMMICAR STUDY 1 of CT053 (*n* = 14, 13.6 months median follow-up), phase I/II clinical trial of CT103A (*n* = 71, 147 days median follow-up), phase I clinical trial of C-CAR088 (*n* = 31, 8 months median follow-up), phase I clinical trial of PHE885 (*n* = 6, 1 month follow-up), phase I clinical trial of CART-ddBCMA (*n* = 16, 155 days median follow-up), phase I clinical trial SGNBCMA-001 of SEA-BCMA (*n* = 20, 12 weeks median follow-up), phase II clinical trial MajesTEC-1 of teclistamab (*n* = 40, 8.2 months median follow-up), phase I clinical trial of REGN5458 (*n* = 68, 2.4 months median follow-up), the dose-escalation cohorts in phase I clinical trial of TNB-383B (*n* = 24, 6.1 months median follow-up), and the patients treated across the efficacious dose range (215–1,000 μg/kg) in part 1 of phase I clinical trial MagnetisMM-1 of elranatamab (*n* = 20, 22 days median follow-up).

in vitro with the 5:1 proportion of the CAR-T cells and the MM cells, the clearance rates of ARP-1 CD24OE and OCI CD24OE cell lines were 99% and 89%, respectively. Unlike the CAR mentioned above targeting two well-defined epitopes, another bispecific CAR targets BCMA and a pan-TAAS simultaneously. The conserved α3 domain of MICA/MICB is the target of the CAR, which could drive antitumor immunoreaction and prevent MICA/MICB shedding at the same time (96). These studies elucidated that both BCMA and the additional antigens could activate those bispecific CAR-T cells targeting them, making these artificial immune cells degranulated to exert their cytotoxicity. Bispecific BCMA-targeted CAR-T-cell therapy is a promising strategy to expand the splash radius of CAR-T cells, which is expected to reduce the resurgence of MM after CAR-T-cell therapy.

3.5.6 The Novel Ameliorative Methods for BCMA-Targeted CAR-T Cell

BCMA-targeted CAR-T-cell therapy also has some other deficiencies (6, 97), which call for reasonable solutions (**Figure 3**). ASH2021 updated several novel engineering improvements to address part of these limitations.

3.5.6.1 CAR-T Cells' Poor Persistence

CAR-T cells' poor endurance is a major cause of disease recurrence after treatment (98). By testing peripheral blood and bone marrow samples from the patients treated with BCMA-targeted CAR-T cells, a study in ASH2021 (99) found that increased BCL-XL expression may enhance CAR-T cells' resistance to the similar effect like activation induced cells death (AICD), and prolong these cells' persistence through responding to CD28 costimulatory signals. Based on this discovery, the researchers designed a second-generation lentiviral CAR (BCMA-BCL2L1-CAR)-armored BCL-XL. In the edited gene of this CAR, classical anti-BCMA scFV-41BBz CAR and BCL2L1 cDNA were linked by a self-cleaving 2A sequence. This kind of modified BCMA-targeted CAR-T cell has a higher BCL2L1 expression, and in MM cell lines (MM1S, OCMY5, and H929) expressing the ligands of FAS death receptor (FASLG), BCMA-BCL2L1-CAR-T cells observably outperformed unarmored BCMA-CAR-T cells in terms of viability and cytolysis activity. Moreover, BCMA-BCL2L1-CAR-T cells with less cells exhaustion showed greater ability to kill the tumor cells under chronic antigenic stimulation, which could cause AICD more easily.

For extending the duration of BCMA-targeted CAR-T cells, a nonvirus transposon system called PiggyBac (PB) has already been put into use. Two novel CAR-T-cell products targeting BCMA manufactured by PB were reported in this meeting. They were named P-BCMA-101 (autologous) and P-BCMA-ALLO1 (allogeneic), respectively (100). This study proved that PB does not only sped up the preparing process of CAR-T cells but also could preserve more desirable stem cell memory T cells (Tscm), whose proportion were closely related to the persistence of BCMA-targeted CAR-T cells (101, 102). Results of the phase I/2 clinical trial for P-BCMA-101 named PRIME (NCT03288493) certified the safety of this agent.

3.5.6.2 CAR-T Cells' Immunogenicity

The nonhuman sequences in scFvs of anti-BCMA CAR have immunogenicity, which can trigger the host versus graft (HvG) response (76). A study reported in ASH2021 constructed a new

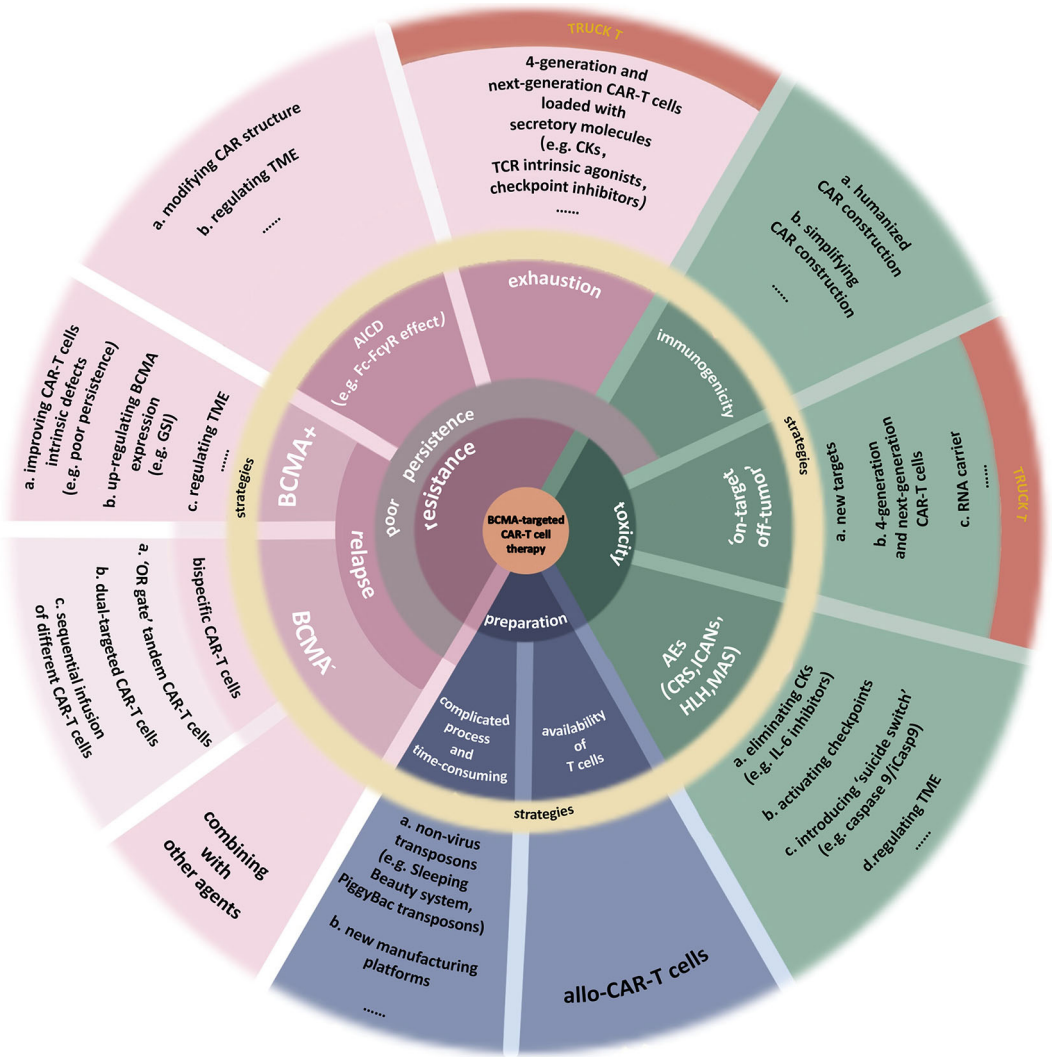

FIGURE 3 | Limitations of BCMA-targeted CAR-T-cell therapy. This image summarizes the deficiencies of BCMA-targeted CAR-T-cell therapy. Also, part of improvements to address these limitations is presented. **(a)** Toxicities. Nonhuman single-chain variable fragments (scFv) in classical CAR construction increases the heterogeneity of CAR-T cell, inducing attack by the immune system of patients. Using humanized materials to prepare CAR or simplifying the CAR construction can reduce the heterogeneity. Finding new targets with higher specificity can reduce the "on-target off-tumor" effect. Fourth-generation and next-generation CAR-T (TRUCK T) cells, fitted with transgenic "payloads" which can express specific secretory molecules or membrane receptors, create a more favorable microenvironment for their function. BiTE-armored and chemokine receptor-armored CAR-T cells can target tumor cells more precisely. The adverse events (AEs) after CAR-T-cell therapy include cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome (ICANs), hemophagocytic lymphohistiocytosis (HLH), macrophage activation syndrome (MAS), or more. The most common of them is CRS. Clearance of excess cytokines (CKs) is the key to addressing these toxicities. **(b)** Resistance. Resistance to CAR-T-cell therapy induces the disease recurrence, including $BCMA^+$ and $BCMA^-$ relapse. Multiple factors, both internal and external of the tumor, may cause malignant downregulation of BCMA, making it insufficient to be recognized by CAR-T cells. Bispecific CAR-T-cell therapies, including "OR gate" tandem CAR-T cells, dual-targeted CAR-T cells, and sequential regimens, have been used to address $BCMA^-$ relapse and the off-target effect. As for the $BCMA^+$ relapse, it can be caused by multiple factors from CAR-T cells, myeloma cells, even TME. A severe problem of existing CAR-T cells is their poor persistence. There are many reasons for this issue, such as the CAR-T-cells' exhaustion or the clearance to these artificial immune cells, which are similar to physiological activation-induced cell death (AICD). The hinge domain of CAR has a similar structure to the Fc domain in Ig. This characteristic induces the Fc-FcγR interactions between CAR-T cells and other immune cells, killing CAR-T cells. Studies have done to improve CAR's structure by modifying the spacer, such as extending the hinge domain or finding a novel hinge with a lower affinity for FcγR so that to avoid immune system cleanup to activated CAR-T cells. In addition, some inhibitors in TME, the expression of specific inhibitory genes, or the increase of terminal $CD45RA^+$ cells all cause the AICD-like effects, thus reducing the persistence of CAR-T cells. T cells stemness is closely associated with the efficacy and exhaustion of CAR-T cells. TRUCK T cells produce specific secretory molecules such as some CKs, which could increase the stemness of cells (e.g., IL-15), TCR intrinsic agonists (e.g., 4-1BB), or checkpoint inhibitors (e.g., PD-1 inhibitors). As a result, the persistence of these next-generation CAR-T cells has been dramatically improved. **(c)** Preparation process. The complicated preparation process of CAR-T cells is time-consuming, and the quality of T cells as the materials sometimes is not up to par. Developing new nonlentivirus transposons such as PiggyBac transposons or culturing platforms can improve manufacturing efficiency. Using allogeneic T cells as the materials can improve the quality of T cells but induce graft-versus-host disease (GvHD).

anti-BCMA CAR (FHVH33-CD8BBZ) that replaced the normal scFv with a smaller fully human BCMA-targeted heavy-chain variable domain (FHVH33) (103). Because of the lack of light chain, artificial linker, and two linker-associated junctions in scFv, FHVH33 may have lower immunogenicity. After infusing this novel CAR-T cell (FHVH33-T), the ORR was 92% (23/25) and 68% of all patients (17/25) got better than VGPR. Up to the date, the DOR was 50 weeks at the highest two-dose levels ($4/6 \times 10^6$ CAR^+ T cells/kg), and the overall median PFS was 78 weeks. Assessing the blood CAR^+ cells confirmed that the median peak blood CAR^+ cell level was 126.5 cells/μl, and the median time postinfusion of peak blood CAR^+ cell levels was 10.5 days. These results suggest that the immunogenicity of CAR-T cells can be reduced to some extent by fully humanizing and reducing the molecular size of CAR, thereby reducing the likelihood of HvG effect and prolonging CAR-T-cell retention *in vivo*.

3.5.6.3 Availability of Autologous T Cells

Various anterior treatments to the patients with RRMM disable T cells and develop adverse phenotypes, such as exhaustion and senescence (104, 105). These, together with the immunosuppressive characteristic of TME (98), reduce the availability of the heavily pretreated patients' T cells to be the materials for CAR-T cells' preparation. To solve this question, a study explored whether T cell materials with better quality derived from a similar preconditioning approach of autologous hematopoietic stem cell transplantation (HSCT) could be used to prepare CAR-T cells (106). The basic process is collecting $CD34^+$ progenitor cells from peripheral blood of patients and mobilizing them by the granulocyte-colony stimulating factor (G-CSF) in earlier stages of MM treatment, then reserving them for the preparation of BCMA-targeted CAR-T cells. The results showed that pretreatment by G-CSF did not have significant negative effects on T cells. It is a pity that there is no mention in this report of CAR-T cells being produced in this way.

3.5.7 Effects of Other Factors on BCMA-Targeted CAR-T-Cell Therapy

3.5.7.1 Corticosteroids

The side-effects of CAR-T cell therapy, such as CRS, ICANS, macrophage activation syndrome (MAS), and hemophagocytic lymphohistiocytosis (HLH), need to be controlled by tocilizumab, corticosteroids, and, or anakinra (107–109). However, steroids not only suppress the excessive inflammatory response but also inhibit T cells' activity and may reduce the efficacy of CAR-T-cell therapy (110). Previous studies have analyzed the effects of steroids on CAR-T-cell therapy in some other hematologic malignancies with impure results (80). A study (111) in ASH2021 compared the therapeutic effects of BCMA-targeted CAR-T cells combined with or without steroids in patients with RRMM. After using steroids 4 days medially, the results showed that there were no significant differences in ORR (95.8% vs. 84.2%), PFS (13.1 vs. 13.2 months), OS (not reached vs. 26.4 months), and time-to-next treatment (TTNT) (10.5 vs. 7.0) between the "experimental group" that received steroids and the "control group." Moreover, these indicators were not affected by steroids obviously at different doses (0, ≤60, and >60 mg). It is worth mentioning that more than 5 days use of steroids may affect PFS and TTNT to some extent (TTNT/PFS after 0, 1–5, and ≥5 days steroids: 22.8, 24.6, and 12.5 months/13.2, 21.4, and 10.6 months). Although this study seems to prove that cortisol use does not affect CAR-T- cell therapy in general, it did not follow the principles of controlled trials strictly, so the conclusions are open for debate.

3.5.7.2 NKTR-255

In addition to radically improving the structure of CAR-T cells, many other attempts have been made to address their poor persistence (112). NKTR-255, a recombinant human IL-15 (rhIL-15) receptor agonist, can activate the IL-15 pathway and promote the proliferation of memory $CD8^+$ T cells and Tscm subsets in tumor-specific T-cell colonies (101, 102). Recently, a phase I study about the influence of NKTR-255 is ongoing, and ASH2021 provided us with the preliminary results (113). The T/CAR-T cell counts and Ki67 expression of six enrolled patients treated with CAR-T/CAR-NK before were evaluated to assess T cells' viability before and after the NKTR-255 administration. After treating by NKTR-255, the peak number of $CD3^+$ CAR-T cells in peripheral blood of three patients increased by 70% compared with the baseline, and the ratio of $CD4^+$:$CD8^+$ CAR-T cells had changed in one patient with a ~2-fold increase in $CD8^+$ compared with $CD4^+$ CAR-T cells. Following one dose of NKTR-255, all patients had an average of ~1.6-fold increase in total $CD8^+$ T cells and an average 9-fold increase in the percentage of $Ki67^+CD8^+$ T cells, standing up for the role of NKTR-255 in saving the prostrated CAR-T cells. This study demonstrated the feasibility of combining drugs to prolong CAR-T-cell persistence.

3.5.7.3 Gamma Secretase Inhibitor

As mentioned above, the formation of sBCMA through gamma secretase reduces the expression of BCMA on MM cells, making them escape from BCMA-targeted CAR-T cells' lethal effect (114, 115). Those sBCMA in circulation may also interfere the therapeutic process of CAR-T cells for patients with RRMM (116). Gamma secretase inhibitors (GSI) can increase BCMA density on the surface of tumor cells and decrease the level of sBCMA, reinforcing the efficacy of the therapies targeting BCMA in murine models with MM (117). Based on these findings, a phase I human trial of GSI (JSMD194) in combination with BCMA-targeted CAR-T-cell therapy has done and was reported in ASH2021 (118). This trial enrolled 18 patients who had received a median of 10 prior LOTs. After three oral doses (25 mg) administered 48 h apart over 5 days of JSMD194 monotherapy, the median number of the receptors on each tumor cell increased from 610 to 9,563, which was 12 times as large as before. These patients were treated with different-dose BCMA-targeted CAR-T cells subsequently. The resulting ORR was 89%, with 44% of all patients achieving CR (including 27% with sCR) and 77% getting better than VGPR. The median PFS reached 11 months with a median of 20 months follow-up. These data illustrated that the combination of GSI and BCMA-targeted CAR-T-cell therapy were safe and tolerable with an improved antitumor effect, even at very low doses of CAR-T cells.

3.5.7.4 Extrinsic and Intrinsic Factors of Tumor

Using BCMA-targeted CAR-T cells to treat RRMM patients with huge differences between individuals can lead the divergent outcomes. Both intrinsic and extrinsic factors of the tumor, such as the expression of tumor genomics or the immunosuppressive elements presented in TME (119, 120), may contribute to the vast gap between these therapeutic results (88). There were two studies in ASH2021 exploring the relationship between these factors and the therapeutic effects of BCMA-targeted CAR-T cell therapy. The first study (121) used mass cytometry (CyTOF) to longitudinally analyze the immunophenotype of peripheral blood mononuclear cells (PBMC, $CD45^+CD66b^-$) from the patients treated with ide-cel and found that the phenotypic changes of PBMCs along with the CAR-T cells' expansion: $CD14^+$ monocytes declined (40% to 13%) while CAR^-CD8^+ T cells, which differentiated towards a $CD8^+$ effector-memory phenotype (EM, $CCR7^-CD45RA^-$), expanded (32% to 43%) from weeks 0 to 4 after the infusion of CAR-T cells. However, the BM samples from the patients who relapsed after CAR-T-cell therapy showed a reversal trend: $CD14^+$ monocytes remained invariable or slightly elevated, but CAR^-CD8^+ T cells decreased instead. This study also analyzed the BM mononuclear cells (BMMC) from patients with ide-cel therapy by unbiased mRNA profiling using single-cell RNA-seq (scRNA-seq). The outcome revealed that patients who relapsed had an altered gene expression, suggesting that the intrinsic tumor factors had an impact on CAR-T-cell therapy. For example, upregulation of gene expression like proinflammatory chemokines (CCL3, CCL4), antiapoptotic genes (MCL-1, FOSB, JUND), and NF-kB signaling genes (NFKBIA) could promote relapse, which may be one of the mechanisms for the resistance to CAR-T therapy. Interestingly, another study (122), which also used scRNA-seq to compare the BM and PBMC samples from the patients who relapsed within 1 year [early relapse (PD)] or more than 1 year [durable response (DR)] after BCMA-targeted CAR-T cells' infusion, showed that the DR patients had more BCMA-high $CD138^+$ cells compared with the PD patients. Moreover, there were two unique clusters in DR patients' $CD138^+$ cells while only one in PD patients. The top marker genes in these three clusters were associated with the pathway of IL-15 signal, BCR signal, and the primary immunodeficiency signal. It should be added that the patients achieving more than VGPR after CAR-T-cell therapy had a higher proportion of $CD8^+$ T cells compared with poor responders (<VGPR) (37% vs. 11%), a lower proportion of $CD14^+$ monocytes (30% vs. 61%) and NK cells (2% vs. 6%) in PB (121).

3.5.7.5 CAR Density

Up to now, the underlying mechanisms of CAR-T cells' dysfunction are not well understood. A part of the studies has proved that the density of CAR can affect the availability and antitumor effectiveness of CAR-T cells. A recent study (123) in ASH2021 performed genomic and functional analyses on the BCMA-targeted second-generation CAR-T cells which have 4-1BB costimulatory domains with different CAR densities (CAR_{High} and CAR_{Low}). The genomic analysis showed entirely different profiles between CAR_{High}-T cells and CAR_{Low}-T cells in both $CD4^+$ and $CD8^+$T-cell subsets, with 3,500-fold difference in gene expression. These genes were related to T-cell activation, and the tonic signaling in CAR_{High}-T cells associated with T-cell proliferation or exhaustion. The functional analysis showed that before encountering the antigens, CAR_{High}-T cells presented intensive tonic signaling, which led to higher activation and more differentiation. After identifying their targets, CAR_{High}-T cells released an increased number of cytokines, indicating that they would exert more potent cytotoxic effects. Moreover, in these CAR_{High}-T cells, the factors about cell proliferation and exhaustion ($PD1^+$/$LAG3^+$/$TIGIT^+$) were increased as well, and these cells presented a higher percentage of terminally differentiated T cells ($CCR7^-$/$CD45RA^+$). The regulons associated with NR4A1 transcription factor that promotes T-cell exhaustion (124) also have been activated in CAR_{High}-T cells. By contrast, the analysis of CAR_{Low}-T cells demonstrated that they had better persistence, in which more $CCR7^+$/$CD45RA^+$/$CXCR3^+$ Tscm were retained. That is to say, increasing CAR density could enhance CAR-T cells' activation, differentiation, and cytotoxicity but reduce their long-term efficacy. Therefore, the CAR density may play a crucial role in CAR-T cells' persistence. It is expected to promote the effectiveness of CAR-T-cell therapy by rationally using the engineered T cells with different CAR densities.

3.6 Prospects for CAR-NK Cell Therapy in MM

Since NK cell activation does not need the prior antigen stimulation and strict HLA matching, CAR-NK cell therapy has shown its unique competitiveness under this era of rapid development of cellular immunotherapy (125). Compared with CAR-T-cell therapy, it seems to have better safety. Because NK cell cytotoxicity is mediated by releasing perforin and granulocytase rather than cytokines such as IL-1, IL-2, IL-6, TNF-α, IL-8, IL-10, and IL-15 released by CAR-T cells, or expressing the apoptosis-inducing ligands including Fas Ligand (FasL) and (TNF)-related apoptosis-inducing ligand (TRAIL), they rarely cause CRS and neurotoxicity (125). In addition, CAR-NK cells are more suitable to be the "off-the-shelf" therapy than CAR-T cells. According to the available studies, allogeneic CAR-NK cells almost never induce GvHD, and the source of NK cells is more extensive, they can be differentiated from peripheral blood (PB) cells, umbilical cord blood (UCB) cells, embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), and specific NK cell lines such as NK92 cells (126). For MM, a variety of CAR-NK cells have been studied in preclinical or clinical trials. They target different targets, including BCMA, CD138, and CS1 (CD319/SLAMF7) (125). Up to now, clinical trials have been conducted on two anti-BCMA CAR-NK cells derived from umbilical/cord blood (CB) (NCT05008536) and NK92 cell line (NCT03940833), but they have not published the relevant data yet. Current studies are focused on optimizing existing BCMA-targeted CAR-NK cells and developing universal CAR-NK cell therapy derived from iPSCs. Existing CAR-NK cell products have been genetically modified by gene editing (127), mRNA electroporation (128) and other techniques, which significantly increased their targeting specificity and tumor killing

effectiveness. A study in ASH2021 (129) creatively combined three antitumor modalities, including CAR, TCR, and CD16 Fc receptor, which is naturally expressed on NK cells. By engineering them into iPSC-derived T cells, they demonstrated the synergistic effect of this tri-modal CAR-iT cell in overcoming tumor cell escape and their heterogeneity. In the future, if we want to promote the clinical application of CAR-NK cells, it is necessary to properly solve or evade their existing limitations such as short life, low toxicity, and the off-target effect.

4 DISCUSSION

Although multiple kinds of BCMA-targeted immunotherapies, including ADCs, BsAbs, and adoptive cell therapies have presented gratifying results of their primeval clinical trials, there are still many hurdles that need to be overcome before they go into real-world service to benefit more suffering patients with RRMM. According to the reports in ASH2021, BCMA-targeted CAR-T-cell therapy seems to show better efficacy than other agents. However, we cannot simply judge the merits of these products. The unique characteristics of these agents not only grant them irreplaceable advantages but also give them inevitable limitations. For instance, anti-BCMA CAR-T-cell therapy with better performance requires more complex preparation conditions and more expensive treatment costs, which are difficult for ordinary families to afford (130). For BsAbs, because of the relatively short half-life period (131), they need to extend the infusion time or improve the medication frequency to maintain its efficacy (132), which also increases the costs of treatment. BiTE depends on the quality of T cells, thus it is mainly used for front-line treatment (133, 134). As for those off-the-shelf ADCs which are cheaper and more convenient, they also have to be administered more frequently because they take effect by internalizing them into the tumor cells and releasing payloads, which are easy to be cleared by the intracellular active substances (135). Luckily, for these agents, the ameliorations for deficiencies are thought in more detail as well. Take the CAR-T products for example, concerning the long-term consumption in preparation of the costly CAR-T cells, multiple new techniques such as Piggy Bac and Sleeping Beauty transposition system have been put into study. Of course, these deficiencies are just a drop in the bucket. Therefore, with continuing the existing studies, we also need to study the underlying mechanisms that influence the curative effect to optimize BCMA-targeted immunotherapies. We have to say that the development of various immunotherapy methods in recent years has changed the treatment landscape of MM to some extent. In the face of so many biological drugs, formulating appropriate medication regimens will be a challenge for clinicians (136). Existing anti-BCMA agents are primarily used to treat those adults with RRMM who have received more than 4 LOTs, but the studies about their front-line application are limited. In fact, the patients who have received three or more LOTs have worse physical conditions, therefore, moving the treatment window forward moderately may be the direction of future clinical studies. Although the prognosis of MM patients has improved greatly, the refractory phenotypes such as EMD are still difficult to overcome. To solve these problems, laboratory research and enriching our clinical experience should continue simultaneously. We believe that with the further research, RRMM patients will eventually go through their winter.

AUTHOR CONTRIBUTIONS

RG is a major contributor in writing the manuscript. WL has made substantial contributions to the conception. RG and XJ drafted the work. YZ, XC, and XJ have substantively revised it. MZ reviewed the draft. 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.

GLOSSARY

MM	multiple myeloma
PI	proteasome inhibitor
IMiD	immunomodulatory drug
mAbs	monoclonal antibodies
BCMA	B-cell maturation antigen
ADCs	antibody-drug conjugates
BITEs	bispecific T-cell engagers
CAR-T cell	chimeric antigen receptor-T cell
ASH	American Society of Hematology
PCs	plasma cells
APRIL	a proliferation-inducing ligand
NF-κB	nuclear factor kappa-B
RAS/MAPK	rat sarcoma/mitogen-activated protein kinase
PI3K-PKB	phosphoinositide-3-kinase–protein kinase B
JNK	c-Jun N-terminal kinase
TEM: tumor microenvironment	H3K36me3
H3K36 trimethylation	sBCMA
soluble BCMA	SLAMF7
signaling lymphocytic activation molecule family member 7	LOT
prior lines of therapy	RRMM: relapsed or refractory multiple myeloma
PD	pharmacodynamics
IRR	infusion-related reaction
DLT	dose-limiting toxicity
VGPR	very good partial responses
ORR	objective response rate
AEs	adverse events
DEX	dexamethasone
PK	pharmacokinetics
BM	bone marrow
BsAbs	bispecific antibodies
BiTEs	bispecific T-cell engagers
TCR	T-cell receptors
GPRC5D	G-protein coupled receptor C family 5D
pts	patients
LEN	lenalidomide
POM	pomalidomide
NA	not applicable
DOR	duration of response
TRAEs	treatment-related AEs
CRS	cytokine release syndrome
Gr	grade
ICANS	immune effector cell-associated neurotoxicity syndrome
TEAEs	treatment-emergent AEs
NK	natural killer
TriTAC	tri-specific T-cell activation constructs
MTD	maximum tolerated dose
RP2D	recommended phase two dose
MMAF	monomethyl auristatin-F
FDA	Food and Drug Administration
MoABs	anti-CD38 antibody
BCVA	best-corrected visual acuity
OS	overall survival
sCR	stringent complete response
cilta-cel	ciltacabtagene autoleucel
MRD	minimal residual disease
RWCP	real-world clinical practice
CR	complete response
ide-cel	idecabtagene vicleucel
HRQoL	health-related quality of life
sAMT	subsequent antimyeloma therapy
scFvs	single-chain variable fragments
GvHD	graft-versus-host disease
TRAC	TCR alpha constant
shRNA	short hairpin RNA
Allo-HSCT	allogeneic hematopoietic stem cell transplantation
TAA	tumor-associated antigens
MGUS	monoclonal gammopathy of undetermined significance
TICs	tumor-initiating cells
AICD	activation-induced cells death
FASLG	FAS death receptor ligand
G-CSF	granulocyte-colony-stimulating factor
PB	PiggyBac
MAS	macrophage activation syndrome
HLH	hemophagocytic lymphohistiocytosis
rhIL-15	recombinant human IL-15
GSI	gamma secretase inhibitors
PBMA	peripheral blood mononuclear cells
BMMC	BM mononuclear cells
scRNA-seq	single-cell RNA-seq
CKs	cytokines
AICD	activation-induced cell death
PFS	progression-free survival
HvG	host versus graft
TALEN	transcription activator-like effector nuclease
MICA/MICB	MHC class I polypeptide-related sequence A/B
FasL	Fas ligand
TRAIL	(TNF)-related apoptosis-inducing ligand
PB	peripheral blood
UCB	umbilical cord blood
ESCs	embryonic stem cells
iPSCs	induced pluripotent stem cells
CB	cord blood
EMD	extramedullary disease

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ORIGINAL RESEARCH
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Generation of Tumor-Specific Cytotoxic T Cells From Blood *via In Vitro* Expansion Using Autologous Dendritic Cells Pulsed With Neoantigen-Coupled Microbeads

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For the past decade, adoptive cell therapy including tumor-infiltrating lymphocytes, genetically modified cytotoxic lymphocytes expressing a chimeric antigen receptor, or a novel T-cell receptor has revolutionized the treatment of many cancers. Progress within exome sequencing and neoantigen prediction technologies provides opportunities for further development of personalized immunotherapies. In this study, we present a novel strategy to deliver *in silico* predicted neoantigens to autologous dendritic cells (DCs) using paramagnetic beads (EpiTCer beads). DCs pulsed with EpiTCer beads are superior in enriching for healthy donor and patient blood-derived tumor-specific CD8+ T cells compared to DC loaded with whole-tumor lysate or 9mer neoantigen peptides. A dose-dependent effect was observed, with higher EpiTCer bead per DC being favorable. We concluded that CD8+ T cells enriched by DC loaded with EpiTCer beads are tumor specific with limited tumor cross-reactivity and low recognition of autologous non-activated monocytes or CD8+ T cells. Furthermore, tumor specificity and recognition were improved and preserved after additional expansion using our Good Manufacturing Process (GMP)-compatible rapid expansion protocol. Phenotypic analysis of patient-derived EpiTCer DC expanded CD8+ T cells revealed efficient maturation, with high frequencies of central memory and effector memory T cells, similar to those observed in autologous expanded tumor-infiltrating lymphocytes. These results indicate that DC pulsed with EpiTCer beads enrich for a T-cell population with high capacity of tumor recognition and elimination, which are features needed for a T-cell product to be used for personalized adoptive cell therapy.

Keywords: neoantigen, tumor-specific antigens, autologous tumor recognition, dendritic cell-mediated activation, personalized cancer immunotherapy

INTRODUCTION

Immunotherapy, including immune-checkpoint inhibitors (ICI) and adoptive cell therapy (ACT), is one of the most prominent and fastest developing fields within cancer treatment. ACT encompasses transfer of tumor-infiltrating lymphocytes (TILs), T cells genetically altered with a TCR or a chimeric antigen receptor (CAR), and NK cells or dendritic cell (DC) vaccines. The usage of genetically modified CAR T cells has been proven very effective against CD19+ hematological cancers while TIL therapies have been proven beneficial in the treatment of several solid tumors (1–4). Unfortunately, the population that benefits from these specific therapies is relatively limited; thus, novel alternative approaches to cancer immunotherapy are needed. One option is to target tumor antigens specifically. However, early studies targeting tumor-associated antigens (TAAs), self-antigens that are overexpressed or have an altered expression pattern, using TAA-directed TCR-transduced T cells or TAA-loaded DC vaccine, reported limited clinical responses or induction of autoimmune toxicity, including cross-reactivity-induced death (5–7). Central and peripheral tolerance against the chosen TAA or loss of antigen presentation on the tumor cell surface likely account for the low clinical efficacy (6).

Personalized immunotherapy using tumor-specific antigens (TSA) has the potential to be an ideal therapy, which maximizes efficacy while minimizing toxicity. Somatic mutations occurring in tumor cells can lead to the generation of novel TSA, which potentially can be recognized as non-self, referred to as neoantigens, which may be presented as tumor-specific peptides by major histocompatibility complex (MHC) molecules on the tumor cell surface. These peptides can be recognized by the immune system without induction of tolerance or risk of "off-target" effects on healthy tissues. Neoantigens are therefore promising targets for ACT and/or DC vaccine-based therapies (8). Numerous clinical trials based on neoantigens have been conducted or are ongoing, including DC loaded with neoantigens or delivered as peptides or mRNA (9).

Neoantigens are identified using next-generation sequencing (NGS), e.g., whole-exome sequencing (WES). Tumor-specific mutations are identified by comparing tumor biopsies to healthy tissues (in general, peripheral blood mononuclear cells) followed by different predictions tools to select for expressed mutations generating a neoantigen, which can efficiently be presented on MHC class I. To date, neoantigen predictions have mainly been focusing on antigens binding to frequent/common MHC class I/HLA class I alleles (10, 11). Computerized MHC-binding neoantigen predictions are in general focusing on MHC-binding affinity, endogenous expression (RNA) and processing, and/or in combination with mass spectrometry (MS). Combining several parameters increases the likelihood to predict neoantigens that are presented on MHC class I on the tumor cell surface. However, to verify if the predicted mutant peptides are true neoantigens, functional T-cell screens are needed.

We have previously shown that it is possible to predict clinically relevant neoantigens from two melanoma patients using free online tools for *in silico* neoantigen prediction (12). Neoantigens were identified by screening for TIL reactivity against custom made 9–10mer peptides. In addition, a DC vaccine-based stimulation method, loading the autologous DC vaccine with mutant 9mer peptides, was established to stimulate tumor-specific CD8+ T cells from the patient's blood. Custom-ordered dextramers, specific for each neoantigen, in combination with CD107a expression (a marker for degranulation), were used to detect neoantigen-specific T cells recognizing the autologous tumor cells. In addition, mass spectrometry was used to verify neoantigen expression on the autologous tumor cell surface.

Others have shown that the usage of longer peptide sequences, ~25mers, to capture all possible "processing variants" of the neoantigen, can be favorable (13–15). Loading these longer peptides or combining several 25mers into (tandem) minigenes onto autologous antigen-presenting cells (APCs) has been shown to trigger efficient autologous T-cell responses measured by cytokine production or activation markers such as 4-1BB or PD1 (13, 16, 17).

Furthermore, longer peptide sequences have also been shown to be favorable when designing therapeutic neoantigen-based vaccines. The longer sequences promote antigen uptake and processing by APC and thereby help to facilitate a stronger T-cell response (18, 19). Due to tumor heterogeneity, targeting one neoantigen can result in outgrowth of tumor cells expressing other neoantigen(s), indicating the importance of targeting multiple neoantigens to lower the risk of immune escape and ensure elimination of all tumor cells (20, 21). In addition, Aurisicchio et al. have shown the importance of targeting multiple neoantigens with a predicted high binding affinity, <50 nM, to induce a poly-specific and poly-functional T-cell response in mice after minigene vaccinations (22). One alternative to neoantigen-based vaccines are DC vaccines loaded with whole tumor lysate (TL), which has been proven effective for certain cancer types (23). Although tumor lysate will contain peptides from all proteins in the cells and therefore "dilute" the neoantigen peptides and their ability to induce an immune response, the usage of tumor lysate circumvents the work with neoantigen prediction and peptide/minigene production.

In the present study, we have investigated several ways to load DC with tumor antigens to enrich for blood-derived tumor-specific CD8+ T cells. To this end, different approaches of tumor antigen administration were compared including whole tumor lysate, neoantigen 9mer peptides, or *via* longer neoantigen peptide sequences coupled to paramagnetic beads (EpiTCer) (24). EpiTCer beads are a novel way of delivering *in silico* predicted and recombinantly expressed neoantigen peptides. EpiTCer beads are paramagnetic beads covalently coupled to a neoantigen protein (NAG), consisting of six 21mer neoantigen peptides linked sequentially to each other. DC were generated according to our established Good Manufacturing Process (GMP) protocol. We have previously used DC generated from this protocol, in the form of DC vaccinations, in combination with TIL in patients with metastatic melanoma (NCT01946373).

We observed that DCs loaded with EpiTCer beads were superior in activating blood-derived autologous CD8+ tumor-

specific T cells compared to DC pulsed with other sources of tumor antigen. Tumor recognition was MHC class I antigen dependent. In addition, patient-derived CD8+ T cells enriched using DC pulsed with EpiTCer beads were tumor specific with limited recognition of healthy cells. Phenotypic analysis of DC-expanded CD8+ T cells revealed efficient maturation of the CD8 + T cells with high frequencies of central memory and effector memory T cells, similar to those observed in TIL. We believe that EpiTCer beads represent an efficient method to pulse neoantigens onto DC, which can either be used to enrich for tumor-specific CD8+ T cells from peripheral lymphocytes in ACT or as a DC vaccine.

MATERIALS AND METHODS

Cells

Patient-derived melanoma cell line, ANRU tumor cells, were generated as previously published (12) and cultured in Roswell Park Memorial Institute (RPMI) with 10%–20% fetal bovine serum (FBS) (Life Technologies, Waltham, USA) supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) (Life Technologies). ANRU CD8+ T cells and monocytes were acquired through leukapheresis fractions 2 and 5, respectively (2018/2254-32). Peripheral blood samples (anonymized blood donations from healthy adult donors) were purchased from Karolinska University Hospital Blood Bank. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy donor buffy coats using density centrifugation with Ficoll® Paque Plus (GE Healthcare). The healthy donor PBMCs were screened and selected on HLA-A2+ donors. Healthy-donor-derived CD8+ T cells and $CD14^+$ monocytes were isolated from PBMCs using positive CD8+ T cells isolation kit or $CD14^+$ microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions.

Neoantigen Predictions

For the identification of tumor-specific variants used to design neoantigen proteins, one melanoma patient, acronym ANRU, tumor tissue, and healthy control cells were used. Exome sequencing and prediction and identification and verification of the neoantigens ETV6 and NUP210 were performed as previously described (12). For the newly predicted neoantigens, the bioinformatics system PIOR (Personalised Immuno-Oncology Ranking, Stockholm, Sweden), developed by NEOGAP Therapeutics AB, was used. PIOR identifies and ranks tumor-specific variants; exome fastq files were processed with fastqc (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to generate quality control parameters. The files were then mapped using bwa (http://bio-bwa.sourceforge.net/). Resulting mapped reads were sorted and deduplicated using samtools (http://www.htslib.org/). Resulting alignments were processed by an ensemble of multiple aligners (Vardict-Java, VarScan, FreeBayes, and Samtools mpileup). The resulting variant calls were combined, and high confidence somatic variant candidates were extracted from the list and then annotated using VEP (https://www.ensembl.org/info/docs/tools/vep/index.html) to establish possible variant effects. Finally, the resulting variants were ranked and presented to the user for visual inspection and selection. Tools used for the bioinformatics analysis are summarized in **Supplementary Table S2**. The mutations found and used as neoantigen sequences loaded on the beads were all single-nucleotide variants (SNVs).

Neoantigen Design

Novel neoantigen proteins (NAGs) were designed as recombinant construct genes. Top-ranked tumor-specific variants identified by PIOR were assembled with the previously validated neoantigens from ETV6 and NUP210, forming ANRU NAG #1 and #2, respectively (see **Figure 1**). Corresponding wild-type (WT) constructs were also generated, replacing the mutated codons with the corresponding WT ones (see **Supplementary Figure S1**). The nucleotide sequences were optimized for expression in *E. coli*; flanking *Bsa*I sites were added to the optimized DNA sequences and directionally cloned into a modified pET28 vector (Merck-Millipore) as previously described (25). When expressed in the modified pET28 vector, the NAG is flanked by a polylysine coupling tail containing K residues flanked by GGS linkers and a W residue at the N-terminus and an eight-histidine purification tag at the C-terminus.

Neoantigen Production

The cloned constructs were transformed into BL21-AI *Escherichia coli* (Thermo Fisher Scientific, Waltham, USA),

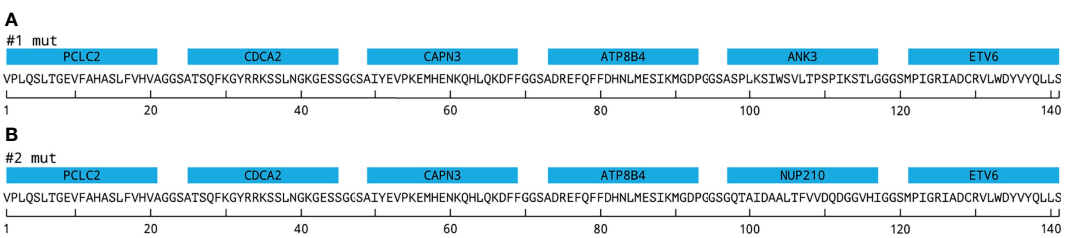

FIGURE 1 | EpiTCer bead constructs. Neoantigen proteins containing six 21mer polypeptides interconnected *via* GGS linkers and covalently coupled to a paramagnetic bead, EpiTCer beads®. **(A)** Displays construct 1 (#1) containing indicated neoepitopes. **(B)** Displays construct 2 (#2) containing indicated neoepitopes. For additional gene, mutation, and sequence information, see *Materials and Methods* and **Supplementary Figure S1** and **Supplementary Tables S1, S2**.

and the NAGs were expressed and purified as described (26), using a modified elution buffer with a pH of 2.0. The eluted and equilibrated NAGs were analyzed for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described, and their concentration was measured using a nanophotometer (Implen, Munich, Germany).

EpiTCer Beads

The NAGs were covalently coupled to 1 µm paramagnetic polystyrene beads [Sera-Mag™ Carboxylate-Modified Magnetic Beads (SpeedBeads, Marlborough, MA, USA), Cytiva], utilizing covalent coupling of primary amines in the neoantigens to the carboxylic groups present on the beads, after EDC/NHS activation, as described (26). Still reactive, non-coupled carboxylic groups were deactivated using a 50-mM Bicine buffer. For endotoxin removal and normalization purposes, the coupled beads were conditioned by four washes with sterile filtered 2M NaOH, followed by four washes with endotoxin-free sterile Dulbecco's phosphate-buffered saline (DPBS) pH 7.4 containing 0.1% Poloxamer 188 (a surfactant that decreases potential beads aggregation). For quality control, NAG load was evaluated by staining coupled beads with Ni-NTA Atto 488 compound, which targets the C-terminal 8His tag on the neoantigens (Sigma-Aldrich). NAG load in the beads was evaluated using flow cytometry (Guava EasyCyte-Luminex Corporation, Saint Louis, USA) and the InCyte analysis software. The number of molecules per bead was calculated to range between 2 and 6 million, based on protein quantification by means of bicinchoninic acid (BCA) assay, flow cytometric determination of bead concentrations, and predicted molecular weight of the purified NAG.

Generation of Neoantigen-Specific CD8+ T Cell From the Blood

Patient- or healthy donor-derived CD14+ monocytes were matured into DC vaccines as previously described (2, 27). Shortly, CD14+ monocytes were cultured in Cellgro® (Cellgenix, Freiburg, Germany) supplemented with IL-4 (20 ng/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (100 ng/ml) (Cellgenix or Peprotech) for 48 h into immature DC (imDC). imDC were harvested and loaded with indicated antigen/neoantigen source (tumor lysates healthy donor, 30 ng/ml; ANRU, 30 µg/ml and beads at indicated bead/DC ratio) and further matured into mature DC by 18 h culture in Cellgro supplemented with IL-4 (20 ng/ml), GM-SCF (100 ng/ml), interferon-gamma (IFNγ) (1,000 IU/ml, Imukin®, Boehringer Ingelheim, Ingelheim, Germany), R848 (2.5 µg/ml, InVivogen, San Diego, US), Poly I:C (20 µg/ml, InVivogen, San Diego, US), and lipopolysaccharide (LPS) (10 ng/ml, InVivogen, San Diego, US). DCs loaded with neoantigen 9mer ETV6, and NUP210, and mature DCs were pulsed with 10 µg/ml (JPT peptides) for 30 min at 37°C and washed before use in co-culture with CD8+ T cells. The DCs were co-cultured with autologous CD8+ T cells in a 1:5 ratio for 10–14 days in CellGro® supplemented with 2% human AB serum (Karolinska University Hospital Blood Bank) and 20 IU/ml IL-2 (Proleukine, Novartis, Basil, Switzerland). All expansions were performed in 96-well U-bottom plates (Cornigen, Corning, New York, USA) if nothing else is stated.

Tumor Lysates

Tumor lysates were generated using our GMP protocol (2, 27).

Flow Cytometry and Functional Assays

All antibodies and FACS reagents were used according to the manufacture's recommendation, if not otherwise stated. All antibodies were titrated for optimal signal-to-noise ratio. Samples were fixed with 2% paraformaldehyde (PFA) (Thermo Scientific) for 15 min before acquisition on a NovoCyte (ACEA Biosciences, San Diego, USA). Compensation was performed using AbC™ Total Antibody Compensation Bead Kit and ArC™ Amine Reactive Compensation Bead Kit (both Invitrogen). FlowJo Software (TreeStar) was used for analysis. All staining protocols included a dead cell marker (LIVE/DEAD® fixable Aqua Dead cell stain (Invitrogen).

T cells were analyzed for anti-CD8 (clone SK1, APC-Cy7), anti-CD3 (clone UCHT1, PE-Cy7), anti-CD45RA-AF488 (clone CI100) (all from BioLegend, San Diego, USA), anti-CCR7-AF647 (clone 3D12, BD), and HLA-A2 (clone BB7.2, PE, BioLegend).

Degranulation/CD107a. Shortly, all long-term co-cultures were harvested and counted, and CD8+ T cells were re-stimulated using ANRU tumor cells, ratio of 1:5, or indicated 9mer peptide (ETV6 or NUP210) 10 µg/ml (JTP peptides). Detection of activated tumor-specific T cells was performed by staining with CD107a (clone H4A3, FITC, BioLegend) antibody, which was added to stimulated T-cell cultures at experiment setup (12, 28). GolgiPlugTM and GolgiStopTM (BD Bioscience) were added after 2 h co-culture, and cells were harvested after an additional 4 h co-culture, harvested and stained for neoantigen specificity (neoantigen specific dextramers, see above) and/or cell surface markers (see above). In experiments where intracellular staining was performed, cells were stained for dead cells, then CD3 and CD8, before fixation and permeabilization using CytoPerm/CytoFixTM (BD Biosciences) and intracellular staining for IFN-γ (clone 4S.B3, PE, BioLegend). When indicated, MHC class I interactions were blocked on ANRU tumor cells for 30 min at 37°C with 20 µg/ml anti-HLA-ABC antibody (clone W6/32, BioLegend) before addition of CD8+ T cells.

RESULTS

Neoantigen Delivery Using EpiTCer® Beads

EpiTCer beads consist of paramagnetic beads in 1 µm size range, onto which neoantigen proteins are covalently coupled. Each construct, comprising six neoantigen polypeptides (21mer), spaced with a three amino acid flexible GGS linker, were coupled to the paramagnetic beads. Previous studies have shown that particles of this size facilitate phagocytosis and efficient antigen processing and presentation by the engulfing

antigen-presenting cell (24, 29–31). In the present study, two different versions of EpiTCer beads have been used, harboring on their surface two different constructs (construct 1 and construct 2), containing in silico-predicted T-cell neoantigens derived from whole exome sequencing from one HLA-A0201 melanoma patient, acronym ANRU (**Figures 1A, B**; **Supplementary Table S1**). The ETV6 and NUP210 neoantigens were previously discovered and validated as 9mers (**Figure 1**) (12).

Efficient Stimulation of Blood-Derived CD8+ T Cells Using EpiTCer Beads® Pulsed DC

To validate the concept of administrating neoantigens to DC through EpiTCer beads, the capability of EpiTCer-loaded DC to stimulate autologous tumor-specific CD8+ T cells from blood was investigated. To this end, HLA-A2+ healthy donor-derived CD14+ monocytes and CD8+ T cells were isolated, and monocytic DC were generated according to our GMP protocol (27). ANRU-derived EpiTCer beads were pulsed onto the immature DC during the second maturation step. ANRU-derived tumor lysate (TL) loaded DC were used as control. For EpiTCer beads, several bead/DC ratios were used. To analyze the efficacy of the tumor antigen-loaded DC to stimulate T cells, long-term co-cultures using autologous DC and blood derived CD8+ T cells was performed. Enriched tumor-specific CD8+ T cells were harvested and re-stimulated with the same melanoma tumor cell line (ANRU) from which the neoantigens and the tumor lysate were derived. Tumor recognition was measured by degranulation (CD107a expression) and cytokine production (IFNγ) using flow cytometry.

We observed that the DC loaded with EpiTCer beads were more potent in enriching and stimulating tumor-specific CD8+ T cells compared to tumor lysate loaded DC, for each of the 1:1–10:1 bead/DC ratios used (**Figure 2A**). A higher EpiTCer bead: DC ratio of 40:1 could further increase tumor recognition and induce an efficient CD8+-mediated antitumoral response for three additional healthy donors (**Figures 2B–D**).

Furthermore, 24-h re-stimulation of the EpiTCer activated CD8+ T cells with ANRU tumor cells resulting in a more efficient tumor recognition than the 6-h re-stimulation, at all tested bead/DC ratios, as measured by degranulation (CD107a) and IFNγ production. In contrast, CD8+ T cells stimulated with DC loaded with tumor lysate, only a marginal difference between 24 vs. 6 h ANRU tumor re-stimulation was noted (**Figure 2**).

Thus, we conclude that DCs loaded with EpiTCer beads, carrying several *in silico* predicted neoantigens, are more efficient in enriching for and activating tumor-specific CD8+ T cells from the blood, compared to tumor-lysate-loaded DC.

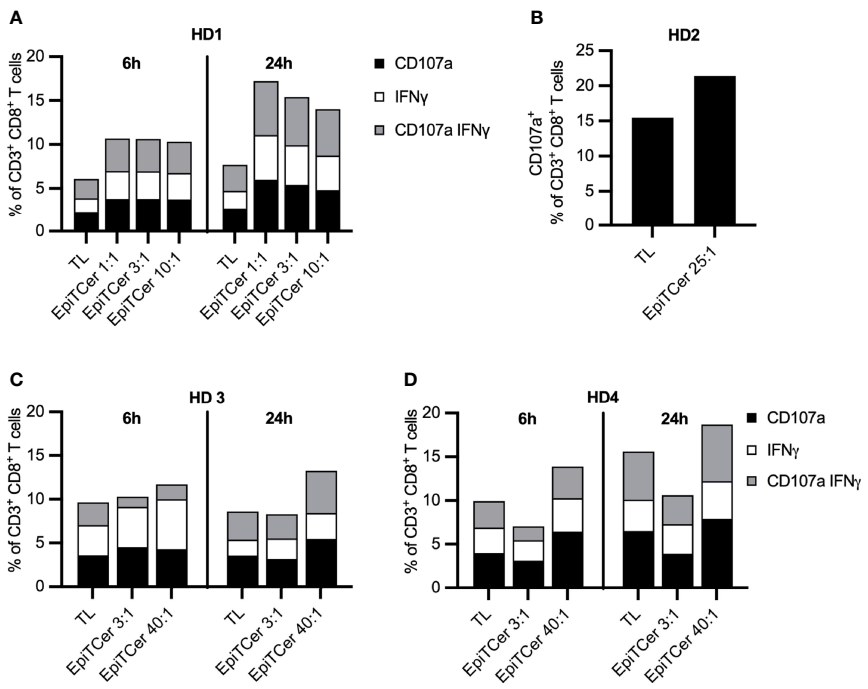

FIGURE 2 | Efficient stimulation of blood-derived CD8+ T cells using EpiTCer® bead pulsed DC. HLA-A2+ healthy donor (HD) blood-derived CD14+ monocytes and CD8+ T cells were isolated; monocytes matured into imDC were loaded with indicated source of ANRU derived tumor antigens and further matured into DC. Long term co-cultures with DC and CD8+ T cells were performed and T cells harvested and re-stimulated with ANRU tumor cells. Tumor recognition was measured by CD107a expression and IFNγ production using flow cytometry. **(A–D)** HD CD8+ T cells were co-cultured with DC pulsed ANRU tumor lysate (TL) or EpiTCer beads #1 at indicated bead/DC ratio. Tumor specificity was measured by re-stimulation with ANRU tumor cells and measured by CD107a expression **(B)** or CD107a expression and IFNγ production **(A**, **C**, **D)**, using flow cytometry. Each donor represents one independent experiment.

Efficient Neoantigen Delivery Through Autologous DC Loaded With EpiTCer® Beads

Next, we investigated which method of tumor antigen loading confers the best capacity of DC to stimulate autologous tumor-specific CD8+ T cells. Healthy-donor-derived DCs were loaded either with several ratios of EpiTCer beads, with tumor lysate or as negative control DC without antigen source (MOCK). As a comparison, DCs pulsed with two validated ANRU neoantigen 9mer peptides, ETV6 and NUP210 (12), which were also included in the EpiTCer constructs, were used. After 14 days of stimulating CD8+ T cells with antigen-loaded DC, tumor reactivity was analyzed by re-stimulation of CD8+ T cells with ANRU tumor cells or with the ETV6/NUP210 9mer neoantigen peptides (only for T cell that had been co-cultured with DC pulsed with the corresponding 9mer peptide). Tumor reactivity was measured by CD107a expression using flow cytometry.

In both healthy donors, an EpiTCer bead/DC ratio dose-dependent increase in the frequency of CD107+ tumor reactive CD8+ T cells was observed (**Figures 3A, B**). In addition, the highest EpiTCer bead/DC ratio (40:1) generated and triggered an increased frequency of tumor-specific CD8+ T cells when compared to DC loaded with either neoantigen 9mer peptides or tumor lysate. However, DC loaded with either neoantigen 9mer peptides, ETV6 or NUP210, or tumor lysate induced increased frequencies of tumor reactive CD8+ T cells when compared to the unloaded DC, MOCK (**Figures 3A, B**). Notably, re-stimulation with ANRU tumor cells triggered a stronger T-cell activation than re-stimulation with the neoantigen 9mer peptide, ETV6, in CD8+ T cells previously enriched by DC loaded with the same peptide (**Figures 3A, B**). This indicates that loading of neoantigen peptides on MHC class I on CD8+ T cells can trigger activation alone but not as strong as when combined with other potential tumor antigens providing co-stimulatory signals expressed on the tumor cells.

Next, we assessed if loading the DC with pooled ETV6 and NUP210 peptides could increase the enrichment for tumor-specific T cells to a similar extent as the one observed with EpiTCer-beads-loaded DC. Notably, there was no additive effect on tumor recognition when stimulating CD8+ T cells with DC loaded with ETV6 combined with NUP210 (**Figures 3C, D**). In both healthy donors, EpiTCer-loaded DC induced an increased frequency of CD8+ tumor-specific T cells compared to ETV6 and NUP210 peptides delivered separately or in pools. Comparable results were observed in a third donor, showing no additional effect by pooling ETV6 and NUP210 (**Supplementary Figure S2A**). These results indicate that there is a dominant neoantigen peptide/epitope, "masking" the response towards additional neoantigens. Furthermore, the

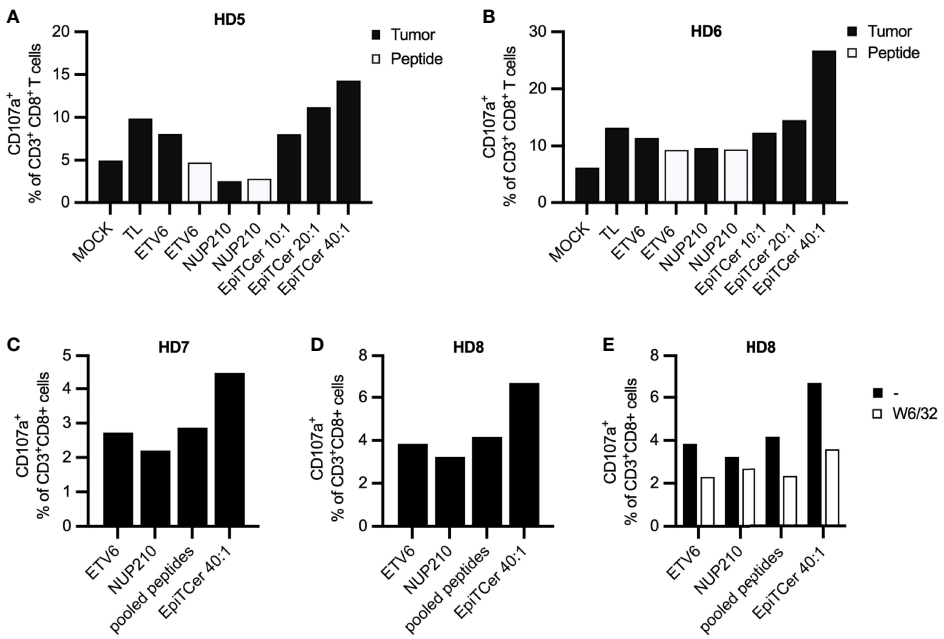

FIGURE 3 | Efficient neoantigen delivery through autologous DC loaded with EpiTCer® beads. HLA-A2+ healthy donor (HD) blood-derived CD14+ monocytes and CD8 + T cells were isolated; monocytes matured into imDC were loaded with indicated source of ANRU derived tumor antigens and further matured into DC. Long-term co-cultures with DC and CD8+ T cells were performed; T cells was harvested and re-stimulated with ANRU tumor cells or 9mer neoantigen peptides. T-cell activation was measured by CD107a expression using flow cytometry. **(A, B)** HD CD8+ T cells were co-cultured with DC pulsed ANRU tumor lysate (TL), indicated 9mer neoantigen peptide or EpiTCer beads #2 at indicated bead/DC ratio. Tumor specificity or T-cell activation was assessed by re-stimulation with ANRU tumor cells or indicated 9mer neoantigen peptide, respectively. **(C–E)** HD CD8+ T cells were co-cultured with DC pulsed with indicated 9mer neoantigen peptide (separately or combined/pooled) or EpiTCer beads #2 at indicated bead/DC ratio. Tumor specificity was assessed by re-stimulation with ANRU tumor cells with **(E)** or without **(C, D)** the presence of MHC class I blockade (W6/32). Values in panels **(D, E)** without W6/32 (–) were obtained within the same experiment. Each donor represents one independent experiment.

dominant neoantigen peptide differs between the healthy donors (HD) investigated, with ETV6 or NUP210 being the dominant epitope in HD 7 and 8 (**Figures 3C, D**) or HD 10 (**Supplementary Figure S1A**), respectively.

To assess MHC class I antigen-dependent tumor cell recognition, CD8+ T cells stimulated with DC pulsed with EpiTCer beads, ETV6 or NUP210 9mer peptides separately or pooled, were re-stimulated with ANRU tumor cells with and without MHC class I blocking antibody (W6/32). All CD8+ T-cell-mediated tumor recognition was MHC class I-antigen dependent, with the strongest influence of MHC class I antigen-dependent presentation observed in the DC pulsed with EpiTCer beads stimulated CD8+ T-cell population (**Figure 3E**).

Efficient Expansion of Patient Blood-Derived Tumor-Specific CD8+ T Cells Using DC Loaded With EpiTCer Beads®

To investigate the clinical relevance of DC loaded with EpiTCer beads, autologous ANRU DC, blood-derived CD8+ T cells, and ANRU tumor cell line were used. ANRU imDC were loaded with tumor lysate, ETV6 or NUP210 9mer peptides, or several ratios of EpiTCer beads. Non-coated EpiTCer beads (empty, E) and EpiTCer beads coated with the corresponding wild-type (WT) sequences were used as controls. Longtime co-cultures of DC-CD8+ T cells were performed, followed by re-stimulation with autologous ANRU tumor cells. Tumor recognition was measured by degranulation (CD107a expression) and IFNγ production analyzed by flow cytometry.

In three independent experiments, DC loaded with EpiTCer beads induced the highest frequency of CD107a+ autologous tumor-specific CD8+ T cells (**Figures 4A, C** and **Supplementary Figure S2B**). In addition, all EpiTCer bead/DC ratios induced a higher frequency of CD107a+ CD8+ T cells compared to more traditional ways to pulse DC with antigens, such as custom made 9mer neoantigen peptides, ETV6 and NUP210, or whole tumor lysate. Furthermore, CD8+ T cells stimulated with EpiTCer beads pulsed DC also displayed the most efficient cytokine production upon ANRU re-stimulation, although the difference to DC loaded with tumor lysate was less than for CD107a (**Figure 4B**). When comparing tumor recognition between all conditions, CD8+ T cells enriched by EpiTCer pulsed DC had a significantly increased frequency of tumor-specific T cells (**Figure 4C**). Notably, all DC conditions, including the control EpiTCer beads and non-coated and wild-type beads, were better at enriching for tumor-specific CD8+ T

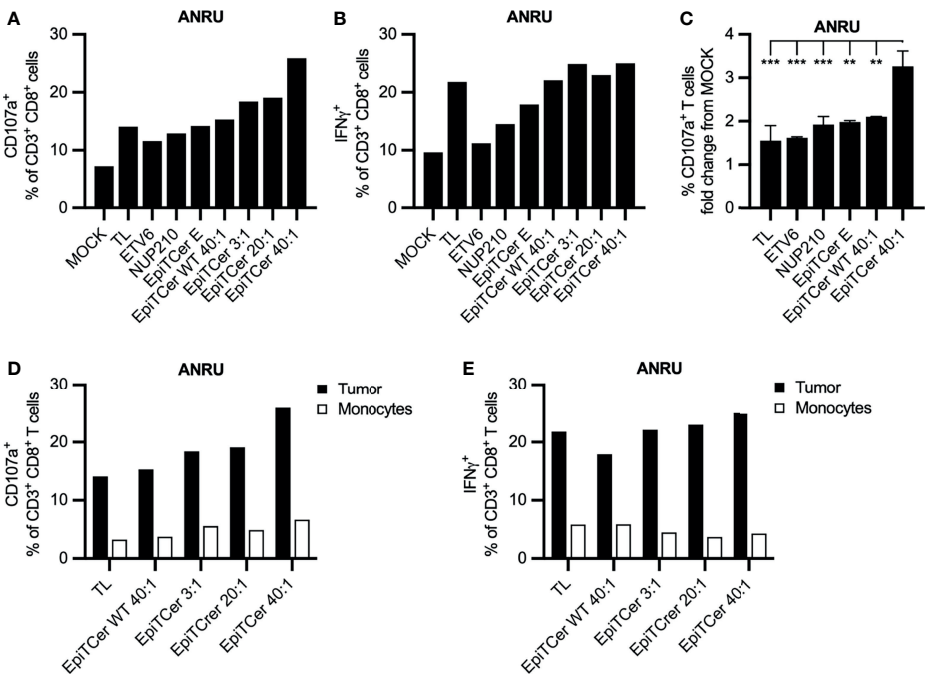

FIGURE 4 | Efficient expansion of patient blood-derived tumor-specific CD8+ T cells using DC loaded with EpiTCer beads®. ANRU blood-derived CD14+ monocytes and CD8+ T cells were isolated; monocytes matured into imDC were loaded with indicated source of ANRU derived tumor antigens and further matured into DC. Long-term co-cultures with DC and CD8+ T cells was performed; T cells were harvested and re-stimulated with ANRU tumor cells or ANRU non-activated monocytes. Tumor recognition or healthy cell reactivity was measured by CD107a expression or IFNγ production using flow cytometry. **(A, B)** ANRU CD8+ T cells were co-cultured with DC pulsed ANRU tumor lysate (TL), indicating 9mer neoantigen peptide, non-coated EpiTCer beads (empty, E), EpiTCer beads carrying the corresponding wild-type sequence (WT), or EpiTCer beads #2. EpiTCer beads were used at indicated bead/DC ratio. Tumor specificity was assessed by re-stimulation with ANRU tumor cells. **(C)** ANRU CD8+ T-cell tumor recognition, presenting values from **Figures 4A** and **5A** and **Supplementary Figure S2B**. **(D, E)** ANRU CD8+ T cells were co-cultured as described in panels **(A, B)**, and T-cell activation was measured by re-stimulation with ANRU tumor cells or non-activated ANRU monocytes. Values in panels **(A, D)** and **(B, D)** for ANRU tumor reactivity were obtained within the same experiment. **(C)** Statistical analysis one-way ANOVA, Tukey's multiple comparison test. Definition of significance: ***$p < 0.001$, **$p < 0.01$.

cells compared to unloaded DC (MOCK). This was the case independently of measuring degranulation or cytokine production (**Figures 4A, B**). This indicates that loading of DC with EpiTcer beads, independently of antigen coupled, was able to stimulate DC to become more efficient in activating blood-derived CD8+ T cells, although this activation was not MHC class I antigen dependent (data not shown).

Most of predicted neoantigens have a single amino acid mutation, while the rest of the sequence remains identical to the wild type. One can argue that the risk of expanding self-reactive T-cell populations may be higher when using longer neoantigen sequences, allowing alternative processing, compared to using pre-determined 9mer peptides. In addition, the EpiTCer beads are coupled to six 21mer long neoantigens interconnected with GGS linkers. Although the linker is designed to not to be similar to any sequence expressed in humans, they could potentially, due to alternative processing of the sequence by the DC, be at risk of stimulating self-reactive T cells. Therefore, the self-reactivity of the T-cell product was investigated by re-stimulating the autologous DC-activated CD8+ T-cell populations with autologous monocytes or CD8+ T cells as target cells.

None of the DC loaded with tumor lysate, EpiTCer beads or EpiTCer WT bead, stimulated CD8+ T cell populations displayed an efficient recognition of the autologous monocytes, measured by degranulation or IFNg production (**Figures 4D, E**). In addition, there was no recognition by the DC-stimulated CD8 + T cells when exposed to autologous non-stimulated CD8+ T cells (data not shown). This shows that DCs loaded with either tumor lysate or any of the EpiTCer bead concentrations do not react to the healthy cells investigated, indicating that the established stimulation method to enhance tumor-specific CD8 + T cells from the blood could potentially be used for adoptive cell therapy. However, further examination of its safety is needed. Comparable results were observed using healthy-donor-derived DC and CD8+ T cells (**Supplementary Figure S2C**).

EpiTCer-Loaded Autologous DCs Enrich for Tumor-Specific CD8+ T Cells Do Not Show Tumor Cross-Reactivity

To assess tumor specificity of DC-stimulated ANRU CD8+ T cell products, autologous ANRU DCs were loaded with tumor lysate, neoantigen 9mer peptides (ETV6 or NUP210) or EpiTCer beads. The DC-stimulated CD8+ T cells were (1) challenged with either autologous ANRU tumor cells or an allogenic melanoma cell line KADA, both HLA-A2+, or (2) further expanded using a GMP-compatible rapid expansion (REP) protocol (2). When directly re-stimulated with either the autologous ANRU or allogenic KADA tumor cells, the CD8+ T cell expanded with EpiTCer-beads-loaded DC displayed a very high tumor selectivity compared to all other stimulation conditions (**Figure 5A**). Notably, CD8+ T cells stimulated with DC MOCK or tumor lysate had an increased reactivity against the allogenic tumor cell line compared to the autologous cell line (**Figure 5A**). When re-stimulating the different REP-expanded CD8+ T cell populations with ANRU or KADA tumor cells, a preserved tumor selectivity was observed (**Figure 5B**). When comparing ANRU recognition with or without rapid expansion, we observed an increased tumor reactivity after REP (**Figure 5C**). These results indicate that tumor selectivity is preserved and enhanced during unspecific stimulation using anti-CD3 and radiated feeder cells.

EpiTCer Pulsed DCs Efficiently Induce Functional Maturation of Blood-Derived CD8+ T Cells

Phenotypic analysis of the long-term DC-stimulated autologous ANRU blood-derived CD8+ T cells was performed to analyze their expression of various maturation and memory markers. Non-DC stimulated ANRU CD8+ T cells and ANRU TIL, expanded using our clinical trial protocol, were used as controls. DC pulsed with EpiTCer beads or tumor lysate efficiently induced maturation of CD8+ T cells into central and effector memory T cells, with a phenotype similar to the one observed for the CD8+ T cells derived from the TIL. CD8+ T cells stimulated with DC loaded with empty EpiTCer beads or EpiTCer beads coated with wild-type sequences displayed a less mature phenotype (**Figure 6A**).

To further investigate the possibility of using DC loaded with EpiTCer beads for expansion of tumor-specific T cells as a cell product for adoptive cell therapy, large-scale expansion was explored.

Healthy donor-derived CD8+ T cell and EpiTCer-loaded DC were used to compare the tumor specificity/reactivity when CD8+ T cells were expanded in small or large scale, using plates or cell culture flasks, respectively. A similar expansion of tumor-specific T cells was observed independently of expansion setup, with a trend of an increased tumor recognition if CD8+ T cells were expanded in large scale using cell culture flasks (**Figure 6B**). Furthermore, blocking MHC class I-mediated recognition using monoclonal antibodies revealed that tumor recognition was antigen dependent for both cell products.

DISCUSSION

In this study, we have demonstrated that EpiTCer beads are an efficient method of loading *in silico* predicted and recombinantly expressed 21mer neoantigens onto autologous DC to enrich for and stimulate tumor-specific CD8+ T cells from peripheral lymphocytes. We observed that autologous DC pulsed with EpiTCer beads were more efficient/significantly better in stimulating both healthy-donor- and patient-derived tumor-specific CD8+ T cells from the blood compared to tumor antigens delivered *via* neoantigen 9mer peptides or tumor lysate (**Figures 1** and **4A**). Tumor specificity was assessed by recognition of the autologous ANRU tumor cell line, from which the neoantigens and the tumor lysate were predicted or generated, respectively. Furthermore, tumor recognition by healthy-donor-derived CD8+ T cells expanded by DC pulsed with tumor antigens, especially when delivered *via* EpiTCer beads, was reduced upon MHC class I blocking (**Figures 3E** and **6C**).

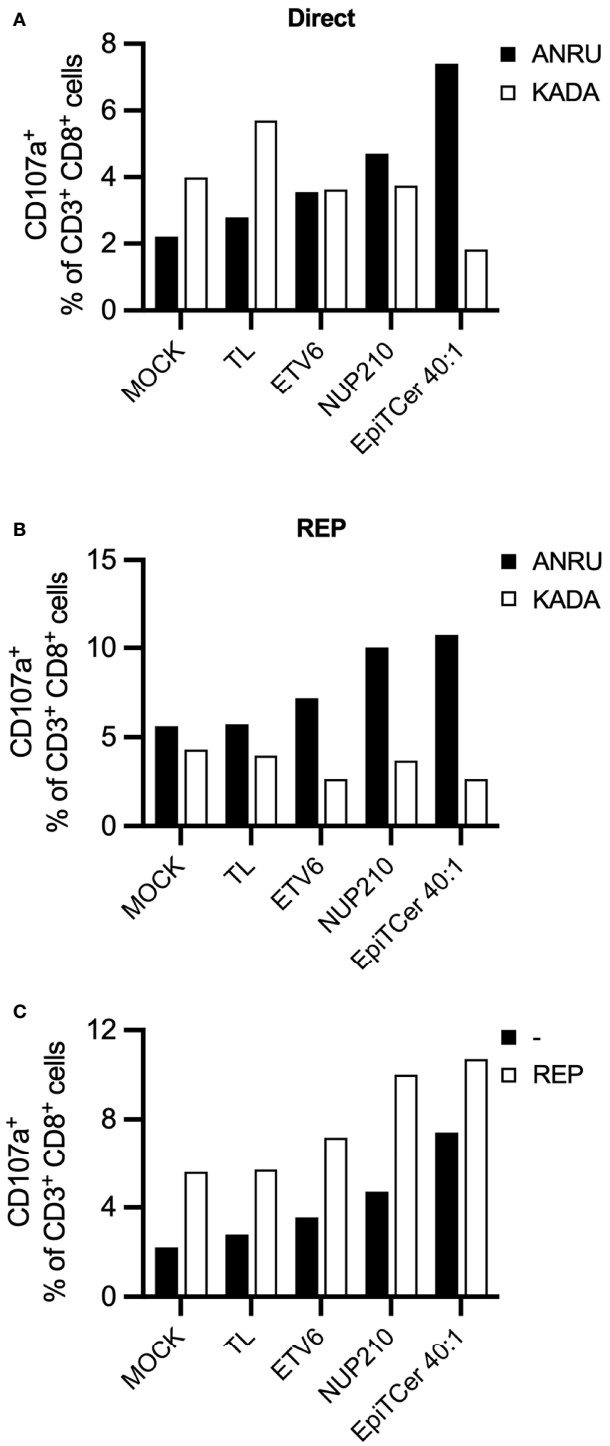

FIGURE 5 | EpiTCer-loaded autologous DC enrich for highly tumor-specific CD8+ T cells with limited tumor cross-reactivity. ANRU blood-derived CD14+ monocytes and CD8+ T cells were isolated; monocytes matured into imDC were loaded with indicated source of ANRU-derived tumor antigens and further matured into DC. Long-term co-cultures with DC and CD8+ T cells were performed: T cells harvested and **(A)** re-stimulated with indicated tumor cell line **(B)** further expanded *via* rapid expansion (REP). Tumor recognition was measured by CD107a expression. **(A)** ANRU CD8+ T cells were co-cultured with DC-pulsed ANRU tumor lysate (TL), indicating 9mer neoantigen peptide or EpiTCer beads #2 at indicated bead/DC ratio. Tumor specificity was assessed by re-stimulation with autologous ANRU tumor cells or allogenic KADA (HLA-A2+ melanoma cell line). **(B)** DC-enriched ANRU CD8+ T cells were further expanded using a REP protocol, harvested and re-stimulated with autologous ANRU tumor cells or allogenic KADA tumor cells. Panel **(C)** shows the CD8+ mediated ANRU tumor recognition observed before (–) and after REP (REP); same values as in panels **(A, B)** are displayed.

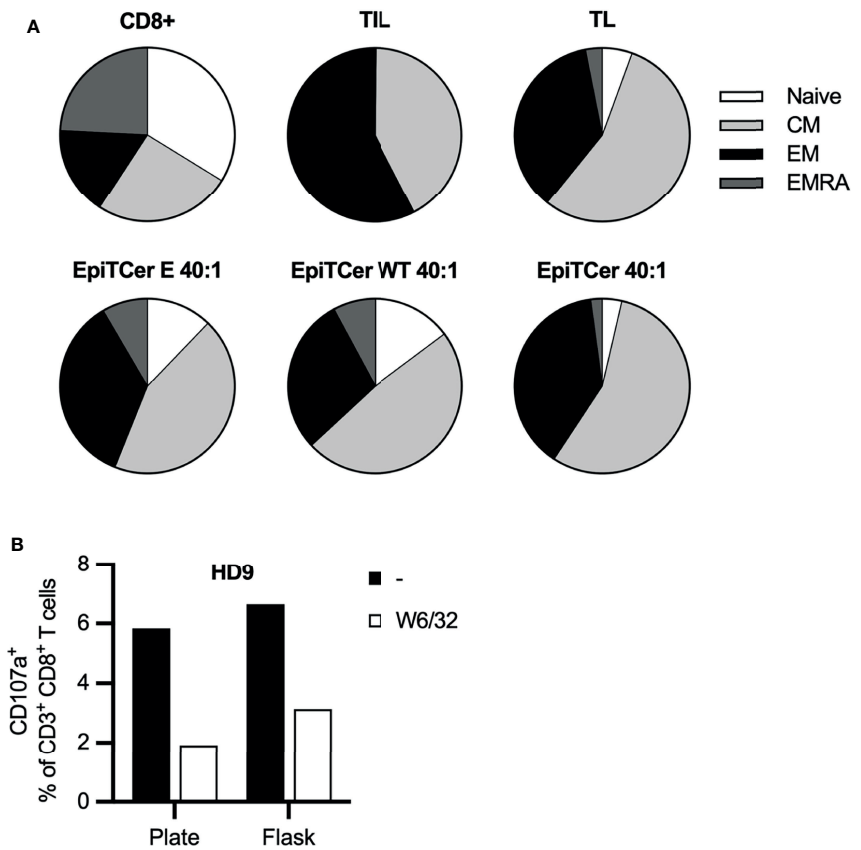

FIGURE 6 | EpiTCer-pulsed DC efficiently induces functional maturation of blood-derived CD8+ T cells. ANRU **(A)** or healthy donor **(B)** blood-derived CD14+ monocytes and CD8+ T cells were isolated, and imDC were loaded with indicated source of ANRU-derived tumor antigens and matured into DC. Long-term co-culture with DC and CD8+ T cells was performed. T cells were harvested and **(A)** phenotypic analysis was performed or **(B)** ANRU tumor reactivity was measured. **(A)** Long-term co-culture with ANRU CD8+ T cells and ANRU DC tumor lysate (TL), non-coated EpiTCer beads (empty, E), EpiTCer wide-type beads (WT) or EpiTCer beads at 40:1 bead/DC ratio was performed. CD8+ T cells were harvested, and phenotypic analysis of maturation status was performed using flow cytometry. Cells were gated on lymphocytes/single cells/live cells/CD3+CD8+ cells, and maturation was investigated *via* CCR7 and CD45RA. For gating strategy, see **Supplementary Figure 3**. **(B)** HD CD8+ T cells were long-term co-cultured with DC loaded with ANRU tumor lysate. Co-culture was performed in plate (96w plates) or in cell culture flasks; CD8+ T cells were harvested and re-stimulated with ANRU tumor cells with and without MHC class I blocking (W6/32). HD, each donor represents one independent experiment.

Numerous methods have previously been used to verify and enrich for tumor and/or neoantigen-specific T cells. In addition, several methods of analyzing, detecting, and defining recognition of tumor and/or neoantigen-specific T cells have been employed. This includes neoantigen-specific tetramers (32, 33) or selection based on activation/antigen-experienced markers, such as PD1+ or 4-1BB+ (13, 17, 34–36). In addition, peripheral lymphocytes recognizing oncogenes, p53 and KRAS, derived neoantigens have been investigated (15, 37, 38). Furthermore, healthy-donor-derived peripheral lymphocytes have been investigated as a source of neoantigens-specific T cells to be used for therapy (39, 40).

We and others have previously shown that it is possible to predict and identify neoantigens that can be used to generate autologous tumor and neoantigen-specific T cells from patient-derived tumor TIL and/or peripheral lymphocytes (12, 17, 32, 34–36). Several other studies have shown that it is possible to identify neoantigens to generate neoantigen-specific T cells within TIL or peripheral lymphocytes populations (13, 15, 33, 37–40). In these studies, neoantigen specificity was assessed by measuring the T cells capability to recognize or become activated upon re-stimulation with the corresponding neoantigen-loaded DC, with neoantigen pulsed/transduced tumor cells or with an allogeneic tumor cell line containing the specific mutation. We have previously shown that recognition of neoantigen-derived peptides does not necessarily mean efficient recognition of autologous tumor cells (12), displaying the importance of assessing recognition of the autologous tumor cells. In the Tumor Neoantigen Selection Alliance (TESLA) consortium, it was found that only 6% of the neoantigen peptides, top ranked by each participants algorithm, were able to bind to the patient's HLA alleles and form a multimeric complex. To our knowledge, no efforts were done to analyze if the T cells recognizing these

peptides were also able to kill the autologous tumors from which they were derived (41, 42).

We observed a relatively high tumor reactivity by the CD8+ T cells enriched by non-coated and wild-type EpiTCer beads (**Figure 4**). This could be explained by the size, ~1 μm, of the EpiTCer beads themselves, which was chosen based on previous studies displaying increased capacity to stimulate antigen processing and presentation (24, 29–31). Non-coated EpiTCer and wild-type beads generated very similar levels of tumor-reactive T cells, indicating that the wild-type sequence or the linker regions did not further enhance the stimulatory capacity.

Furthermore, peripheral tumor-specific ANRU CD8+ T cells enriched using autologous DC loaded with EpiTCer beads, ANRU tumor lysate, or EpiTCer wild-type beads, displayed limited recognition of autologous healthy tissue/cells, monocytes, and unstimulated CD8+ T cells. These results indicate that alternative processing of either the neoantigen sequences or the linker regions does not produce peptides that stimulate autoreactive T cells. The implications from these findings are however limited, and to further exclude the risk of autoreactivity of the T cells, a more extensive screening of a panel of normal tissues will have to be performed. Unfortunately, there were too few CD8+ T cells enriched *via* non-coated EpiTCer-pulsed DC to analyze healthy tissue cross-reactivity.

In line with previously published reports (13, 32), an efficient distinction between autologous, ANRU, and allogeneic KADA tumor cells was observed in all DC-enriched conditions (**Figure 5**). Furthermore, the tumor specificity was maintained during rapid expansion, with an increased autologous tumor recognition (**Figures 5B, C**).

It has previously been demonstrated that naive CD8+ T cells within peripheral lymphocyte populations can have reduced effector functions, low proliferation, and low TCR avidity, and are unlikely to engage in TCR–antigen interactions when compared to memory T cells, T_{CM} and T_{EM} (43, 44). Central memory T cells (T_{CM}) have been shown to possess a greater capacity to persist *in vivo*, while effector memory T cells (T_{EM}) have immediate effector functions although with lower proliferative capability (45, 46). We observed that DC loaded with EpiTCer beads were more efficiently matured CD8+ peripheral lymphocytes into central and effector memory T cells, when compared to DC loaded with non-coated EpiTCer or wild-type beads (**Figure 6A**). These results are consistent with the tumor recognition analysis and with the evidence that EpiTCer-loaded beads are efficient in expanding a T-cell population with high capacity of tumor recognition and elimination, as measured by CD107a expression.

The data presented in this study strongly support the potential of using EpiTCer-beads-loaded DC as a method for enriching for patient-derived tumor-specific T cells from peripheral blood and/or as a DC vaccination approach. Neoantigen-loaded therapeutic DC vaccines have been shown to enrich for pre-existing neoantigen-specific T cells *in vivo* in patients with cutaneous melanoma or advanced lung cancer (9, 47). It has also been shown that peptide/mRNA-based neoantigen vaccines can stimulate neoantigen-specific T-cell populations *in vivo*, leading to improved clinical outcome in melanoma patients (48, 49). However, there are also clinical trials showing a limited or no beneficial effect of neoantigen-based vaccines as monotherapy or in combination with checkpoint inhibitors (50–54). These reports indicate that cancer/neoantigen vaccines cannot completely eradicate the disease (55). Most likely, cancer vaccines will have to be combined with approaches targeting the immune suppressive microenvironment to eliminate MDSC and T regs and other suppressive mechanisms, thereby allowing the effector functions of tumor-specific CD8+ T cells. It is likely that DC, when optimally activated with methods such as loading them with beads, may be able to activate both tumor-specific T cells and non-specific immune mechanisms.

These questions have been addressed in mouse models with conflicting results. Salvatori et al. investigated different therapeutic combinations of checkpoint inhibitors (ICI), α-CTLA4 or α-PD1, and neoantigen-based cancer vaccines (56). They found that the combination of α-CTLA4 and a neoantigen vaccine had a large impact on tumor growth, while monotherapy using α-CTLA4 had no effect on tumor growth using a CT26 tumor model. In addition, the combination of α-CTLA4 and a neoantigen vaccine significantly reduced MC38 tumor growth, while the addition of α-PD1 had no effect on tumor growth. However, Li et al. investigated neoantigen-specific T cells in combination with α-CTLA4 or α-PD1 in Lewis lung carcinoma and observed an expansion of neoantigen-specific CD8+ TIL after ICI therapy but no effect on tumor regression (57). When combining a neoantigen vaccine with α-CTLA4 and α-PD1 therapies, a specific expansion of neoantigen-specific CD8+ TIL was detected but no effect on tumor growth. These results indicated the complexity of designing an efficient treatment protocol and how it should be evaluated. The presented study is built on neoantigens predicted from patient material. We have investigated the option of performing *in vivo* elimination assays based on inoculation of immune compromised NSG mice. These experiments were done with ANRU tumor cells to assess tumor elimination/regression after injection of autologous CD8+ T cells stimulated with DC loaded with the different antigen sources. Unfortunately, ANRU tumor was found to grow poorly in NSG mice, and *in vivo* elimination assays could not therefore be performed.

To date, the neoantigen prediction methods have mainly been focusing on MHC class I-binding peptides, and therefore, CD8+ T-cell responses and the usage of neoantigen-specific T cells for ACT has been skewed towards investigating the effect mediated by CD8+ T cells. However, longer, 15-30mer, neoantigen peptides incorporating a 9–11mer CD8+ T-cell neoantigen peptide can also serve as a neoantigen presented on MHC class II for CD4+ T cells. This has been demonstrated in clinical trials exploring neoantigen vaccination where a clear CD4+ T-cell dominated response has been observed (38, 49). In line with other studies targeting neoantigens to produce a T-cell product for ACT, we focused on the CD8+ T-cell mediated antitumoral

response. However, Arbelaez et al. showed that vaccination utilizing nanoparticle-delivered neoantigen peptides stimulated both a CD4+ and CD8+ T-cell-mediated response with a CD8+ T-cell-dependent antitumoral response (58). In contrast, vaccination with naked peptides only triggered a CD4+ T-cell response without any antitumoral effect (59). These results encourage further investigations asking if EpiTCer-bead-delivered neoantigens can trigger a stronger antitumoral effect if a combined CD4+ and CD8+ T cell response is initiated. Furthermore, Wei et al. have shown that formation of a neoantigen cancer vaccine consisting of polymerized synthetic peptides through a reversible polycondensation reaction resulted in an improved antigen delivery to lymph nodes (LN) and facilitated efficient activation of antigen-presenting cells compared to a naked peptide vaccine (58). The advanced formula for rapid release of neoantigen peptides in response to intracellular reduction activity upon internalization facilitated the delivery of peptide antigens and enabled cross-presentation by APC, triggering an increased CD8+ T-cell-mediated response and CD8+ T-cell maturation. In the present study, six different 21mer neoantigen polypeptides, each comprising 6 neoepitopes, were coupled separately to paramagnetic beads. The covalent linking of the designed neoantigen constructs offers several benefits. Directional coupling *via* the polylysine domain is a feature for direct conjugation, and the eight-histidine tag is used for purification and coupling quantification. In addition, the EpiTCer bead size induces activation and natural antigen presentation when phagocytosed by APC. Yet, the coupling method is not hampered by inaccurate disulfide bonds. Taken together, the covalent linking allows efficient coupling of the diverse repertoires of personalized neoantigens. In accordance with Wei et al., an efficient CD8+ T-cell maturation and antitumoral response was observed when delivering neoantigen peptides *via* EpiTCer beads.

To our knowledge, we have shown here for the first time an efficient enrichment of tumor-specific CD8+ peripheral lymphocytes using DC loaded with personalized neoantigens delivered *via* paramagnetic beads. We suggest that a novel therapy using *in vitro* expanded tumor-specific peripheral blood-derived T cells using EpiTCer-pulsed DC combined with a DC vaccine could be a possible option for patients with inoperable tumors and could be considered for patients with tumor types that do not allow efficient TIL production.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Local Ethics Committee Stockholm, Sweden (no. 2015/18-62-32). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

AK, KR, SR, and SLW. carried out the experiments and data analysis. SLW designed the experiments. ON performed neoantigen predictions, and LN produced the neoantigen proteins and coupling to EpiTCer beads. SLW wrote the manuscript with input from all the co-authors. HG and RK helped supervise the project. HG, RK, and SLW conceived the original idea. SLW supervised the project. All authors contributed to the article and approved the submitted version.

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REVIEW
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CRISPR Gene Editing of Human Primary NK and T Cells for Cancer Immunotherapy

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Antitumor activity of immune cells such as T cells and NK cells has made them auspicious therapeutic regimens for adaptive cancer immunotherapy. Enhancing their cytotoxic effects against malignancies and overcoming their suppression in tumor microenvironment (TME) may improve their efficacy to treat cancers. Clustered, regularly interspaced short palindromic repeats (CRISPR) genome editing has become one of the most popular tools to enhance immune cell antitumor activity. In this review we highlight applications and practicability of CRISPR/Cas9 gene editing and engineering strategies for cancer immunotherapy. In addition, we have reviewed several approaches to study CRISPR off-target effects.

Keywords: NK cells, CRISPR, T cell, immunotherapy, off-target analysis, CRISPR screening, CAR-T cells, CAR-NK cell

INTRODUCTION

In recent years, adoptive T cell and NK cell therapies and immune checkpoint blockades have been successfully used in the clinic to improve immunotherapy for cancer. Immunotherapies with T and NK cells aim to overcome tumor-mediated immunosuppression and augment immunity against cancer (1–3). Adoptive T cell cancer immunotherapies comprehend tumor-infiltrating lymphocytes (TILs), transgenic T cell receptor (TCR)- T cell and chimeric antigen receptors (CAR)-T cell therapies (1). NK cell immunotherapies with cytokine stimulation, antibodies, and gene CAR-NK cells have been studied to overcome immunosuppression in cancers (2, 4). Although advancement in immunotherapy has been significant and durable, most cancer patients fail to respond to immunotherapy due to resistant tumor nature. Thus, we urgently need to find novel immunotherapies for cancer patients.

CRISPR/Cas9 gene-editing technology application has been widely studied and used in cancer immunotherapy research (5, 6). CRISPR method offers precise and powerful gene-editing efficiency in cancer and immunotherapy research. It has been used to identify essential genes as immune checkpoint targets, generate CAR-T and CAR-NK cells, construct TCR, understand signaling pathways, and screen for new druggable targets in immunotherapy (1, 7–10).

In this review, we describe the fundamentals of CRISPR gene editing in primary human T cells and NK cells. In addition, we highlight the applications of CRISPR/Cas9 technology in engineered T cells and NK cells and how it improves the immune cell function against cancers. Furthermore, several approaches to study off-target effects of CRISPR has been discussed.

CRISPR GENE EDITING

CRISPR are classes of repeated DNA sequences that act in coordination with CRISPR-associated (Cas) genes to devote bacterial and archaeal immunity against foreign raider phages and plasmid DNA (11). This system has been tested in several human cells including primary immune cells such as T-cells and NK cells. CRISPR consists of three elements: tracer-RNA, crispr-RNA (complementary to the target gene) and the Cas nuclease protein (12). Recognition of the target gene by guideRNA (Tracer-RNA + crispr-RNA) bound to Cas protein results in double stranded break (DSB) (5, 13, 14). DSBs can be repaired by one of the two highly conserved competing repair mechanisms, named as nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathways (15). NHEJ results in insertion/deletion (indel) of nucleotides at the Cas9 targeting site and causes a frame shift in coding region and introduces gene knock-out (15). On the other hand, HDR is essential for insertion of a transgene such as a DNA template encoding a CAR into the Cas9 targeting site through homology repairs when homologous arms for the flanking region of Cas9 targeting site are provided in the DNA template (5). The best approach to deliver CRISPR elements and the DNA template depends on the target tissue or cell, packaging capacity, immunotoxicity, tropism, and integration site (5). Viral delivery has been widely used for human cells. Some of them are non-integrative, like the adeno-associated viruses (AAV) and adenoviruses (AdV), while some are integrative, such as Retroviridae family (MLV; murine leukemia virus or HIV; human immunodeficiency virus) (16, 17). Stable expression of the CRISPR in human primary cells is challenging due to the activation of anti-viral activity of the cells especially in NK cells and expressing a big protein like Cas9 results in low efficiency (18, 19). Therefore, delivery of pre-transcribed gRNA and pre-translated Cas9 as Cas9/Ribonucleoprotein (Cas9/RNP) has been favorable in immune cells (20, 21). Generation CAR expressing immune cells by site-directed gene insertion has been shown to be successful in both NK and T-cells. In this approach the DNA encoding a CAR is delivered as an HDR template by AAV vectors following electroporation of Cas9/RNP (22, 23). Providing optimal homology arms for Cas9-targeting site in the HDR template would be challenging as AAV has a small packaging capacity (less than 5 kb) (24). We have shown that a minimum of 300bp homology arms is required for high efficiency of the transgene integration into the Cas9 targeting site (23).

INTRODUCTION TO T CELLS AND THEIR ROLE IN CANCER IMMUNOTHERAPY

T cells are one of the most prominent components of the adaptive immune response. They can be distinguished from other lymphocytes by possessing TCR on their cell surface. T cells are developed in the thymus, and they recognize the antigen peptides presented by major histocompatibility complexes (MHC) class I and class II. T cells have two major CD8+ and CD4+ subtypes. CD8+ T cell refers to killer T cells, and CD4+ T cell refers to helper T cells. CD8+ killer T cells are involved in directly eradicating the virally infected cells as well as cancer cells. Even though T cells incredibly work and eliminate the most frustrating cancers, cancer remains one of the most devastating diseases globally and the leading cause of death. Conventional treatment options such as chemotherapy, radiotherapy and surgery have not been very effective in treating cancers. Recently, cell-based therapies, checkpoint blockades, cancer vaccines, oncolytic viruses and other forms of immunotherapies have shown promising clinical outcomes. T cell-based therapies are among the most efficient immunotherapies for cancer patients due to their eminent clinical efficacy (25). These new immunotherapies rely on the ability of T cells to eradicate tumors (26, 27). To enhance their antitumor activity and specificity, great interest in CAR- T cells has been evolved and have been used to treat hematologic malignancies and solid tumors. In autologous CAR-T cell-based therapies, the patient's own T cells are genetically engineered to express a single-chain CAR which includes an antibody extracellular binding domain that recognizes a tumor cell surface antigen. Tumor antigen is recognized by extracellular domain of the CAR. Signaling activation is achieved by both costimulatory molecule such as CD27, CD28, 41BB and CD3zeta which contains ITAM motives (28). Thus, the engineered CAR-T cells can bind to tumor antigens and lyse the tumor cells independently from MHC, whereas normal T cells require TCR binding to an MHC class peptide antigen for their activation (19). Although CAR-T cell immunotherapies have been shown to be the most promising FDA approved cell based treatments, several challenges remain to be tackled (29). There has been some severe adverse events associated with CAR T cell toxicities (30–37). For example, most of the clinical trials use autologous T cells isolated from patients' blood. This results in cell manufacturing failures from the early phase of the trial, due to low T cell quality and lymphocyte counts in some of the heavily treated patients (38). Manufacturing of autologous CAR T cell is a time-consuming process, therefore delaying the treatment in patients (33, 34). Additionally, when apheresis product is used for CAR-T cell production, sometimes failure in the process causes unsuccessful CAR-T cell manufacturing and poor response to treatment (30, 39–41). To overcome the problems related to autologous CAR-T cells, allogeneic CAR-T cell therapies has become alternative to autologous CAR-T cells (42–44). However, allogeneic CAR-T cell recognize and attack the recipient's tissues causing graft-versus-host disease (GvHD) therefore limiting their use in the clinic (45–48). In addition to that, in both autologous and allogeneic CAR-T cells, side effects such as cytokine release syndrome (CRS) and neurologic toxicity in patients remains a challenge to overcome (34–37, 49–55). Efforts in gene-editing technologies such as CRISPR gene editing aid as a potential tool for overcoming the barriers in CAR-T immunotherapies (**Figure 1**) (27, 38, 56–62).

EXAMPLES OF CRISPR EDITED T CELLS

Genome editing technologies facilitate remarkable, highly efficient, and specifically targeted genomic modifications. CRISPR/Cas9 technology has been the most practical and efficient gene-editing method among other strategies for editing the T cells (63–66). Producing off-the-shelf universal CAR- T cells, overcoming T cell exhaustion, and suppressive TME become significant obstacles which CRISPR can be a suitable tool to tackle those issues (**Figure 1**) (44, 63). Several groups have reported successful gene editing of T-cells using Cas9/RNP (66, 67). Electroporation of Cas9/RNP to edit T-cells has been very efficient and been successfully used in the clinic to treat cancers (68). To solve the limitations of antigen-specific and HLA-matched T cells and generate universal allogeneic CAR-T cells, genetically engineered TCR complexes were developed for immune therapy. Targeted gene editing in T cells has major advantages over lentiviral transduction platforms. For example, lentiviral transduction of TCR leads to variable transgene copy numbers and untargeted transgene integration and therefore initiates variable TCR expression and functionality. Oppositely, TCR editing with CRISPR/Cas9 allows high-efficient gene targeting and avoids random integration (63, 64). CRISPR/Cas9 strategy has also been used to target PD-1, CTLA-4, LAG-3, and TIM-3 inhibitory molecules to overcome tumor mediated immune suppression and enhance CAR-T cell function (22, 69, 70). It also has been shown that diacylglycerol kinase (DGK) CRISPR-Cas9 KO improves the anti-tumor activity of CAR-T cells (71). TGF-β receptor II (TGFBR2) KO with CRISPR/Cas9 was also shown to reduce CAR-T exhaustion and increase the anti-tumor activity of CAR-T cells (72). Inhibition of CD7 and TRAC using CRISPR/Cas9 enhances CAR-T cell-killing activity and prevents fratricide against T-ALL. Sterner et al. (73) showed that CRISPR/cas9 KO of granulocyte-macrophage colony-stimulating factor (GM-CSF) decreased the side effects like cytokine release syndrome and neuroinflammation of CAR-T cell therapies and also improved the CAR-T cell anti-tumor activity *in-vivo* (73). CRISPR/Cas9 gene not only used for KO, it has been also utilized for gene insertion of exogenous DNAs. Site directed gene knock-in (KI) has improved CAR-T cell antitumor efficiency (74, 75). Several approaches have been developed to deliver the DNA template encoding CARs. Schumann et al. introduced a HDR template into the CXCR4 gene locus by electroporation of a plasmid DNA and Cas9/RNP, and demonstrated successful site directed KI (75). Moreover, insertion of CD19 specific CAR expressing DNA into the TRAC locus has been achieved with the CRISPR/Cas9 method and improved CAR-T cell efficiency. To generate these cells, T cells were electroporated with Cas9 mRNA and gRNA. Next, the HDR template encoding CD19 CAR was delivered to the cells *via* AAV6 transduction (60). In the T cell engineering era, insertions or deletions of short sequences with CRISPR/Cas9 technology have been very effective, precise, and routinely used. However, it has also been possible to KI longer sequences using ssDNA inserts called the Easi-CRISPR method with high efficiency (74). Cas9 is the most used endonuclease protein in CRISPR systems, but other Cas proteins such as Cas12 or Cpf1 is also used to generated CAR-T cells when combined with AAV gene delivery (22). To generate CAR-T cells with simultaneous KO of checkpoints and knock-in of double CARs, a method called KIKO has been developed. This method uses AAV-Cpf1 to generate KO and double knock-in KIKO-CAR-T cells (22, 76).

INTRODUCTION TO NK CELLS

Natural Killer Cells (NK cells) are type of innate lymphocytes mediates anti-viral and anti-tumor activity. NK cells develop in

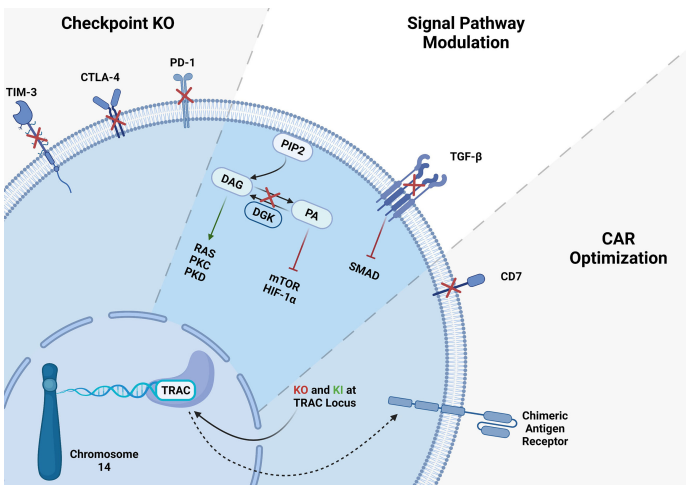

FIGURE 1 | CRISPR gene editing in T-cells. Several gene KO and KI have been tested in T-cells, here we summarized the targeted genes. T cell checkpoint inhibitory receptor KO such as TIM3, CTLA-4 and PD-1 KO resulted in higher antitumor activity of T-cells. CAR-T cell signaling modulation *via* inhibition of immunosuppressive TGF-β signaling showed significant improvement of CAR-T cells. Integration of CAR-T in TRAC locus may solve the mentioned problems with allogeneic CAR-T therapies.

the bone marrow (BM) and secondary lymphoid tissues such as, tonsils, spleen and lymph nodes (LNs) and they represent 5-20% of circulating lymphocytes in humans (77, 78). NK cells are distinguished from the other immune cells by possessing $CD3^-$ and $CD56^+$ phenotype. Human NK cell subsets express also CD16 molecule, which is involved in antibody dependent cellular cytotoxicity (ADCC). NK cells are effector cytotoxic cells, they recognize and destroy their target without prior sensitization. Unlike T cells, they do not need MHC class presentation to enact their cytotoxic properties. Unlike T cells, NK cells recognize and kill tumor in an HLA-independent manner which result in being known as a great candidate for allogeneic anti-tumor cell-based therapies, as they do not cause acute GvHD (79–81). NK cells use KIR receptor and ligand mismatch to recognize cancer cells from self-cells, therefore mediating enhanced engraftment, anti-tumor response, and safe clinical outcomes (79, 81–85). NK cell killing of target cells accomplished with a balance of activating and inhibitory signals engaged around the cell. NK cell activating receptors includes, killer cell's immunoglobulin-like receptors (KIRs), KIR2DS2, KIR2DS5 KIR3DS1, CD94/NKG2C, NKG2D, NKp30 NKp40, NKp44 and NKp46 recognize ligands present on target cells. NK cells have the ability of recognize non-self by NKp80, SLAM, CD18, CD2 and TLR3/9 receptors. Some of the NK cell inhibitory ligands are PD-1, TIGIT, TIM-3 and LAG-3. Inhibitory KIR ligands, KIR2DL1, 2DL2, and 2DL3 interact with highly polymorphic human leukocyte antigen (HLA). There are three HLA groups, group 1, 2 and HLA-Bw4, which usually bind inhibitory KIR and have long extracellular immunoglobulin structure. It has been shown that patients who receive NK cell immunotherapy containing haplo-mismatched NK cells they have anti-leukemic effects without the risk of GVHD. In hematopoietic stem cell transplant (HSCT) patients, infusions of haplo-mismatched KIR and HLA NK cells has shown benefits of survival and lower relapse rates. If the infused NK cells are identical, they only show benefit if the KIR receptors are activating (86, 87). NK cells can be isolated from peripheral blood, umbilical cord, and induced pluripotent stem cells (iPSCs) (88–91). Once isolated from their primary source, feeder cells, such membrane-bound IL-21 K562s, used to expand NK cells ex-vivo (92). They can be cultured anywhere from 14-21 days in most protocols and can proliferate remarkably over hundreds of folds (92). Cytokines such as IL-2, IL-12, IL-15, IL-18, and IL-21 are also added in NK cell cultures to enhance NK cell proliferation and activation (86, 87). NK cells have several mechanisms to eradicate their targets. One of the main mechanisms is perforin and granzyme induced apoptosis. Granzymes which are serine/proteases, packaged along with perforin and when they release by NK cells, they initiate target apoptosis *via* caspase-3 pathway. In addition to that, NK cells *via* Fas ligand and tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) pathways can destroy their targets (93–95).

EXAMPLES OF CRISPR EDITED NK CELLS

CRISPR editing of NK cells has been challenging, however we and others have shown that using electroporation of Cas9/RNP can solve the issue of low viral transduction efficiency of NK cells (18, 23, 96–103). Gene editing in NK cells in a short period since its invention has been used for serval applications such as to improve their metabolic function, knocking-out checkpoint molecules, improving antibody therapies and generation of CAR-NK (96). One great example of gene engineered NK cells is CD38 knock-out NK cells. NK cells highly express CD38 on their surface. Patients treated with daratumumab (Dara, hereafter), a monoclonal antibody targeting CD38 on multiple myeloma, showed a decrease in NK cells number. This is a result of NK-NK recognition through CD16 biding to Dara coated CD38+ NK cells, referred to as "fratricide." Beyond the role of the structural marker, CD38 is well described to be associated with a large diversity of physiological and pathological conditions. Our group and others successfully developed NK cells lacking CD38 by introducing the CRISPR/Cas9 as Cas9/RNP *via* electroporation (96, 101). In particular, CD38 is an NAD-degradation enzyme in mammalian tissues (104–110). Our data demonstrated that CD38KO NK cells have more prominent metabolic profile, increased killing mediated by ADCC against $CD38^+$ multiple myeloma cell lines and patient derived samples and are protected from fratricide mediated by daratumumab (96, 101).

Another important target to improve the NK cell's function is CISH encoded by CIS gene. CISH has a critical impact on NK cells, and its activation is known to disable JAK-STAT downstream signaling pathways including a decline in NK cell ability to kill malignant cells (111, 112). Different groups have shown that CISH is overexpressed in the presence of IL-2 and IL-15 (113–115). IL15 was previously described as an important factor potentiating NK cells cytokine production and cytotoxicity activity (116–118). Felices et al. have demonstrated that prolonged administration of IL15 can unleash NK cells exhaustion *via* metabolic failure (119). Delconte et al. showed that *CISH* was quickly activated after IL15 stimulation in a mouse model, supporting that using gene-editing in NK cells to delete CISH seems to be advantageous (120). Using CRISPR/Cas9 on human iPSC to generate iPSC-CISH knockout NK cells displayed prolonged persistence *in vivo* and enhanced antitumor activity for acute myeloid leukemia (121, 122). NK cell checkpoint blockade has been used as a promising therapy for liquid and solid tumors. Other candidate for gene editing in NK cells is NKG2A which is an immune checkpoint in CD8+ αβ T cells, natural killer T cells (NKT) and CD56hi NK cells. Upon activation of immune cells, NKG2A leads to decreased effector function (123, 124). Data from the literature have shown that NKG2A drives NK cells to fatigue when highly expressed, and it can be predictive of poor prognosis in liver cancer patients (125). Thus, the blockage of the NKG2A receptor enhances NK cell's effector function for immunotherapy (126–128). Similarly, Berrien-Elliot et al., have shown that gene-editing using CRISPR/CAS9 to delete NKG2A from human NK cells was able to increase NK cell ability to control $HLA\text{-}E^+$ K562 leukemia when compared to control NK cells demonstrating a substantial inhibitory function for NKG2A (129). Additionally, $NKG2A^{KO}$ NK cells did not affect their persistence in NSG mouse model

(129), however, the role of NKG2A in NK cells licensing may cause development of unlicensed NK cells with lower cytotoxic activity (130). It is very well established that the PD1/PD-L1 axis has an inhibitory function that can impair many T cells' functions. This fact has been validated in preclinical models where the inhibition of this signaling cascade is used for cancer treatment (131). Indeed, high expression of PD1 ligand I or II in cancer cell lines impairs cytotoxic function on CD8+ T cells. On the other hand, the absence of a functional PD1 was responsible for tumors rejection in the murine model (132, 133). The blockage of the PD1/PD-L1 axis with monoclonal antibodies repair these effects and unleash T cells to effectively kill tumor cells (132–134). Recently it has been shown that in different malignancies, human NK cells also express PD-1 (135–139). Like T cells, blockade of the PD1/PD-L1 axis was able to activate NK response (140). However, such strategies present limitations, especially regarding off-target toxicity (102). Pomeroy et al. could generate $PD1^{KO}$ NK cells by electroporating mRNA Cas9 and gRNA (102). They demonstrated that $PD1^{KO}$ NK cells showed notably enhanced cytotoxicity and cytokine secretion *in vitro* and *in vivo*, decreasing tumor burden that culminated with survival (102). Another promising target for gene editing to boost cancer immunotherapy is the Suppressor of cytokine signaling 3 (SOCS3). The protein SOCS3 is one among eight members of the Suppressor of cytokine signaling family (SOCS1–7 and CIS). Those proteins downregulate cytokine signaling *via* the JAK/STAT signaling cascade. Murine NK cells upregulated SOCS3 expression after IL-15 stimulation (120). SOCS3 impair inflammation by inhibiting pro-inflammatory signaling pathways, including IL-12 inducing IL-12Rβ2 subunit blockage *via* the SH2 domain and its signaling pathway mediated by STAT4 (122). The absence of SOCS3 does not impact NK cells function upon IL15 stimulation in murine models. In humans NK cells, our group successfully generated $SOCS3^{KO}$ NK cells using Cas9/RNP and showed higher cell proliferation and enhanced NK cells anti-tumor activity (100). Suggesting $SOCS3^{KO}$ NK cells could be an excellent target for gene-editing to boost cancer immunotherapy. Another novel target is ADAM17, this gene has well described as a membrane-associated protease responsible for cleaving a large variety of membrane molecules, including CD16 (102, 141–144). Blocking ADAM17 activity leads to improvement in cytokine production of human NK cells due to maintaining their CD16 on the cell surface and activating higher ADCC when combined with antibodies (145). Pomeroy et al. have demonstrated that CRISPR-edited $ADAM17^{KO}$ NK cells are prevented against CD16 shedding compared to WT NK cells (102). Additionally, those data are similar to ADAM17 inhibitors where treated groups presented enhanced killing through ADCC. Similarly, Yamamoto et al. showed that ADAM17 gene-edited iPSCs derived NK cells have enhanced ADCC (102, 141, 144–146).

To improve immune cell recognition and killing towards tumor cells, immune cells, including T cells and NK cells are engineered to express chimeric antigen receptors (CARs) (147–149). In one of the first clinical trials using iPSC CD19-CAR NK cells, the patients treated with the CAR-expressing NK cells showed some improvements in their clinical outcomes (150). Generation of CAR-NK cells have been challenging due to the low efficient viral transduction including CAR-NK cells used in the trial mentioned above. Our group recently showed that we could efficiently combine Cas9/RNP approach with self-complementary (sc) Adeno-associated virus (AAV) or single-stranded gene delivery for generating highly efficient human primary CAR-NK cells (98). Using this approach, we developed CD33 CAR-NK cells (98). These CAR-NK were efficiently able to kill AML cells and showed improvement on their activation markers (98). Similar data were obtained when CD33-CARNK cells co-culture with patient samples (97, 98). Recently, Daher et al. showed that CRISPR edited CIS-KO NK cells expressing CAR-IL-15 construct could boost CAR-NK cell function *in vitro* and xenograft models by increasing aerobic glycolysis (121). This double enhancement of CAR-IL-15/CIS-KO signaling is significantly beneficial in the TME (151). Overall, gene editing of NK cells has been challenging but the recent successes in using CRISRP by electroporating Cas9/RNP helped to improve the outcome of the NK cells therapy (**Figure 2**) (18, 101, 103, 152, 153). There has been some evidence showing that Polymer-stabilized Cas9 nanoparticles and modified repair templates can increase genome editing efficiency. These modified nanoparticles improved knock-out and knock-in efficiency of the CRISPR gene editing in several primary cells such as NK and T cells (16).

Clinical Trials Using CRISPR Edited NK Cells and T Cells

Advancements in immunotherapy and gene therapy opened a new era for clinical trials to treat some hematological malignancies and solid tumors. Along with other platforms, CRISPR/Cas9 technology was adapted and brought up to the clinic to correct some mutations and boost immune responses. CRISPR/Cas9, as a precise gene-editing tool with minimal cytotoxicity and off-target effects, has become a promising approach to treat complex and refractory diseases. However, due to some limitations, including transduction efficiency, off-target mutations, ethical questions, and the deficiency in scientific risk assessment, CRISPR/Cas9 gene-editing clinical trials have not been prevalent, especially for T and NK cells. However, CRISPR has opened its way to the clinic. One of the first in human phase 1 clinical trial of using CRISPR engineered T cell have been used for patients with refractory cancers in the U.S. (clinicaltrials.gov; trial NCT03399448) (68). In this trial, endogenous TCR and immune checkpoint molecule PD-1 were targeted in T cells with CRISPR/Cas9 to improve immunotherapy in several refractory cancers. Two patients with advanced refractory myeloma and one with metastatic sarcoma were treated with these CRISPR-edited cells (68). The results of this trial demonstrated the safety of infusing CRISPR-edited ex-vivo expanded CAR-T cells in patients (151). Examples of some clinical trials with the CRISPR/Cas9 method in T cells are presented in **Table 1**. However, there are no registered CRISPR/Cas9 transduced CAR-NK cell clinical trials in the United States.

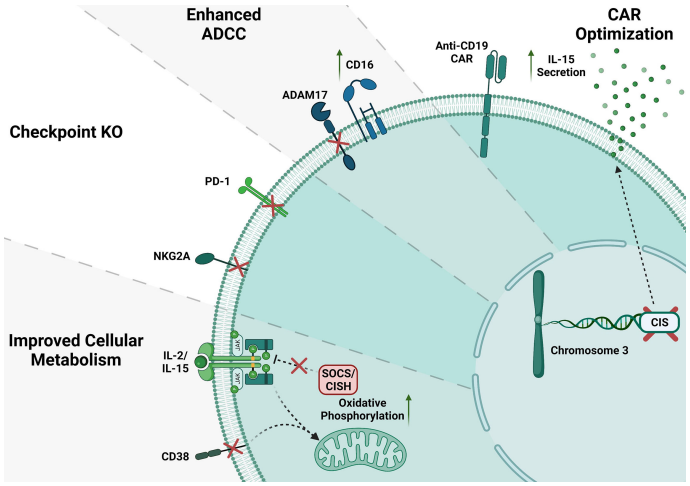

FIGURE 2 | CRISPR gene editing in NK cells. Several gene KO in NK cells have been done to improve NK cell function; here, we show some of the NK cell gene modifications. CD38 and SOCS/CISH KO can improve metabolism in NK cells. Inhibitory checkpoint receptor KO such as NKG2A and PD-1 KO. ADAM17 KO enhance CD16 mediated ADCC. Anti-CD19 CAR NK cells increase IL-15 production and enhance NK cell anti-tumor activity.

OFF-TARGET ANALYSIS OF CRISPR EDITED IMMUNE CELLS

Recently by the promises of Cas9 endonuclease, researchers can target multiple genes in immune cells, including T cells and Natural killer (NK), to improve cancer immunotherapy. For these applications that lead to clinical cancer immunotherapy, the induced mutations by CRISPR-Cas9 should be highly precise and specific for the targeted loci with high on-target efficiency and low or no off-target activity. However, rare off-target events are inescapable during the manipulation of the gene of interest. This phenomenon requires scrutiny identification, especially in clinical applications to cure cancers and avoid adverse effects during cancer immunotherapy such as introduction of an oncogene. By developing next-generation sequencing (NGS) a survey of new functional and non-functional variations during gene manipulation became possible (154, 155). NGS has been broadly applied by researchers and employed in clinical trials due to its development in data acquisition with speedy and high-quality recognition (156, 157). Analyzing these NGS-generated data is even more critical to optimize and manage the workflow to fill the gap between massive data and scientific exploration. To date, several methods have been invented to analyze NGS data and off-target effects of CRISPR mediated mutations, such as GUIDE-seq, SITE-Seq, CHANGE-seq, Cas-OFFinder and Churchill (158–162). Some of them like GUIDE-seq, SITE-Seq and CHANGE-seq are based on the PCR amplification of pre-selected potential sites, which predicted by CRISPR/Cas9 design tools, and sequencing the PCR amplicons utilizing Sanger or NGS technologies (158–160, 163). For instance, Schumann et al.,

TABLE 1 | Examples of clinical trials with CRISPR/Cas9 gene edited T cells (151).

National Clinical Trial Number	Cancer	CRISPR target gene	T cell source	Technique	Country
NCT04037566	Relapsed or refractory ALL and B-cell lymphoma	HPK1	Autologous T cells	Rnp Electroporation	China
NCT03399448	Multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma	TCRα, TCRβ and PD-1	Autologous T cells	Rnp Electroporation	USA
NCT03545815	Solid tumors	Endogenous TCR and PD-1	T cells (unknown source)	N/A	China
NCT04244656	Refractory multiple myeloma	B2M gene and TCR	Allogeneic T cells	N/A	USA and Australia
NCT03747965	Solid tumors	PD-1	T cells (unknown source)	N/A	China
NCT04035434	B-cell malignancies	B2M gene and TCR	Allogeneic T cells	N/A	USA and Australia
NCT03166878	B-cell leukemia and lymphoma	B2M gene and TCR	Allogeneic T cells	Rnp Electroporation	China
NCT03044743	EBV related diseases	PD-1	EBV CTL from autologous source	N/A	China

N/A stands for non-applicable.

used a 2-step PCR method and sequenced with the amplicons with Illumina HiSeq, and identified indel mutations and their spatiality distribution in the target region in primary human T cells (75). In another study the efficiency and indel rates in the created CAR-T cells, using CRISPR-Cas9-mediated multiplex gene editing, was quantified by both surveyor assay and tracking of indels by decomposition (TIDE) analysis (58). Stadtmauer et al. utilized iGUIDE, a modified method of GUIDE-seq, for the Cas9-mediated cleavage specificity analysis in the engineered T cells to cure refractory cancer and found no clinical toxicities (68, 158, 164). Although these methods are simple and available to most molecular biology laboratories, they are not always precise as they are based on the predictions of potential off-target sites by CRISPR/Cas9 design tools in the genome of interest and therefore result in studying limited loci. As a matter of fact, DSBs happened beyond the predicted sites and may be ignored and caused detrimental side effects during the process of clinical cancer immunotherapy (163). This major disadvantage of off-target mutations identification by PCR based methods have been resolved by whole genome sequencing (WGS) which is unbiased and has been used to screen for off-target mutations induced by CRISPR/Cas9 in different cells including human inducible pluripotent stem cells, primary T cells, CAR-T cells (163, 165–167). Using this method, researchers can recognize both small indels and SNPs as well as major deletions, inversions, duplications and, rearrangements (163, 166). The only restriction of whole genome sequencing is missing the most low-frequent off-targets that happens to a small number of clones (163, 168). Cas-OFFinder algorithm have been invented in order to search for potential off-target sites in any sequenced genome regions (161). In a clinical trial, the safety and feasibility of CRISPR–Cas9 PD-1-edited T cells were confirmed after analyzing all the potential off-targets using Cas-OFFinder method in the treatment of lung cancer (169). More recently, as an ultra-fast, definite, highly scalable, and balanced parallelization strategy for discovering human genetic variation in clinical and population-scale genomics, Churchill has been applied for the analysis of next-generation sequencing data (162). We reported the high efficacy of Churchill analysis in verifying off-target events after deletion of CD38 in NK cells *via* Cas9/RNP and showed low off-target effects of Cas9/RNP (96). It has successfully revealed all the existing mutations and categorized them as missense and non-frameshift and moderate or high impact (96). Overall, WGS can provide more precise landscape of the off-target effects in CRISPR-edited cells. Here, we summarize and compare the current methods in off-target effects analyses of CRIPR edited immune cells (**Table 2**).

CRISPR SCREENING IN PRIMARY IMMUNE CELLS

Genome wide CRISPR screen has been used in several cancer cells to discover novel targets for cancer immunotherapy. CRISPR screening approach has not been extensively used in human primary immune cells due to several technical challenges. However, some studies have shown successful screening approaches in human primary T cells and Cas9-expressing transgenic mice in recent years (19, 170–172).. In general, to perform a CRISPR screen we need to introduce Cas9 and gRNA pool library into the cells (173). These molecules usually delivered to the target cells *via* lentiviral transduction. However, expressing large proteins such as cas9 using LV vectors in immune cells such as NK cells and T-cells has been challenging and results in low transduction efficiency. Shifrut

TABLE 2 | Current methods in off-target analyses of CRISPR edited immune cells.

Off target analysis method	Definition	Pros	Cons
Cas-OFFinder (161)	It is an algorithm that searches for possible off-target sites that can be found in an already sequenced genome.	- It is not limited by the number of mismatches and the PAM sequence. - It allows alterations in PAM sequences which are differentiable with Cas9. - a rapid and highly assorted off-target searching tool available at http://www.rgenome.net/cas-offinder	- it relies on a computational method, which may result in ignoring some potential off-targets sites. - it is biased due to the assumption that off-target sequences are affiliated with the on-target site which may cause missing off-target sites in any loci throughout the genome.
SITE-Seq (selective enrichment and identification of tagged genomic DNA ends by sequencing) (159)	It is a biochemical method, using Cas9 and single-guide RNAs (sgRNAs), to recognize all the Cas9-mediated cut site sequences inside the genomic DNA.	- It allows retrieval of off-target sites with different cleavage sensitivity by utilizing a vast range of sgRNP concentrations from very low to high. - Provides guidance for precise and plenary inspection of possible off-target sites in cells by gaging the incidence of mutations and their functional cellular effects. - Production of sequencing libraries which are highly enriched for	- DNA-repair machinery does not have a role in the process as it is performed on high molecular weight DNA.

(Continued)

TABLE 2 | Continued

Off target analysis method	Definition	Pros	Cons
		sgRNP cut sites, providing unique profiling with minimal read depth.	
GUIDE-seq (genome-wide, unbiased identification of DSBs enabled by sequencing) (158)	It is a PCR-based method that relies on the enteral of double-stranded oligodeoxynucleotides into the DSB caused by RNA-guided nucleases (RGN) without contributing to off-target site.	- Enables to turn out universal specificity perspective for different RGNs - Identifies the hotspots in DNA breakpoints that can take part together with RGN-induced DSBs in higher-level genomic alterations such as translocations. - Its performance on living cells enables capturing of DSBs that occur over a more extended period, thereby making it a more delicate and plenary assay.	-Relies on an integration of donor sequences, which usually happens in a low frequency. - mispriming may occur due to the annealing of PCR primers to DNA sequences apart from the ODN, resulting in PCR products that are not differentiable from products formed by primers binding to the ODN.
iGUIDE (improvement of the GUIDE-seq method) (164)	GUIDE-seq method allows mis priming artifacts to be recognizable from credible ODN integration sites by using a larger ODN (46 nt versus 34 nt).	- by using larger ODN, PCR primer binding sites can be back off from the junction of the ODN in the final PCR product and can cause mis priming events.	-It is tough to scale due to individual transfections for each target or cell source.
ChIP-seq (chromatin immunoprecipitation sequencing) (158)	It identifies the off-target binding sites by using catalytically dead Cas9 (dCas9)-gRNAs complex.	- Important for the identification of the genome-wide binding sites with dCas9 fusion proteins.	-It rarely indicates the off-target sites of cleavage caused by active Cas9 nuclease. -not effective for recognition of genome-wide, off-target cleavage sites for catalytically active RGNs.
CHANGE-seq (circularization for high-throughput analysis of nuclease genome-wide effects by sequencing) (160)	It is a high-throughput procedure for determining the genome-wide operations of CRISPR-Cas9 nucleases based on Tn5 mediated gDNA tagmentation *in vitro*.	- A simplified, susceptible, and scalable approach. - It can elucidate the genome-wide perspective of genome editing activity exquisitely sensitive. - elaborated to efficiently procreate circularized genomic DNA libraries for elucidating the genome-wide activity of genome editors by leveraging a new Tn5 tag mentation-based workflow.	-cost and availability -it relies on the Tn5 tagmentation of donor sequences. - Similar to SITE-Seq, the DNA repair machinery is ignored.
Churchill (162)	In clinical and population-scale genomics provides fast, decisive, scalable, and balanced parallelization tactic for the detection of human genetic mutation.	- It uses a robust comparison based on whole genome sequencing data comparing wildtype and CRISPR edited cells. - The procedure is highly scalable, authorizing full resolution of the 1000 Genomes raw sequence dataset utilizing cloud resources in a week. - It eliminates the bottlenecks of the computational sequence analysis impasse *via* the avail of cloud computing resources. - It matches with the amplitude of genomic data.	- Limited access to the platform and the algorithm is not publicly available yet.

et al; tested a hybrid approaching which the Cas9 was introduced to the gRNA library expressing cells *via* electroporation (19). They developed Single guide RNA (sgRNA) lentiviral infection with Cas9 protein electroporation (SLICE) and resulted in discovery of novel genes important in activation and expansion of CD8 T-cells (19). A similar approach was used by other groups to perform CRISPR screening in CAR-T cells (174). To date, there is no publication on CRISPR-screening on NK cells. Our

group is investigating some new approaches to overcome issues related to lentiviral transduction of NK cells.

CONCLUSION

CRISPR gene editing technology has shown to be a very versatile tool for improving anti-tumor activity of NK cells and T-cells. We reviewed here some of the CRISPR edited cells used for cancer immunotherapy. We also reviewed ways to determine the off-target effects of CRISPR and emphasized that Cas9/RNP approach results in low off-target effects. We also mentioned how important information can be discovered by CRISPR screening approach and there are a lot to do the efficiently optimize this method to be used in NK cells and T cells. Overall, CRISPR gene editing shows promising clinical outcome and have potentials to be used more broad Clinical applications such as cancer immunotherapy using NK cells and T cells.

AUTHOR CONTRIBUTIONS

The corresponding authors MNK and DAL supervised the authors for manuscript completion. The first author EE contributed to manuscript writing and revising. All authors contributed to the article and approved the submitted version.

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Expression of CD274 mRNA Measured by qRT-PCR Correlates With PD-L1 Immunohistochemistry in Gastric and Urothelial Carcinoma

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Programmed death-ligand 1 (PD-L1) immunohistochemistry (IHC) is widely used to predict the clinical responses to immune checkpoint inhibitors (ICIs). However, PD-L1 IHC suffers from the complexity of multiple testing platforms and different cutoff values caused by the current one drug-one diagnostic test co-development approach for ICIs. We aimed to test whether PD-L1 (CD274) mRNA expression levels measured using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) can represent PD-L1 IHC and predict responses to ICI. The FDA-approved PD-L1 IHC results with 22C3 pharmDx (gastric cancer) and SP142 (urothelial carcinoma) were compared with *CD274* mRNA expression levels via qRT-PCR using the same formalin-fixed, paraffin-embedded tissue blocks from 59 gastric cancer and 41 urothelial carcinoma samples. *CD274* mRNA expression was identified using three independent sets of primers and TaqMan® probes targeting exon 1–2, exon 3–4, and exon 5–6. *CD274* mRNA levels in spanning exon 1–2, exon 3–4, and exon 5–6 junctions of *CD274* correlated well with PD-L1 expression (r^2=0.81, 0.65, and 0.59, respectively). The area under the curve of exon 1–2 was the highest (0.783), followed by exon 3–4 (0.701), and exon 5–6 (0.671) of the *CD274* gene against the PD-L1 combined positive score cutoff of 10. When *CD274* mRNA expression was matched for response to immunotherapy, the overall response rate was higher in patients with high *CD274* mRNA levels with a cutoff of 0.0722 (gastric cancer) and 0.0480 (urothelial carcinoma) than in those with low *CD274* mRNA expression ($P < 0.001$ and P = 0.018, respectively). These results show that *CD274* mRNA levels predicted ICI responses in patients with gastric or urothelial carcinomas and could be used as alternatives for PD-L1 IHC.

Keywords: mRNA expression, immunotherapy, gastric, urothelial, CD274, carcinoma

INTRODUCTION

In 2017, the Food and Drug Administration (FDA) granted accelerated approval to pembrolizumab for patients with recurrent locally advanced or metastatic, gastric or gastroesophageal junction adenocarcinoma whose tumors express programmed death-ligand 1 (PD-L1) as determined by an FDA-approved test based on the clinical results of KEYNOTE 059 (NCT02335411) (1). In advanced gastric or gastroesophageal junction adenocarcinoma, PD-L1 expression is assessed using the FDA-approved PD-L1 IHC 22C3 pharmDx assay and a combined positive score (CPS) (2). In 2016, FDA gave accelerated approval to atezolizumab injection (Tecentriq) for the treatment of patients with locally advanced or metastatic urothelial carcinoma who have disease progression during or following platinum-containing chemotherapy or have disease progression within 12 months of neoadjuvant or adjuvant treatment with platinum-containing chemotherapy. FDA approved Ventana PD-L1 (SP142) assay to measure PD-L1 expression in urothelial carcinoma. With FDA approvals, PD-L1 immunohistochemistry (IHC) is popular for predicting therapeutic responses to immune checkpoint blockade (ICB) (3). While this method measures PD-L1 protein levels, antibody clones, staining platforms, and interpretations differ. For instance, whereas that in metastatic non-small cell lung cancer (NSCLC) samples relies on tumor proportion scores (TPS) instead of CPS (4). The Ventana SP142 assay is used to analyze urothelial carcinoma (UC) and to count immune cells (IC) within the tumor microenvironment (5). This variability in scoring methods has contributed to confounding results across clinical trials and in clinical practice, leading to uncertainty regarding the universal value of PD-L1 protein levels as a biomarker across tumor types (6, 7). Furthermore, the use of formalin-fixed, paraffin-embedded (FFPE) archival tumor tissues prepared, fixed, and stored in non-standardized ways might not generate predictable and intended results for adequate PD-L1 antigen retrieval. This could potentially increase the heterogeneity of IHC intensity, extent, and topography of staining (3). All these factors complicate the reliability of PD-L1 levels assessed by IHC to predict clinical responses to ICB (8).

Assays of FFPE tissues based on RNA are currently employed clinically to classify or predict recurrence risk in patients affected by various types of tumors (9, 10). Gene expression assays based on RNA include microarray, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR), and RNA sequencing (11–13). The qRT-PCR assays are popular for quantifying genes due to a large dynamic range, high sensitivity, high specificity, little to no post-amplification processing, and increased sample throughput (14, 15). The use of specific primers targeting stably expressed genes provides high specificity and sensitivity, allowing for the simultaneous measurement of several targets, including genes, for sample quality control purposes. Gene expression profiling by qRT-PCR has minimal input requirements and could be far more cost-effective than IHC. Furthermore, close concordance between qRT-PCR and IHC has validated qRT-PCR analyses, even for challenging FFPE tumor samples (16). Therefore, gene-specific reverse transcription might considerably increase the success rate of molecular classifier validation in FFPE sample cohorts.

The present study aimed to develop a more rapid qRT-PCR assay to measure *CD274* mRNA expression that closely correlates with PD-L1 IHC and save archival tumor tissues for other IHC assays in the same patient. Therefore, we designed three qRT-PCR primers and compared their results with those of PD-L1 IHC, then clinically validated the results in patients with GC and UC treated with ICIs.

MATERIALS AND METHODS

Patients and Data Collection

We collected retrospective data from 100 patients with advanced GC (n = 59) or UC (n = 41) that were treated with palliative chemotherapy (n = 100) and anti-programmed death 1 (PD-1)/PD-ligand (L)-1 immunotherapy (n = 49) at Samsung Medical Center between December 2016 and January 2020. The median age was 61.0 (33–81) years and 30 (61.2%) patients were male. All the patients present with GC were stage IIB–IV disease at diagnosis and have experienced local recurrence or metastasis at treatment for ICI. For UC patients, they were all locally advanced stage II–IIIb disease stages (**Supplementary Table S1**). Responses of the 49 patients treated with immunotherapy were assessed every 6–12 weeks according to the Immune Response Evaluation Criteria in Solid Tumors (iRECIST) (17). Data from patients with at least 6 weeks of follow up were included. The primary clinical endpoint was the objective response rate (ORR), defined as a complete (CR) or partial (PR) response. Patients with progressive (PD) or stable (SD) disease were classified as non-responders. Clinicopathological data were retrospectively extracted from electronic medical records. This study proceeded in accordance with the Institutional Review Board guidelines (IRB No. 2018-09-041-001) for data analysis and investigational treatment, and written informed consent from the patients was also obtained to analyze their innominate data.

RNA Extraction and qRT-PCR

Total RNA was isolated from FFPE tumor tissues using the ReliaPrep^{TM} FFPE Total RNA Miniprep System (Promega Corp., Madison, WI, USA), and a amplified using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) as described by the manufacturer. Target genes were analyzed using a gene expression assay with forward and reverse primers and an Applied Biosystems FAM-labeled MGB TaqMan^{TM} probe (Thermo Fisher Scientific Inc.) as we previously described (18). We found that the PD-L1 IHC results correlated with those of NanoString nCounter assays (19), we used *CD274* TaqMan probes spanning exon 1–2 (assay ID; Hs01125296_m1), 3–4 (assay ID; Hs00204257_m1), and 5–6 (assay ID; Hs01125301_m1) boundaries for qRT-PCR (**Supplementary Figure S1**). These sequences were amplified by PCR in triplicate under the following conditions using

QuantStudio 6 (Thermo Fisher Scientific Inc.): 2 min at 50°C and 10 min at 94°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Threshold cycle (Ct) values for each sequence were calculated for each and averaged, and normalized to the mean of the reference gene *GUSB2* (assay ID: Hs99999908_m1), which was stably expressed (18). The mRNA expression of each gene was measured using the $2^{\wedge -\Delta Ct}$ ($\Delta Ct = \Delta Ct_{target\ gene} - \Delta Ct_{GUSB2}$) method.

Immunohistochemical Detection of PD-L1

Gastric FFPE tissue blocks were cut into 4-μm sections and stained using an Autostainer Link 48 system and Dako PD-L1 IHC 22C3 pharmDx kits (both from Agilent Technologies Inc., Santa Clara, CA, USA) (2). A rabbit anti-human PD-L1 monoclonal antibody (clone SP142; Ventana Medical Systems, Tucson, AZ, USA) was used as described for UC samples (20). The CPS of PD-L1 expression was calculated as the number of PD-L1-stained GC tumors and ICs divided by the total number of viable tumor cells, multiplied by 100. The concordance rate between qRT-PCR and IHC was evaluated using CPS cut-offs of 1 and 10 for GC. Infiltrative ICs covering ≥ 5 of a UC tumor area were defined as PD-L1-positive. For positive control, we used positive cell lines provided by PD-L1 IHC 22C3 pharmDx and tonsil tissues. For negative control, we used MCF-7 cell lines provided by PD-L1 IHC 22C3 pharmDx. Benign human tonsil is tissue control as it contains both positive and negative staining epithelial and immune cells and can serve as both a positive and negative tissue control for VENTANA PD-L1 (SP142) Assay staining (21).

Statistical Analyses

We used CPS ≥1 and ≥ 10 for GC, and IC ≥5 for UC to compare IHC with qRT-PCR. To calculate the sensitivity, specificity, positive (PPV) and negative (NPV) predictive values, and accuracy, a positive IHC result was considered as CPS ≥1 or ≥ 10 for GC, and IC ≥ 5 for urothelial carcinoma. Predicted responses based on tumor type, IHC results, and qRT-PCR results were evaluated using logistic regression.

The ORR (CR/PR) and disease control rate (DCR; CR/PR/SD) were compared with the *CD274* mRNA qRT-PCR results using two-tailed unpaired Student t-tests. The diagnostic values of panels were assessed by calculating the area under the receiver operating characteristics (ROC) curve (AUC). Kaplan–Meier estimates of progression-free (PFS) and disease-specific survival (DSS) were compared using log-rank tests. All graphs were generated using GraphPad Prism v. 9.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $P < 0.05$. All data were statistically analyzed using SPSS software version 27.0 (IBM Corp., Armonk, NY, USA).

RESULTS

Comparison of IHC and qRT-PCR Results

The 22C3 pharmDx assay identified PD-L1 positivity with CPS ≥ 1 and ≥ 10 in 32 (54.2%) and 13 (22 %) of 59 GC samples, respectively. The mean PD-L1 CPS in GC was 9.24 (0–95). The Ventana SP142 assay identified PD-L1 positivity with IC ≥ 5 in 12 (29.3%) of 41 UCs. The mean PD-L1 IC in urothelial carcinomas was 10.46 (0–95) (**Figure 1**).

The mean RQ (range) of relative *CD274* mRNA expression spanning exons 1–2, 3–4, and 5–6 were 0.1004 (0–2.4897), 0.2371 (0–7.5214), and 0.0928 (0–3.7064), respectively. These values closely correlated (Spearman correlations: $r^2 = 0.92$ for exons 1–2 and 3–4; $r^2 = 0.89$ for exons 1–2 and 5–6, and $r^2 = 0.99$ for exons 3–4 and 5–6; **Figure 2A**). The PD-L1 scores in 100 evaluated samples closely correlated with *CD274* mRNA expression spanning exons 1–2 ($r^2 = 0.81$), 3–4 (r^2=0.65), and 5–6 ($r^2 = 0.59$; **Figure 2A**). In GC, The PD-L1 CPS score with 22C3 pharmDx significantly correlated with the exon 1–2 ($r^2 = 0.81$), 3–4 ($r^2 = 0.67$), and 5–6 ($r^2 = 0.62$) junctions of *CD274* (**Figure 2B**). The Ventana SP142 PD-L1 IC score was significantly associated in UC with exon 1–2 ($r^2 = 0.93$), exon 3–4 ($r^2 = 0.82$), and exon 5–6 ($r^2 = 0.76$) junctions of *CD274* (**Figure 2C**).

The RQ cutoffs of *CD274* mRNA expression in exon 1–2, 3–4, and 5–6 junctions were evaluated as the AUC based on PD-L1 CPS cut-offs of 1 and 10 for GC and PD-L1 IC cut-offs of 5 for UC (**Supplementary Table S2** and **Supplementary Figure S2**). At a CPS cutoff of 10, the highest AUC in GC was 0.783, obtained from *CD274* mRNA expression at the exon 1–2 junction with a cut-off of 0.0722 ($P < 0.0001$). The highest AUC of UC based on PD-L1 IC cut-offs of IC 5 was 0.781, obtained from *CD274* mRNA expression in the exon 1–2 junction with a cut-off of 0.0480 ($P < 0.0001$).

IHC and qRT-PCR Results Predicted Responses to Anti-PD-1/PD-L1 Inhibitor

Between May 2018 and October 2020, 49 patients were treated with anti PD-1/PD-L1 agents, and treatment responses to treatment with pembrolizumab (n = 16), nivolumab (n = 16), atezolizumab (n = 13), and durvalumab (n = 4) were evaluated during > 6 weeks of followup (**Supplementary Table S1**). The median number PD-1/PD-L1 cycles was 8.9 (range, 1–37) as of May 20, 2021, and the patients were followed up for a median of 11.3 months. **Table 1** summarizes the clinicopathological characteristics of the patients treated with anti-PD-1/PD-L1.

Anti-PD-1/PD-L1 responders (CR/PR, n = 16) and non-responders (PD/SD, n = 33) were identified using the iRECIST category of ORR. The expression of PD-L1 ($P = 0.010$) and high *CD274* mRNA expression ($P < 0.001$) were significantly associated with the response to immunotherapy. The ROC curve for the predictive performance of PD-L1 IHC and mRNA expression of *CD274* at exon 1–2 was discriminatory. The AUC and 95% confidence intervals (CIs) were 0.76 (0.61–0.91) for PD-L1 and 0.75 (0.59–0.91) for mRNA expression of *CD274* exon 1–2. These findings were similar using the iRECIST category of DCR (CR/PR/SD, n = 30 and PD, n = 19). Furthermore, PD-L1 expression ($P = 0.015$) and high *CD274* mRNA expression ($P = 0.038$) predicted responses to immunotherapy with AUCs of 0.70 (0.55–0.86) and 0.68 (0.53–0.83), respectively. In GC, the expression of PD-L1

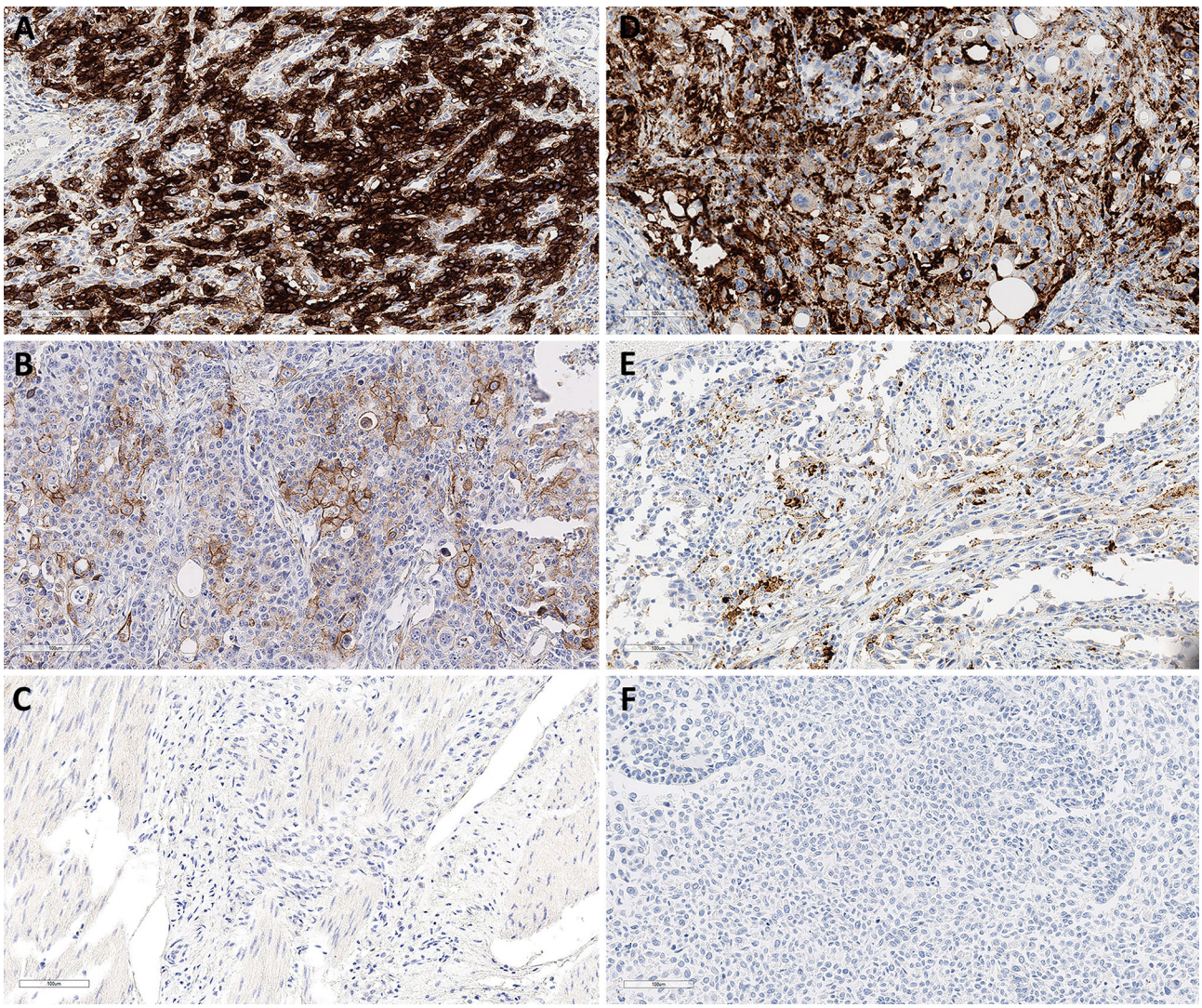

FIGURE 1 | Representative PD-L1 immunohistochemical staining in GC and SP142 in UC. Combined positive scores of 95 **(A)**, 25 **(B)** and 0 **(C)** in GCs with 22C3 pharmDx. Immune cell scores of 40 **(D)**, 20 **(E)** and 0 **(F)** in UCs with Ventana PD-L1 (SP142) assay. Magnification in all images, 20×. GC, gastric cancer; UC, urothelial carcinoma.

(P = 0.002) and high CD274 mRNA expression (P=0.041) were significantly associated with the response to immunotherapy. In UC, the expression of PD-L1 (P = 0.147) and high CD274 mRNA expression (P=0.008) did not reach statistical significance in predicting response to immunotherapy (**Figure 3A**). The ROC curve for the predictive performance of PD-L1 IHC and mRNA expression of CD274 at exon 1–2 was discriminatory. In GC, the AUC and 95% confidence intervals (CIs) were 0.80 (0.63–0.97) for PD-L1 and 0.69 (0.47–0.92) for mRNA expression of CD274 exon 1–2. In UC, the AUC and 95% Cis were 0.68 (0.36–0.99) for PD-L1 and 0.87 (0.67–1.00) for mRNA expression of CD274 exon 1–2 (**Figure 3B**). These findings were similar using the iRECIST category of DCR (CR/PR/SD, n = 15 and PD, n = 18) in GC. PD-L1 expression (P = 0.008) and high CD274 mRNA expression (P = 0.017) predicted responses to immunotherapy with AUCs of 0.73 (0.56–0.90) and 0.71 (0.53–0.90), respectively, in GC. In UC, anti-PD-1/PD-L1 responders (n = 15) and non-responders (n = 1) were identified using the iRECIST category of DCR. PD-L1 expression (P = 0.375) and high CD274 mRNA expression (P = 0.250) predicted responses to immunotherapy with AUCs of 0.67 (0.43–0.91) and 0.90 (0.71–1.00), respectively (**Figures 3C, D**).

Correlations Between Survival and PD-L1 Immunohistochemical and qRT-PCR Results

The PFS was closely associated with PD-L1 expression (P = 0.018) and high CD274mRNA expression spanning the exon 1–2

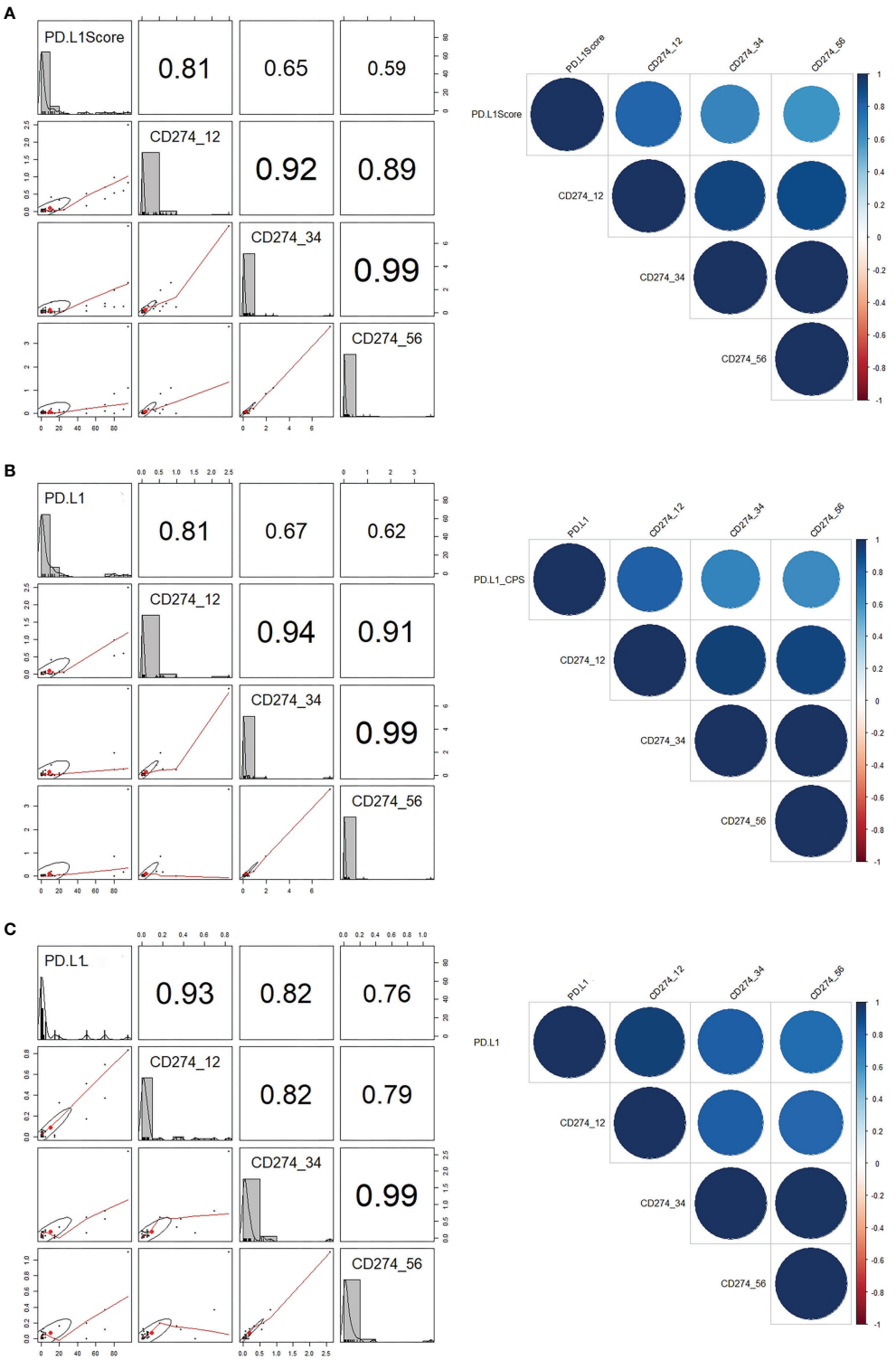

FIGURE 2 | Correlations between PD-L1 scores and *CD274* mRNA expression. **(A)** Correlations between (A) PD-L1 scores and *CD274* exons 1–2, 3–4, and 5–6 in all GC and UC. **(B)** PD-L1 combined positive score and *CD274* mRNA expression in GC. **(C)** PD-L1 immune scores and *CD274* mRNA expression in UC. GC, gastric cancer; PD-L1, programmed death-ligand 1; UC, urothelial carcinoma.

TABLE 1 | Clinicopathological characteristics of patients treated with anti-programmed death 1 (PD-1)/programmed death-ligand 1 (PD-L1) therapy.

	Anti-PD-1/PD-L1 patients, No (%)	Overall response rate (CR/PR), (%)	P-value	Disease control rate (CR/PR/SD), (%)	P-value
Overall	**49**	**16 (32.7%)**		**30 (61.2%)**	
Age			0.261		0.043
<65	30 (61.2%)	8 (26.7%)		15 (50%)	
≥ 65	19 (38.8%)	8 (42.1%)		15 (78.9%)	
Sex			0.045		0.001
Male	30 (61.2%)	13 (43.3%)		24 (80%)	
Female	19 (38.8%)	3 (15.8%)		6 (31.6%)	
Treatment line of immunotherapy			0.929		0.003
1	20 (40.8%)	7 (35%)		18 (90%)	
2	12 (24.5%)	4 (33.3%)		5 (41.7%)	
≥3	17 (34.7%)	5 (29.4%)		7 (41.2%)	
Immunotherapy regimen			0.196		0.002
Pembrolizumab containing	16 (32.7%)	8 (50%)		12 (75%)	
Nivolumab containing	16 (32.7%)	5 (31.3%)		5 (31.3%)	
Atezolizumab containing	13 (26.5%)	3 (23.1%)		12 (92.3%)	
Durvalumab containing	4 (8.1%)	0 (0%)		1 (25%)	
Gastric cancer	**33**	10 (30.3%)		15 (45.5%)	
PD-L1 CPS cutoff 1	18	9 (50%)	0.007	12 (66.7%)	0.007
qRT-PCR cutoff 0.0276	15	6 (40%)	0.269	10 (66.7%)	0.025
PD-L1 CPS cutoff 10	8	5 (62.5%)	0.023	5 (62.5%)	0.266
qRT-PCR cutoff 0.0722	5	5 (100%)	<0.001	5 (100%)	0.008
Urothelial carcinoma	**16**	6 (37.5%)		15 (93.8%)	
PD-L1 IC cutoff 5	6	3 (50%)	0.424	5 (83.3%)	0.182
qRT-PCR cutoff 0.0480	5	4 (80%)	0.018	5 (100%)	0.486

qRT-PCR, quantitative reverse transcription-polymerase chain reaction; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; CPS, combined positive score; Bold, a statistically significant correlation with a p-value less than 0.05.

junction (P = 0.010) in GC (**Figure 4A**). The association was also similar between DSS and PD-L1 expression (P = 0.047). However, DSS was not significantly associated with mRNA expression (P = 0.134); **Figure 4B**). The expression of PD-L1 was significantly associated with PFS (P = 0.016) and DSS (P = 0.009) in UC, whereas the CD274mRNA expression at exon 1–2 junction did not significantly correlate with PFS and DSS (**Supplementary Figure S3**).

Clinical Value of PD-L1 IHC and qRT-PCR

The clinical value of PD-L1 assessment with IHC and qRT-PCR was compared using the standard parameters of sensitivity, specificity, PPV, NPV, and accuracy (**Table 2**). We used two cut-offs for GC samples (CPS ≥ 1% and 10%; RQ ≥ 0.0276 and ≥ 0.0772) to ensure the optimal performance to predict responses for immunotherapy. The CPS ≥ 1% for PD-L1 was the most sensitive (90%), and qRT-PCR with a RQ cutoff of 0.0722 was the most specific (100%) in GC. The sensitivity was highest in GC samples with CPS ≥ 1 (90%) although the PPV was very low (50%). The sensitivity (66.7%) and specificity (90%) of detecting UC were higher with qRT-PCR and the AUC values higher than those in PD-L1 IHC.

DISCUSSION

The expression of PD-L1 is one of the most studied biomarkers to predict the responses to ICI and one of the most controversial biomarkers to be introduced into clinical practice (3). Despite evidence showing that technological and histological variability limit clinical its utility (2, 22), four IHC-based tests have been approved for guiding treatment decisions regarding patients with multiple tumor types. The wide range of FDA-approved assays with differential sensitivity and scoring systems (23) and the lack of harmonization among them (24) have led to confusion in pathology laboratories (25). In GC, pembrolizumab exhibited favorable efficacy in PD-L1-positive patients (KEYNOTE-059) (26). Owing to the results, pembrolizumab was approved for PD-L1-positive GC patients in second- or later-line treatment by the FDA. However, the predictive value of PD-L1 expression in GC was challenged by other clinical trials (27–29). In UC, five PD-1/PD-L1 inhibitors are approved for treatment of locally advanced or metastatic UC. Due to restrictions by the FDA, first-line treatment with Atezolizumab and Pembrolizumab in platinum-ineligible patients requires PD-L1 IHC. In the second-line setting, all drugs are approved without PD-L1 IHC testing (30). PD-L1 IHC tests used in clinical trials of UC immunotherapy include the 28-8 pharmDx (Nivolumab), the 22C3 pharmDx (Pembrolizumab), Ventana SP142 (Atezolizumab), and the Ventana PD-L1 SP263 assays (Durvalumab). Here, we measured PD-L1 mRNA expression using qRT-PCR and compared the results with FDA-approved PD-L1 IHC assays for GC and UCs. We found that CD274 mRNA expression spanning exon 1–2 closely correlated with PD-L1 IHC and predicted responses to ICIs.

Although PD-L1 IHC measured by IHC is a predictive biomarker of responses to ICIs (22), whether an alternative

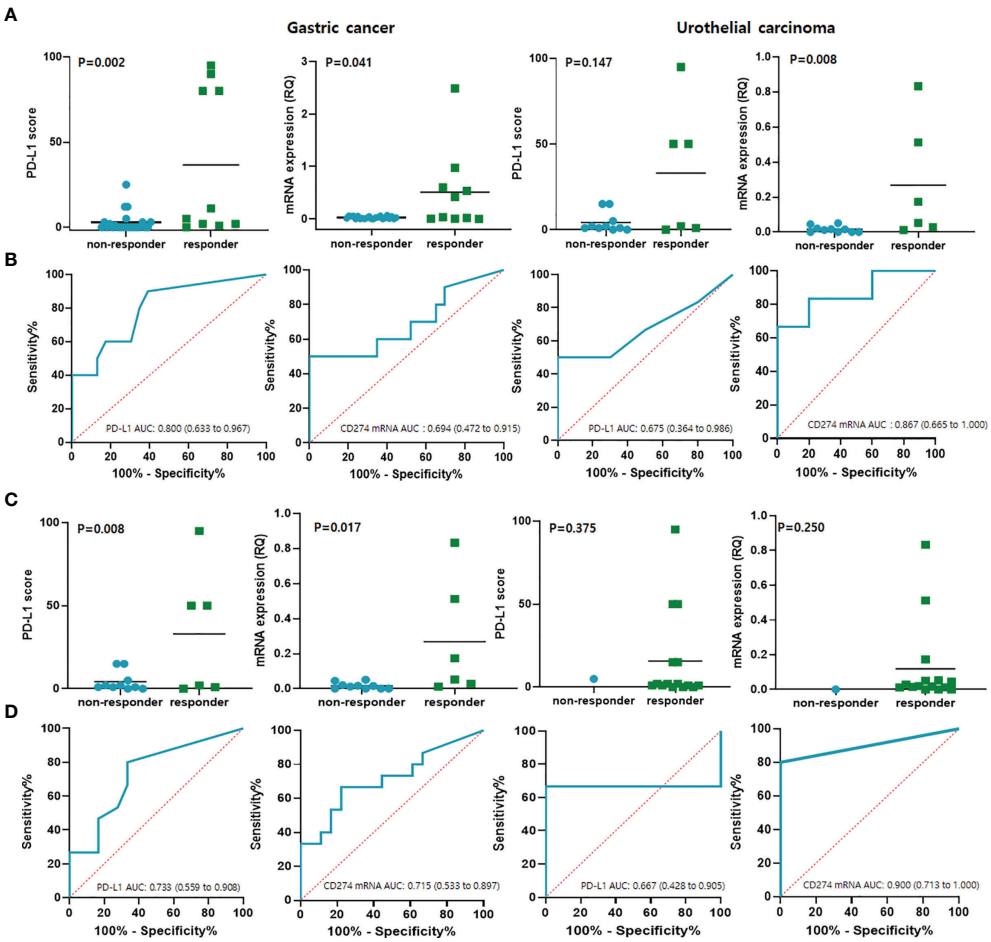

FIGURE 3 | Results of qRT-PCR predicted responses to anti- PD-1 checkpoint blockade in GC and UC. **(A)** PD-L1 and CD274 mRNA expression per iRECIST ORR categories of responders (CR/PR) and non-responders (PD/SD). **(B)** Predictive performance of PD-L1 and CD274 mRNA expression determined from ROC curves in terms of ORR categories. **(C)** PD-L1 and CD274 mRNA expression levels per iRECIST DCR category of responders (CR/PR/SD) and non-responders with SD. **(D)** Predictive performance of PD-L1 and CD274 mRNA expression determined from ROC curves in terms of DCR category. CR, complete response; GC, gastric cancer; iRECIST, immune Response Evaluation Criteria in Solid Tumors; ORR, objective response rate; PD, progressive disease; PD-L1, programmed cell death ligand 1; PR, partial response; ROC, receiver operating characteristics; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; SD, stable disease; UC, urothelial carcinoma.

methodology could validate PD-L1 utility as a predictive biomarker has remained unclear (3). Much effort has been directed towards evaluating whether RNA-based PD-L1 assays could replace PD-L1 IHC as a biomarker to predict responses to ICI (**Table 3**) (3, 31–33, 35–38). Unlike IHC, qRT-PCR or RNA sequencing quantifies the number of mRNA transcripts expressed in an entire tumor without subjective scoring methods or cell type discrimination (3). Recently, various omics-based approaches have been undertaken to identify both tumor intrinsic and extrinsic factors which can serve as predictive biomarkers to ICB (39). Wu et al. reported that high-throughput gene expression data would further help prioritize important biomarkers and potential therapeutic targets for combination treatments with anti-PD-1 therapy for a given cancer type (39). Chen et al. also found that gene expression profiles between responder and non-responder are not significantly different for pre-treatment samples, but much more significantly for on-treatment samples (40). Our results also confirmed that *CD274* mRNA expression measured by qRT-PCR closely correlated with PD-L1 IHC measured using FDA-approved assays. Kowanetz et al. also showed that *CD274* mRNA expression had predictive value for responses to atezolizumab in UC (41). Although our patient cohort was small, we found that high *CD274* mRNA expression determined by qRT-PCR predicted the responses of all 49 patients to immunotherapy with an AUC of 0.75, which was similar to that of PD-L1 IHC (0.76). Objective qRT-PCR assays are operator independent, and can resolve major disadvantages associated with PD-L1 IHC such as assay variance between vendors, subjective assessment by pathologists, and operator-dependent variations in results (42).

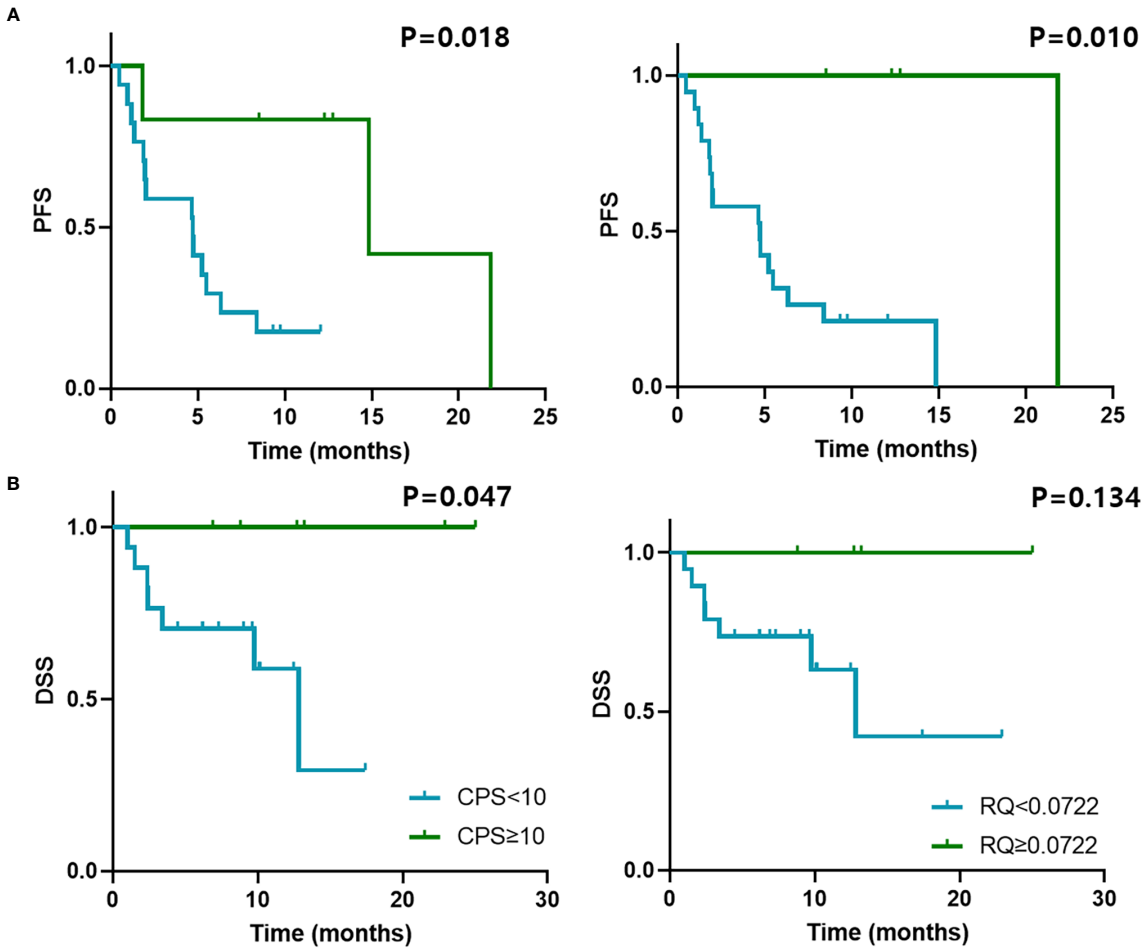

FIGURE 4 | Survival outcomes and qRT-PCR results of GC treated with anti-PD-1/PD-L1. Kaplan–Meier curves of **(A)** PFS and **(B)** DSS of patients with GC treated with anti-PD-1/PD-L1 according to PD-L1 CPS cut-off 10 and *CD274* mRNA expression determined by qRT-PCR with cut-off 0.0722. PFS, progression-free survival; DSS, disease-specific survival.

Therefore, evaluating *CD274* mRNA expression by qRT-PCR has potential as a diagnostic test with easy standardization and a rapid turnaround time.

One limitation of this study is that it is a single-institutional retrospective investigation of a relatively small sample of patients treated with immunotherapy. We plan to validate our results in a prospective study. Another limitation is that we analyzed patients with GC and UC treated with various individual and combined immunotherapeutic agents in the same cohort. Although gastric and urothelial carcinomas are quite different in their nature, however, in predicting responses for immunotherapy using PD-L1 IHC, CPS is used in interpretation and both cancers were approved relatively early for immunotherapy. Therefore, we decided to study both gastric and urothelial carcinomas. Future studies could address this issue by evaluating patients with GC and UC who receive uniform treatment.

TABLE 2 | Comparison of clinical applicability between IHC PD-L1 and qRT-PCR results.

Prediction Method	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)
Gastric cancer IHC ≥ 1%	90.0%	60.9%	50.0%	93.3%	0.75 (0.58-0.93)
Gastric cancer RQ ≥ 0.0276	60.0%	60.9%	40.0%	77.8%	0.60 (0.39-0.82)
Gastric cancer IHC ≥ 10%	50.0%	87.0%	62.5%	80.0%	0.69 (0.47-0.90)
Gastric cancer RQ ≥ 0.0772	50.0%	100.0%	100.0%	82.1%	0.75 (0.54-0.96)
Urothelial carcinoma IHC ≥ 5%	50.0%	70.0%	50.0%	70.0%	0.60 (0.30-0.90)
Urothelial carcinoma RQ ≥ 0.0480	66.7%	90.0%	80.0%	81.8%	0.78 (0.52-1.00)

AUC, area under ROC curve; IHC, immunohistochemistry; ROC, receiver operating characteristics; RQ, relative quantification; qRT-PCR, quantitative real-time polymerase chain reaction.

TABLE 3 | Comparison of published CD274 mRNA expression and PD-L1 IHC data.

Study No.	Authors	Years	Cancer Type	Method	Immune checkpoint inhibitor	AUC response to ICI	Cutoff	Patients (n)
	Present study	2021	GC and UC	qRT-PCR and IHC (22C3 and SP142)	Nivolumab, pembrolizumab, and atezolizumab	Overall AUC 0.75 ORR vs. 0.76 by IHC	CPS cutoff 1, 10%, and IC 5%	49
1	Tsimafeyeu et al. (31)	2020	NSCLC	qRT-PCR and IHC (22C3, SP142, SP263)	NA	NA	IHC TC cutoff 10%	437
2	Xiao et al. (32)	2019	ccRCC	qRT-PCR and IHC (E1L3N)	NA	PFS and OS only	IHC TC cutoff 5%	242
3	Conroy et al. (3)	2019	Melanoma, RCC and NSCLC	RNAseq and IHC (22C3, 28-8)	Nivolumab, pembrolizumab, and atezolizumab	Overall 73% ORR vs. 56% by IHC	TPS cutoff 1, 50%, and CPS 1%	209
4	Duncan et al. (33)	2019	NSCLC, HNSCC, and UC	IHC (SP263) and RNAscope	NA	NA	IHC TC 25%	86
5	Vannitamby et al. (34)	2019	NSCLC	qRT-PCR, ddPCR and IHC	NA	NA	IHC TC cutoff 1%	28
6	Tretiakova et al (35)	2018	Bladder carcinoma	RNAscope and IHC (22C3, 28-8, E1L3N, and SP142)	NA	NA	NA	156
7	Erber et al. (36)	2017	NSCLC	qRT-PCR and IHC (E1L3N and 28-8)	NA	NA	IHC TC cutoff 50%	22
8	Shent et al. (37)	2014	Osteosarcoma	qRT-PCR and IHC (B7-H1)	NA	NA	NA	38

AUC, area under the receiver operator characteristics curve; ccRCC, clear cell renal cell carcinoma; GC, gastric cancer; HNS CC, head and neck squamous cell carcinoma; ICI, immune checkpoint inhibitor; IHC, immunohistochemistry; NA, not applicable; NSCLC, Non-small cell lung cancer; qRT-PCR, quantitative real-time polymerase chain reaction; SCC, squamous cell carcinoma; TC, tumor cell; UC, urothelial carcinoma.

In conclusion, *CD274* mRNA expression measured by qRT-PCR closely correlated with PD-L1 IHC measured using FDA-approved assays and predicted the responses of patients with GC or UC to ICBs.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board guidelines of the Samsung Medical Center (IRB 2018-09-041-001). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SK and K-MK designed and supervised the study. SK, YH, GK and K-MK collected tissue samples and clinical data and performed histopathological examination. SK, YH, and K-MK analyzed the data. SK, YH, GK and K-MK conducted the experiments. SK and K-MK wrote the draft. SK, YH, GK and K-MK revised the manuscript. All authors reviewed and approved the final version of the manuscript.

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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.2022.856444/full#supplementary-material

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