MODULATION OF HUMAN IMMUNE PARAMETERS BY ANTICANCER THERAPIES

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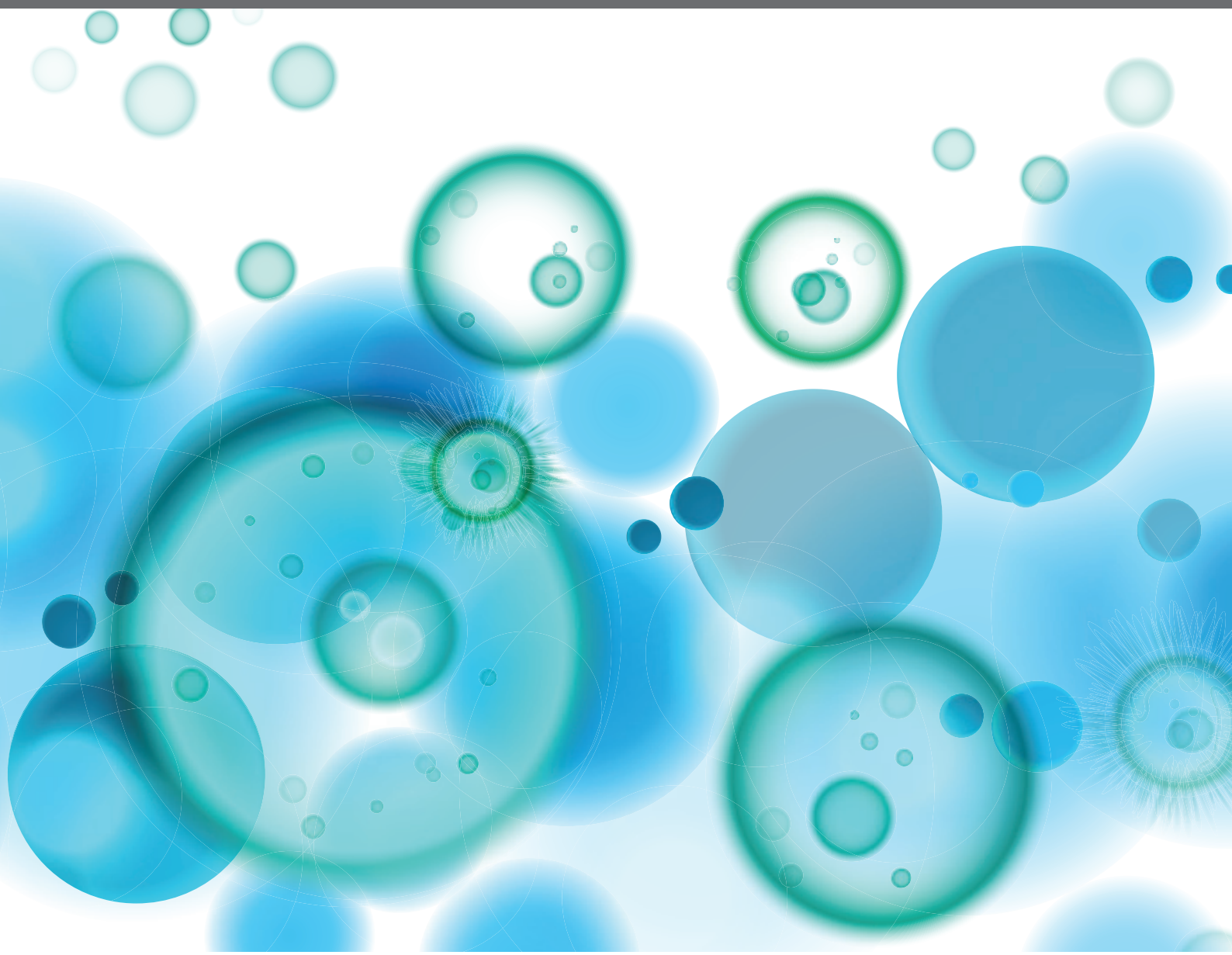

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MODULATION OF HUMAN IMMUNE PARAMETERS BY ANTICANCER THERAPIES

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Editorial: Modulation of Human Immune Parameters by Anticancer Therapies

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Keywords: immunoncology, flow cytometry, checkpoint inhibition/blockade, immune modulation, tumor-immune cell interaction

Editorial on the Research Topic

Modulation of Human Immune Parameters by Anticancer Therapies

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Immunoncology is among the most important hallmarks of immunotherapy revolution of cancer medicine. Here, we compiled reviews and original research articles reflecting current developments in immunoncology.

Novel therapies modulate the complex interaction between tumor and immune system (**Figure 1**). Multiparametric flow cytometry (FCM) is a key analytical tool contributing over 1,000 research articles/year to the field. As a quantitative single-cell technology, FCM reliably and reproducibly identifies rare populations, detects subtle changes in modulatory signals, and assesses time-sensitive antigenic expression patterns. State-of-the-art equipment, fast sophisticated software, and flexibly labeled monoclonal antibodies allow rapid analyses with high sensitivity and specificity, even in routine applications. Lambert et al. explain how new analytes are added to the portfolio of diagnostic and research laboratories. Sample preparation, antibody titration, and appropriate controls are central in cytometric analysis and must be controlled with the necessary rigor and reproducibility (1).

Although tumor cell analysis is a key application of cytometry (2, 3), this research topic is dedicated to the modulation of immune parameters, and we only included work focusing on tumor-immune-cell interaction and its disease-course impact.

Dendritic cells (DCs) are crucial in tumor protection (4). Lu et al. dissect the interaction of DCs with non-small cell lung cancer (NSCLC) cells, which can induce an immunosuppressive microenvironment and evade immune surveillance. Analysis of costimulatory molecules and pro-/anti-inflammatory cytokines reveals new subpopulations of CD1c+ DCs in coculture with NSCLC. Particularly, the expression of signal molecules and pro-inflammatory cytokines are suppressed, whereas the secretion of anti-inflammatory cytokines by DCs is upregulated, suggesting that NSCLC can induce tolerogenic DCs, blocking DC-mediated anti-tumor immunity.

Chemokines and their corresponding receptors play a pivotal role in orchestrating trafficking of immune cells to fulfill their next tasks. CXCL10 has been associated with T cell recruitment into

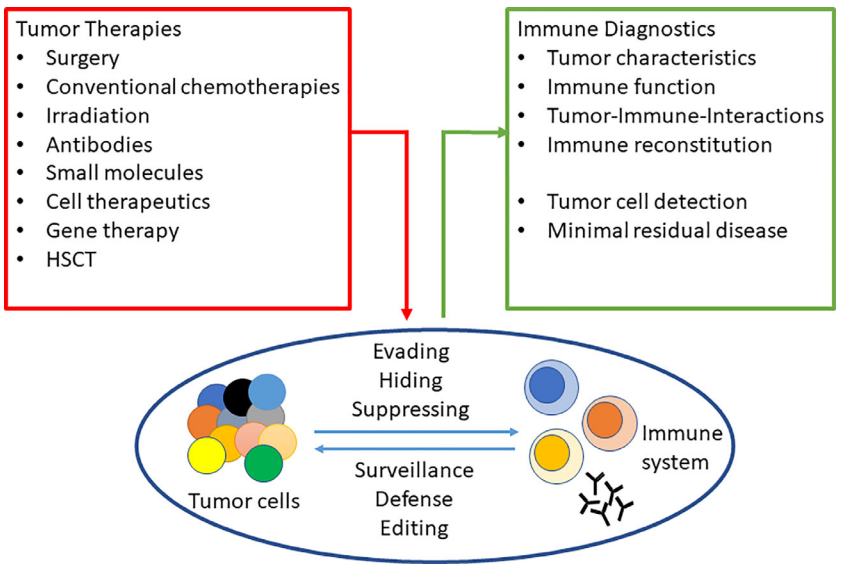

FIGURE 1 | Tumor-immune-interactions and investigation by flow cytometry.

tumors. Meng et al. link an increased CXCL10 expression and T cell infiltration with autophagy inhibition in gastric cancer (GC). Since autophagy was associated with GC cell survival and therapy resistance, autophagy inhibition is considered a potential GC treatment strategy, which might also favorably effect T cell recruitment into the tumor.

Inflammation is central in tumorigenesis underlining close interactions between immune system and tumor. Besides adaptive immunity, innate immunity is crucial in tumor defense (5). Stein et al. address the role of the inflammatory cytokine TNF-α in colorectal cancer (CRC). CRC has commonly good prognosis, if detected early. With distant metastasis, 5-year survival rate drops below 10% with little therapeutic progress. The metastasis-associated oncogene in CRC 1 (MACC1) is involved in CRC metastasis, induces cell proliferation and motility, supports cell survival, and redirects metabolism. Also, in several other solid cancers, MACC1 is a potential target for late forms of metastasis. The authors demonstrate that TNF-α triggers upregulation of MACC1 mRNA and protein *via* induction of c-Jun expression, resulting in promoted CRC-cell migration. MACC1 induction was successfully inhibited by MACC1 and c-Jun knockdown as well as anti–TNF-α and anti-TNFR1 blocking antibodies, providing potential therapeutic targets for treating inflammation-associated CRC.

Tumor-immune-cell interactions are decisive in the disease course but not yet fully understood and addressed by Plesca et al. In various cancers, high densities of CD45RO+ T-helper1 cells and CD8+ T cells are associated with improved outcome, M2 macrophages rather with worsened prognosis (5). This can also be applied to the expected response to anti-programmed cell-death-protein 1 (PD-1). Anti–PD-1 therapy affects an increased density of tumor-infiltrating T cells in responders, and increased frequency of melanoma-infiltrating TCF7+CD8+ T cells. However, tumor-infiltrating PD-1+CD38hi CD8+ T-cells are associated with anti–PD-1 resistance that favors implementation of immunoprofiling before checkpoint inhibition therapy.

Based on the manifold relationships between immune system and tumor, the numerous parallels between immunopathology and tumor therapy are not surprising. Khoy et al. present Natalizumab in multiple sclerosis (MS) as a typical example for immune therapies and precursor of today's antibody therapies for tumors. MS is a chronic demyelinating disease of the CNS with an autoimmune component. Among the recently available disease-modifying therapies, Natalizumab, a monoclonal antibody against VLA-4 integrin, effectively inhibits cell migration to tissues including the CNS, thereby inhibiting disease progression. Since also immune function is impaired, immunomonitoring during therapy is important to detect adverse effects.

Klöß et al. report on challenging examples that bridge between treatment of cancer and immune-mediated diseases, major hurdles are suitable experimental models reflecting the complex tumor-immune-interactions during treatment for identifying new therapies. In addition to patient-derived tumor xenotransplants (PDX) (humanized) mouse models, *ex vivo* approaches to cancer modeling like microfluidic human organs-on-chips are shown. Better understanding of treatment mechanisms and side effects permitted the development of novel targeted cell-, drug-, and biological-based therapies. Progress of our knowledge about inhibitory and stimulatory immune mechanisms associated with autoimmune diseases enable novel strategies to tackle autoimmunity using regulatory CAR-T cells (CAR Treg) of natural killer cells (NK) (6, 7).

Cell-based therapies, particularly CAR-T or CAR-NK cells redirected against aggressive leukemia and lymphoma, have taught lessons to improve immunoncology. Identification of

tumor-associated antigens and the respective target-to-effector interaction and understanding how to overcome the immunosuppressive tumor-microenvironment are #1 challenges as addressed in previous Frontiers in Immunology (8, 9). Current development in CAR-NK cells for leukemia treatment (10–12) must be applied also to solid tumors.

Gibellini et al. review single cell approaches to profile the response to immune checkpoint inhibitors. Since tumor cells are highly variable, single-cell analysis like polychromatic FCM, single-cell sequencing, or high-resolution imaging can be employed to examine rare tumor cells. These methods allow analyses in unprecedented detail, fostering understanding of molecular and cellular interactions between cancer and the immune system.

Unfortunately, analysis of tumor cells and immune signatures is not per-se successful. For many cancer types, finding cancer stem cells (CSCs) is essential for therapy optimization as Walcher et al. highlight. They review the most used CSC markers focusing on lung, gastric, liver, breast, and colon cancer and myeloid leukemias. CSCs are an integer part of tumors, drive tumor initiation and can cause relapses. To date, several biomarkers characterizing CSCs have been identified and correlated with diagnosis, therapy, and prognosis. However, CSCs have a high plasticity altering their phenotypic and functional appearance. Such changes are induced by chemo- and radiotherapy as well as by senescent tumor cells, modifying the tumor microenvironment. One source of CSCs is circulating tumor cells that are not part of this issue but are addressed in recent overviews (13, 14).

The last article reports on drug actions immunomonitored by high-content FCM (15, 16). Parry et al. investigated long-term Ibrutinib therapy in B-Cell Chronic Lymphocytic Leukemia (CLL). CLL is associated with immunosuppression and susceptibility to infection. Investigating virus-specific CD8+ T cells, authors could demonstrate a reduction in PD-1 expression and increased cytokine production following stimulation. The results suggest that Ibrutinib therapy is associated with recovery of pathogen-specific T cells in B-CLL thus contributing to reduced risk of infection.

In summary, we hope that this research topic adds important facets to the picture of immunoncology.

AUTHOR CONTRIBUTIONS

US, AT, FP, and IN developed the topic, identified the authors, supported the publication process, and wrote this editorial. UK gave advice, supported selection of authors, and co-edited this editorial. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Non-small Cell Lung Cancer Cells Modulate the Development of Human CD1c$^+$ Conventional Dendritic Cell Subsets Mediated by CD103 and CD205

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Advanced non-small cell lung cancer (NSCLC) leads to a high death rate in patients and is a major threat to human health. NSCLC induces an immune suppressive microenvironment and escapes from immune surveillance *in vivo*. At present, the molecular mechanisms of NSCLC immunopathogenesis and the immune suppressive microenvironment induced by NSCLC have not been fully elucidated. Here, we focus on the effect of NSCLC cells on the development and differentiation of human CD1c$^+$ conventional dendritic cell (DC) subsets mediated by CD205 and CD103. The peripheral blood mononuclear cells (PBMCs) were isolated from NSCLC patients and healthy donors. DCs were induced and cocultured with primary NSCLC cells or tumor cell line H1299. DCs without incubation with tumor cells are control. The protein expression of costimulatory molecules such as CD80 and CD86, HLA-DR, pro-/anti-inflammatory cytokines such as IL-10 and IL-12, and CD205 and CD103 on CD1c$^+$ DCs was detected by flow cytometry. Our data revealed two new subpopulations of human CD1c$^+$ DCs (CD1c$^+$CD205$^+$CD103$^+$ and CD1c$^+$CD205$^+$CD103$^-$ DC) in healthy donors and NSCLC patients. NSCLC cells modulate the development of the CD1c$^+$CD205$^+$CD103$^+$ DC and CD1c$^+$CD205$^+$CD103$^-$ DC subpopulations *in vitro* and *ex vivo*. NSCLC cells also suppress the expression of signal molecules such as CD40, CD80, CD86, and HLA-DR on CD1c$^+$ DCs. In addition, the production of pro-inflammatory cytokines, including IL-12 and IL-23, is downregulated by NSCLC cells; however, the secretion of anti-inflammatory cytokines, such as IL-10 and IL-27, by CD1c$^+$ DCs is upregulated by NSCLC cells. Our results suggest that NSCLC cells may induce immune tolerogenic DCs, which block DC-mediated anti-tumor immunity in NSCLC patients. Our data may be helpful in revealing new cellular mechanisms related to the induction of tolerogenic CD1c$^+$ DCs by NSCLCs and the development of an immune suppressive microenvironment that causes tumor cells to escape immune surveillance. Our results indicate a potential role for CD1c$^+$ DC subsets mediated by CD205 and CD103 in DC-mediated immunotherapy to target NSCLC in the future.

Keywords: dendritic cell, immune tolerance, immunotherapy, non-small lung cancer, CD1c$^+$ cDCs

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a major type of lung cancer (1–3). The survival rate of late-stage NSCLC is very low (4). At present, the immunopathogenesis of NSCLC has not been fully elucidated (5). NSCLC cells escape from immune surveillance *in vivo* and induce a tumor immune suppressive microenvironment (6). The molecular mechanisms involved in the NSCLC-induced tumor immune suppressive microenvironment are still unknown (7). We focused on the effect of NSCLC cells on dendritic cell (DC)-mediated immune function in this research project. We propose that NSCLC cells may induce specific immune tolerogenic DCs and suppress DC-mediated immune responses *in vivo*. Our results will show that NSCLC cells inhibit the expression of signal molecules such as CD40, CD80, and CD86 on DCs. In addition, NSCLC cells also regulate the production of multiple pro- and anti-inflammatory cytokines, such as IL-6, IL-10, IL-12, IL-23, IL-27, and TGF-β, in DCs. NSCLC cells may affect the immune function of DCs mediated by these signal molecules and cytokines *in vivo*.

DCs are major regulatory immune cells that are necessary for adaptive and innate immunity (8, 9). DCs comprise at least two typical types: conventional DCs (cDCs) and plasmacytoid DCs (pDCs) (10, 11). In addition, DCs can also be divided into inflammatory and tolerogenic DCs according to their different immune functions (12, 13). There are at least three subsets of DCs in human peripheral blood mononuclear cells (PBMCs): $CD1c^+$ (cDCs), $CD141^+$ (cDCs), and $CD303^+$ DCs (pDCs) (14). Their immune functions have not yet been fully elucidated. In this project, the effect of NSCLC cells on the expression of signal molecules and cytokine production in $CD1c^+$ DCs was investigated. Our results suggest that NSCLC cells may induce immune tolerogenic DCs through modulating the expression and production of signal molecules and cytokines in $CD1c^+$ DCs, which play an important role in anti-tumor immunity and immune tolerance *in vivo*.

$CD1c^+$ DCs are cDCs in human peripheral blood (15). At present, the functions of the $CD1c^+$ DC subsets in humans have not been fully elucidated (16). It is still unknown whether NSCLC cells can modulate the development and differentiation of $CD1c^+$ DC subsets, although Stankovic et al. investigated DC composition in NSCLC patients (17). Tabarkiewicz et al. reported that the percentage of $CD1c^+$ DCs in NSCLC patients is lower than that in healthy donors (18). It is unclear whether NSCLC cells affect the development and differentiation of $CD1c^+$ DC subpopulations. In this study, two new subsets of $CD1c^+$ DCs with activity mediated by CD205 and CD103 were found in both healthy donors and NSCLC patients. NSCLC cells modulate the development and differentiation of $CD1c^+$ DC subpopulations, and this is mediated by CD205 and CD103. Our results imply that NSCLC cells may affect the immune function of $CD1c^+$ DC subsets via regulating the expression of CD205 and CD103 on $CD1c^+$ DCs. This is likely one aspect of the cellular mechanisms involved in the NSCLC-induced immune suppressive microenvironment *in vivo*.

Abbreviations: APC, Allophycocyanin; CD, Cluster of differentiation; COPD, Chronic obstructive pulmonary diseases; DC, Dendritic cell; FCS, Fetal Calf Serum; Fig, Figure; GM –CSF, Granulocyte-macrophage colony-stimulating factor; IL, Interleukin; Lin, Lineage; LPS, Lipopolysaccharide; 2-ME, 2-mercaptoethanol; NSCLC, Non-small cell lung cancer; PBS, Phosphate-buffered saline; PBMCs, Peripheral blood mononuclear cells; SD, Standard deviation; SEM, Standard error of arithmetic mean; Th, Helper T cells; T_{regs}, Regulatory T cells.

MATERIALS AND METHODS

Patients and Healthy Donors

All patients and healthy donors were recruited via the CAS Lamvac Biotech Co., Ltd. registry and provided informed consent. PBMCs were obtained from seven patients and seven healthy donors. The cells have been collected and studied since 2017. The details of the characteristics of the NSCLC patients and healthy donors are summarized in **Supplementary Table 1**. All samples were tested in the CAS Lamvac Biotech Co., Ltd. Animal and Human Care facilities, and all experimental procedures were approved by the Institutional Animal and Human Care and Use Committee of Cas Lamvac Biotech Co., Ltd.

Isolation of Human PBMCs

Human blood samples (5 ml blood obtained from each person) were centrifuged at 300 g for 20 min at room temperature (RT). The plasma was transferred into a clean, labeled 15-ml conical tube for each sample with a 5-ml pipet after centrifugation. The buffy coat, including lymphocytes, was then transferred into a new clean 15-ml conical tube with a 2-ml pipet using a circular motion. The buffy coat was diluted 1:3 with 1× sterilized PBS and inverted at RT. The diluted buffy coat was then slowly and carefully transferred into 3 ml of Lympholyte-H (Cedarlane Laboratories Limited, Burlington, ON, Canada) with a 10-ml pipet at RT. The cells were then centrifuged at 800 *g* for 20 min at RT. The cells in the lymphocyte layer were transferred into a new 50-ml conical tube by using a 2-ml serological pipet. The lymphocytes were then diluted with 40 ml staining buffer (5% fetal bovine serum, FCS, Gibco, Grand Island, NY, USA, and 0.1% azide in 1 × sterilized PBS). The cells were then centrifuged twice at 500 *g* for 10 min at RT. The supernatant was decanted. The PBMCs were then diluted with 5 ml of media A (40% heated inactive human AB serum in RPMI 1640 medium, Sigma, St. Louis, MO, USA) for the FACS assay.

Freezing and Thawing of PBMCs

The total PBMCs were counted, and 3×10^6 cells were placed into each cryo-vial tube along with 0.5 ml of media A. Then, 0.5 ml of media B (20% DMSO in RPMI 1640 medium, Sigma) was added to each cryo-vial tube. The cryo-vial tubes were then sealed and placed into a cell freezing container containing isopropanol. The cells were kept at −80°C for 24 h and then put into a liquid nitrogen (LN2) canister with LN2.

When thawing frozen PBMCs, the frozen cells were quickly thawed at 37°C for 1 min. Cells were resuspended in RPMI 1640 complete medium with benzonase (25 U/ml) (Sigma). The PBMCs were then centrifuged twice at 300 *g* for 8 min. Finally, the cells were resuspended in 1 ml of complete RPMI 1640 medium (Gibco) without benzonase for counting, and the cell

concentration was adjusted with complete RPMI 1640 medium without benzonase for the flow cytometry assay.

Human DC Culture

A total of 1×10^7 PBMCs in 5 ml of RPMI 1640 complete medium were placed into T_{25} flasks and incubated at 37°C with 5% CO_2 for 4 h. The floating cells were removed, and the attached mononuclear cells were incubated with DC culture medium (complete medium with 1,000 IU/ml GM-CSF and 500 IU/ml IL-4, PeproTech, Rocky Hill, NJ, USA) at day 0. Half of the DC culture medium was removed on days 3 and 6. The DCs were then centrifuged twice at 300 g for 5 min. The supernatant was decanted, and the cells were resuspended in the same amount of fresh DC culture medium and placed into the same DC culture flask. The DCs were harvested at day 8 for the flow cytometry assay.

Tumor Cell Line and Primary NSCLC Cell Culture

Tumor tissues and para-carcinoma tissues were resected and sterilized. The histologically malignant tissue and para-cancerous tissue were washed with PBS three times. The tissues were cut and ground using a sterilized sieve ($d = 0.075$ mm). The primary human tumor cells and human H-1299 non-small lung cancer cells (Cell Bank, Chinese Academy of Sciences, P.R. China) were resuspended in RPMI 1640 complete medium for the flow cytometry assay.

Flow Cytometry Assay

For surface staining, 5×10^5 DCs were either incubated with living tumor cells or were not cocultured with tumor cells, and all cells were stained with BV 480-human CD40 (Becton Dickinson, BD; Franklin Lakes, NJ, USA), BV 650-human CD80 (Biolegend, San Diego, CA, USA), BV 605-human CD86 (BD), APC-Cy7-human CD1c (Biolegend), BV 711-human CD103 (Biolegend), BV 421-human CD205 (BD), AF 700-human HLA-DR (eBiosciences, Grand Island, NY, USA), and BV 510 lineage antibodies (Lin) (Biolegend) for 24 h at 4°C. The cells were washed twice with staining buffer (Biolegend) at 300 g for 5 min. The DCs were fixed with 0.3 ml of fixation buffer (Biolegend) per sample for 15 min in a dark room at RT. The cells were then centrifuged twice with a permeabilization buffer (Biolegend) at 800 g for 10 min. Finally, the cells were resuspended in 0.1 ml of permeabilization buffer per sample for intracellular staining.

For intracellular staining, DCs were incubated with FITC-human IL-6 (Biolegend), Pacific Blue-human IL-12 (Biolegend), BV 786-human IL-10 (BD), PE-CF594-human TGF-beta1 (BD), PE-human IL-27 (Biolegend), and eFluor 660-human IL-23p19 antibodies (eBiosciences) for 24 h at 4°C. The cells were centrifuged twice with permeabilization buffer at 800 *g* for 5 min and resuspended in 0.3 ml of staining buffer per sample. The cells were analyzed by a Cytek Aurora flow cytometry instrument (Cytek Biosciences Inc., Fremont, CA, USA). The flow cytometry assay data were analyzed using Flow Jo software (TreeStar, Ashland, OR, USA).

Statistical Analysis

Experimental data were analyzed by Prism software 6.0 (GraphPad Software, San Diego, CA, USA), and *t*-tests were conducted. The results were regarded as indicating a significant difference if the *P*-value was <0.05.

RESULTS

1. The development of the $CD1c^+CD103^+CD205^+$ DC subset is suppressed in NSCLC patients.

Since CD103 and CD205 expression on DCs play an important role in DC-mediated immune function, NSCLC cells may affect the biological function of DCs through modulating the expression of CD103 and CD205 on DCs. To investigate whether NSCLC cells regulate the expression of CD103 and CD205 on $CD1c^+$ DCs, PBMCs were isolated from NSCLC patients and healthy donors. The expression of CD103 and CD205 on $CD1c^+$ DCs was detected by flow cytometry. Our experimental results demonstrated that the number of $CD1c^+CD205^+$ DCs obtained from NSCLC patients was less than the number of $CD1c^+CD205^+$ DCs isolated from healthy donors (**Figure 1A**). In contrast, the number of $CD1c^+CD103^+$ DCs in NSCLC patients was similar to the number of $CD1c^+CD103^+$ DCs in healthy donors (**Figure 1B**). In addition, the population of the $CD1c^+CD103^+CD205^+$ DC subset in NSCLC patients was also less than the population of the $CD1c^+CD103^+CD205^+$ DC subset in healthy donors (**Figure 1C**). In contrast, there was no significant difference between healthy donors and NSCLC patients in the $CD1c^+CD205^+CD103^-$ DC subpopulation (**Figure 1D**). This implies that NSCLC cells may modulate the development of the $CD1c^+$ DC subset mediated by CD205 and CD103 *in vivo*.

2. H-1299 tumor cells regulate the development of $CD1c^+$ DC subsets derived from NSCLC patients mediated by CD205 and CD103.

Our data indicated that co-culture with H-1299 tumor cells modulates the development of $CD1c^+$ DC subpopulations, which is mediated by CD205 and CD103, derived from healthy donors (**Supplementary Figure 6**). We proposed that H-1299 tumor cells may also regulate the differentiation of $CD1c^+$ DC subsets isolated from NSCLC patients. To investigate this hypothesis, DCs isolated from three NSCLC patients were incubated with H-1299 tumor cells or were incubated without tumor cells as a control. The protein expression of CD205 (**Figure 2A**) and CD103 (**Figure 2B**) on $CD1c^+$ DCs was detected by flow cytometry. Our data show that coculture with H-1299 cells upregulated the expression of CD205 but downregulated the expression of CD103 on $CD1c^+$ DCs compared with that of those on $CD1c^+$ DCs that were not cocultured with H-1299 tumor cells (**Figures 2A,B**). In addition, incubation with H-1299 tumor cells suppressed the development of the $CD1c^+CD205^+CD103^+$ DC subset, but it facilitated the differentiation of the $CD1c^+CD205^+CD103^-$ DC subpopulation when compared with that of DCs that were not cocultured with H-1299 cells (**Figures 2C,D**). It can be concluded that coculture

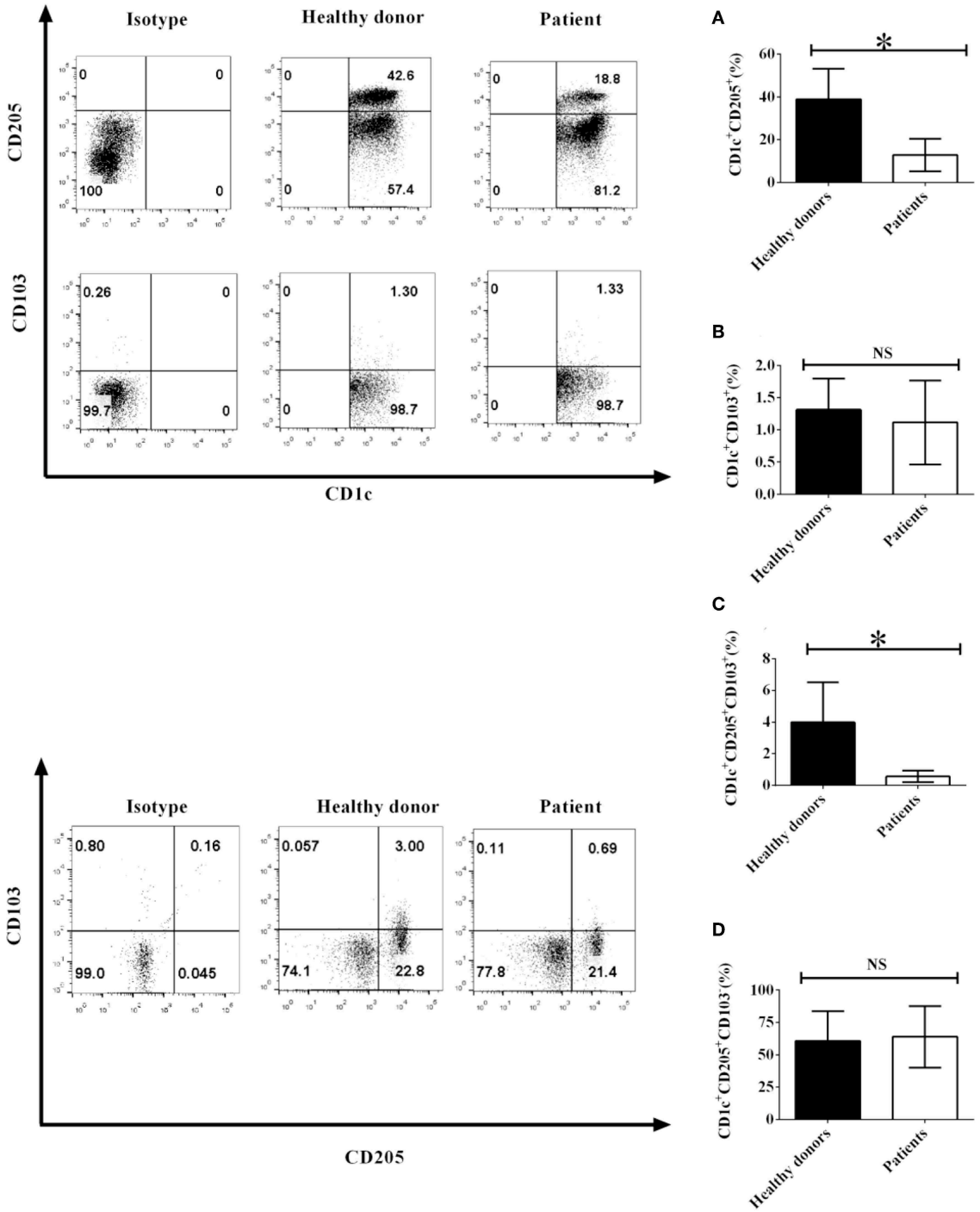

FIGURE 1 | The phenotypes of CD1c$^+$ DC subsets mediated by CD103 and CD205 in NSCLC patients and healthy donors. PBMCs from NSCLC patients and healthy donors were collected and stained with human CD1c, CD103, CD205, and lineage (Lin) antibodies. Lin$^-$CD1c$^+$ cells were gated like those shown in **Supplementary Figure 1**. Protein expression of CD205 **(A)** and CD103 **(B)** on CD1c$^+$ DCs and the frequencies of CD1c$^+$CD205$^+$CD103$^+$ DCs **(C)** and CD1c$^+$CD205$^+$CD103$^-$ DCs **(D)** were determined. The error bars shown in this figure represent the mean and SD of quadruplicate determinations from one experiment (*$P < 0.05$, $n = 4$, t-test).

with H-1299 tumor cells modulates the development of CD1c$^+$ DC subsets derived from NSCLC patients mediated by CD205 and CD103.

3. Primary NSCLC cells modulate the development and differentiation of CD1c$^+$ DC subsets derived from NSCLC patients mediated by CD205 and CD103.

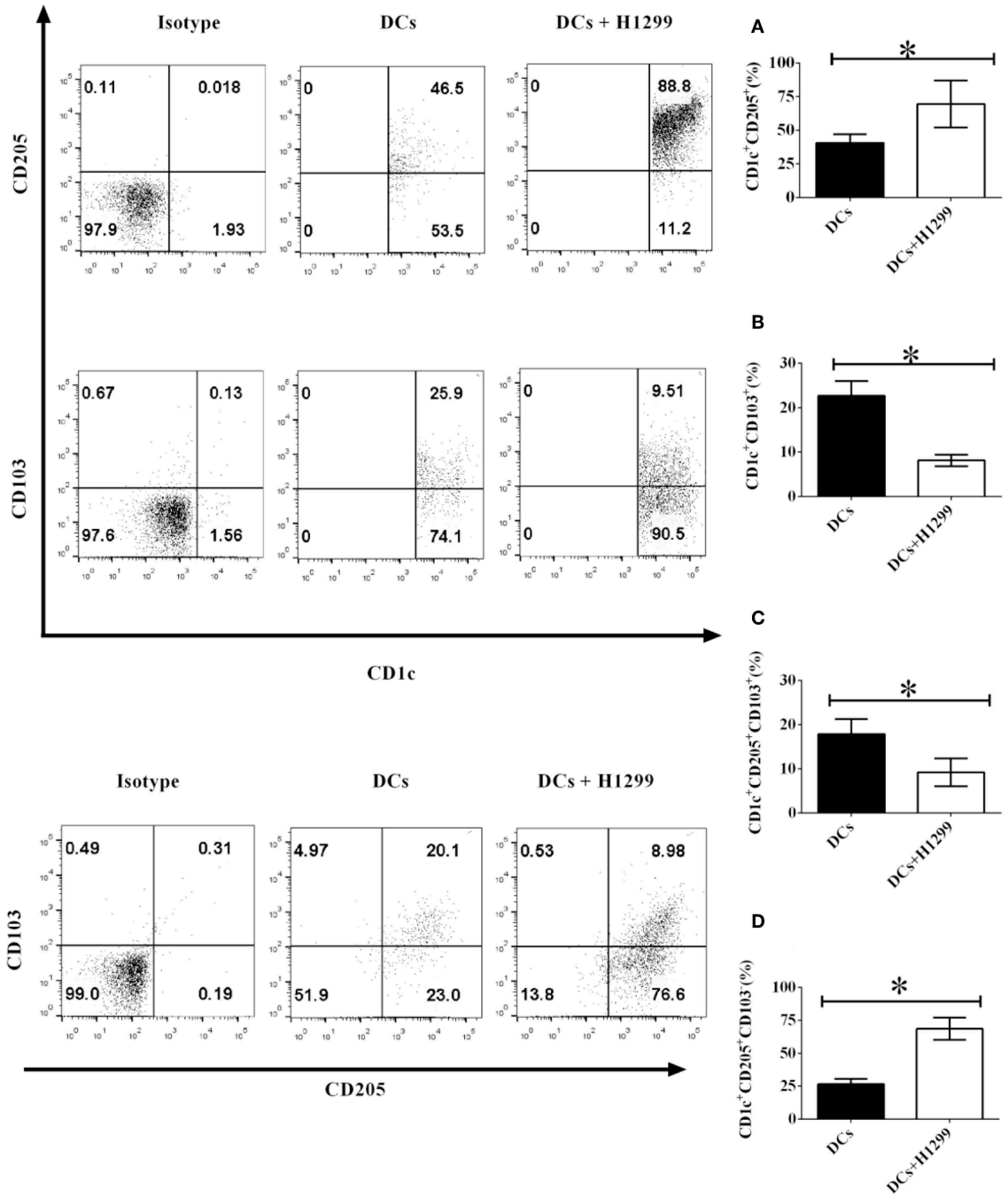

FIGURE 2 | H-1299 tumor cells regulate the development of CD1c$^+$ DC subpopulations derived from NSCLC patients mediated by CD205 and CD103. PBMCs from three NSCLC patients were collected and stained with human CD1c, CD103, CD205, and lineage antibodies. Lin$^-$CD1c$^+$ cells were gated. Protein expression of CD205 **(A)** and CD103 **(B)** on CD1c$^+$ DCs was tested by flow cytometry. The frequencies of the CD1c$^+$CD205$^+$CD103$^+$ DCs **(C)** and CD1c$^+$CD205$^+$CD103$^-$ DCs **(D)** were determined. The error bars shown in this figure represent the mean and SD of triplicate determinations of the frequency of CD1c$^+$ subpopulations in three independent experiments (*$P < 0.05$, $n = 3$, *t*-test).

Our data showed that the NSCLC cell line H-1299 can modulate CD1c$^+$ DC subset development mediated by CD205 and CD103 when they are cocultured with DCs derived from NSCLC patients (**Figure 2**). We hypothesized that primary NSCLC cells may also regulate the development of CD1c$^+$ DC subpopulations through modulating the expression of CD205 and CD103 on DCs. To investigate this hypothesis, primary NSCLC cells were isolated from the cancer tissue from two NSCLC patients and

cocultured with DCs derived from the same patients. The protein expression of CD205 (**Figure 3A**) and CD103 (**Figure 3B**) on $CD1c^+$ DCs treated with primary tumor cells or without incubation with primary NSCLC cells was detected by flow cytometry. The experimental data indicate that the expression of CD205 on $CD1c^+$ DCs was increased after coculture with primary tumor cells compared with that on $CD1c^+$ DCs without incubation with primary NSCLC cells (**Figure 3A**). In contrast, CD103 expression on $CD1c^+$ DCs incubated with primary NSCLC cells was downregulated compared with that on $CD1c^+$ DCs without coculture with primary tumor cells (**Figure 3B**). In addition, coculture with primary NSCLC cells downregulated the differentiation of the $CD1c^+CD205^+CD103^+$ DC subset compared with that of DCs without incubation with primary tumor cells (**Figure 3C**); however, incubation with primary tumor cells facilitates the development of the $CD1c^+CD205^+CD103^-$ DC subpopulation compared with that without coculture with primary NSCLC cells (**Figure 3D**). It can be concluded that primary NSCLC cells also modulate the development and differentiation of $CD1c^+$ DC subsets derived from NSCLC patients mediated by CD205 and CD103.

4. H-1299 tumor cells suppress the expression of signal molecules on $CD1c^+$ DCs derived from NSCLC patients.

Since our results indicate that H-1299 tumor cells downregulate the expression of CD40, CD80, CD86, and HLA-DR on $CD1c^+$ DCs isolated from healthy donors (**Supplementary Figure 4**), we proposed that H-1299 cells may also block the expression of costimulatory molecules on $CD1c^+$ DCs derived from NSCLC patients. To investigate this hypothesis, DCs isolated from three NSCLC patients were incubated with H-1299 tumor cells or were not cocultured with H-1299 cells as a control. The protein expression of CD40 (**Figure 4A**), CD80 (**Figure 4B**), CD86 (**Figure 4C**), and HLA-DR (**Figure 4D**) was detected by flow cytometry. Our results demonstrated that the expression of CD40, CD80, CD86, and HLA-DR was downregulated after coculture with H-1299 tumor cells compared with that on $CD1c^+$ DCs that were not incubated with H-1299 cells (**Figure 4**). It can be concluded that H-1299 tumor cells also suppress the expression of signal molecules on $CD1c^+$ DCs derived from NSCLC patients, similar to their effect on $CD1c^+$ DCs isolated from healthy donors (**Supplementary Figure 4**).

5. Primary NSCLC cells also inhibit the protein expression of signal molecules on $CD1c^+$ DCs derived from NSCLC patients.

Our results demonstrated that coculture with H-1299 NSCLC cells leads to the downregulation of the expression of signal molecules, such as CD40, CD80, CD86, and HLA-DR, on $CD1c^+$ DCs (**Figure 4**); however, H-1299 is a tumor cell line, and we are not certain whether primary NSCLC cells also suppress the expression of costimulatory molecules on DCs. To investigate whether primary NSCLC cells modulate the expression of signal molecules on $CD1c^+$ DCs, primary tumor cells were isolated from tumor tissues of two NSCLC patients, and the primary tumor cells were incubated with DCs induced with PBMCs derived from the same patients. DCs without coculture with primary tumor cells served as a control. The protein expression of CD40 (**Figure 5A**), CD80 (**Figure 5B**), CD86 (**Figure 5C**), and HLA-DR (**Figure 5D**) on $CD1c^+$ DCs was detected by flow cytometry. The experimental data showed that the protein expression of CD40, CD80, CD86, and HLA-DR on $CD1c^+$ DCs was downregulated after co-culture with primary NSCLC cells compared with that on $CD1c^+$ DCs that were not cocultured with tumor cells (**Figures 5A–D**). It can be concluded that primary NSCLC cells are able to downregulate the expression of CD40, CD80, CD86, and HLA-DR on $CD1c^+$ DCs after incubation with DCs derived from the same NSCLC patients.

6. H-1299 tumor cells modulate the production of pro- and anti-inflammatory cytokines in $CD1c^+$ DCs isolated from NSCLC patients.

Our data showed that H-1299 cells regulate the secretion of pro- and anti-inflammatory cytokines in $CD1c^+$ DCs derived from healthy donors compared with those that were not cocultured with H-1299 cells (**Supplementary Figure 5**). We hypothesized that H-1299 cells may also affect the production of pro- and anti-inflammatory cytokines in $CD1c^+$ DCs isolated from NSCLC patients. To test this hypothesis, DCs derived from three NSCLC patients were incubated with H-1299 tumor cells. DCs without incubation with H-1299 cells served as a control. Our results demonstrate that coculture with H-1299 tumor cells leads to the upregulation of IL-6, IL-10, and IL-27 production by $CD1c^+$ DCs compared with that by $CD1c^+$ DCs that were not cocultured with H-1299 cells (**Figures 6A,B,E**). In contrast, incubation with H-1299 cells causes the downregulation of IL-12 and IL-23 production by $CD1c^+$ DCs compared with that by $CD1c^+$ DCs that were not cocultured with H-1299 cells (**Figures 6C,D**). Moreover, H-1299 cells do not affect the production of TGF-β in $CD1c^+$ DCs compared with that in $CD1c^+$ DCs that were not cocultured with H-1299 cells (**Figure 6F**). These results are the same as those obtained with $CD1c^+$ DCs derived from healthy donors, as shown in **Supplementary Figure 5**. It can be concluded that H-1299 tumor cells can modulate the production of pro- and anti-inflammatory cytokines by $CD1c^+$ DCs derived from both healthy donors and NSCLC patients.

7. Primary NSCLC cells modulate the production of pro- and anti-inflammatory cytokines by $CD1c^+$ DCs derived from NSCLC patients.

Since our data show that the NSCLC cell line H-1299 regulates the secretion of multiple cytokines by $CD1c^+$ DCs (**Figure 6**), we propose that primary NSCLC cells may also affect the production of pro- and anti-inflammatory cytokines by DCs *in vivo*. To test this hypothesis, primary NSCLC cells were isolated from tumor tissue and cocultured with DCs derived from the same NSCLC patients. DCs that are not cocultured with primary tumor cells served as a control. The production of the cytokines IL-6 (**Figure 7A**), IL-10 (**Figure 7B**), IL-12 (**Figure 7C**), IL-23 (**Figure 7D**), IL-27 (**Figure 7E**), and TGF-β (**Figure 7F**) by $CD1c^+$ DCs was detected by flow cytometry. Our results indicate that coculture with primary NSCLC cells downregulates the production of IL-6, IL-12, and IL-23 by $CD1c^+$ DCs compared with that of $CD1c^+$ DCs that are

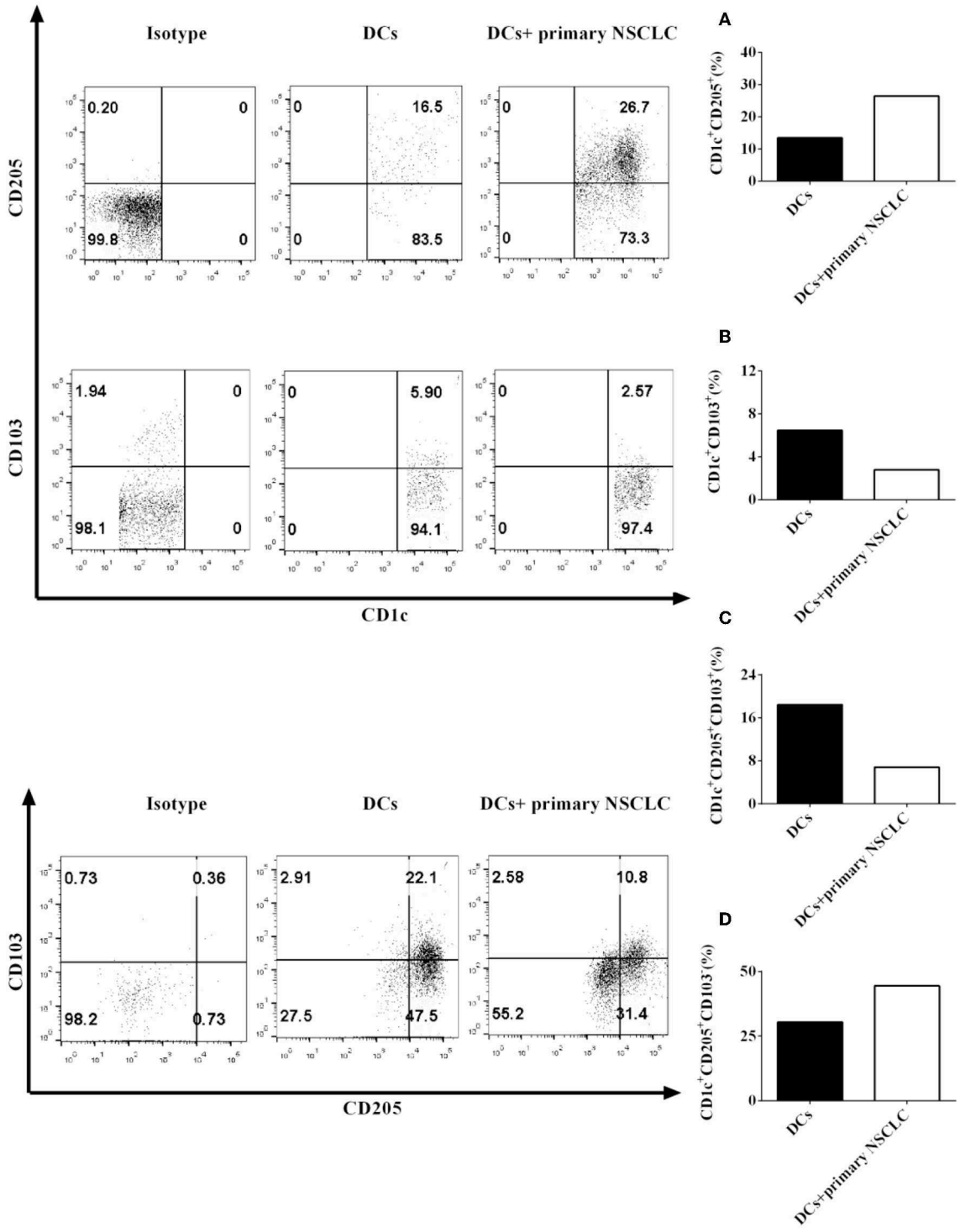

FIGURE 3 | Primary NSCLC cells regulate the development of CD1c$^+$ DC subpopulations derived from NSCLC patients mediated by CD205 and CD103. Primary tumor cells and PBMCs from two NSCLC patients were collected and stained with human CD1c, CD103, CD205, and lineage antibodies. Lin$^-$CD1c$^+$ cells were gated. Protein expression of CD205 **(A)** and CD103 **(B)** on CD1c$^+$ DCs was detected by flow cytometry. The frequencies of the CD1c$^+$CD205$^+$CD103$^+$ DC subset **(C)** and the CD1c$^+$CD205$^+$CD103$^-$ DC subpopulations **(D)** were determined. The statistical figure shows the mean of determinations of the frequency of the CD1c$^+$ subpopulations in two independent experiments ($n = 2$).

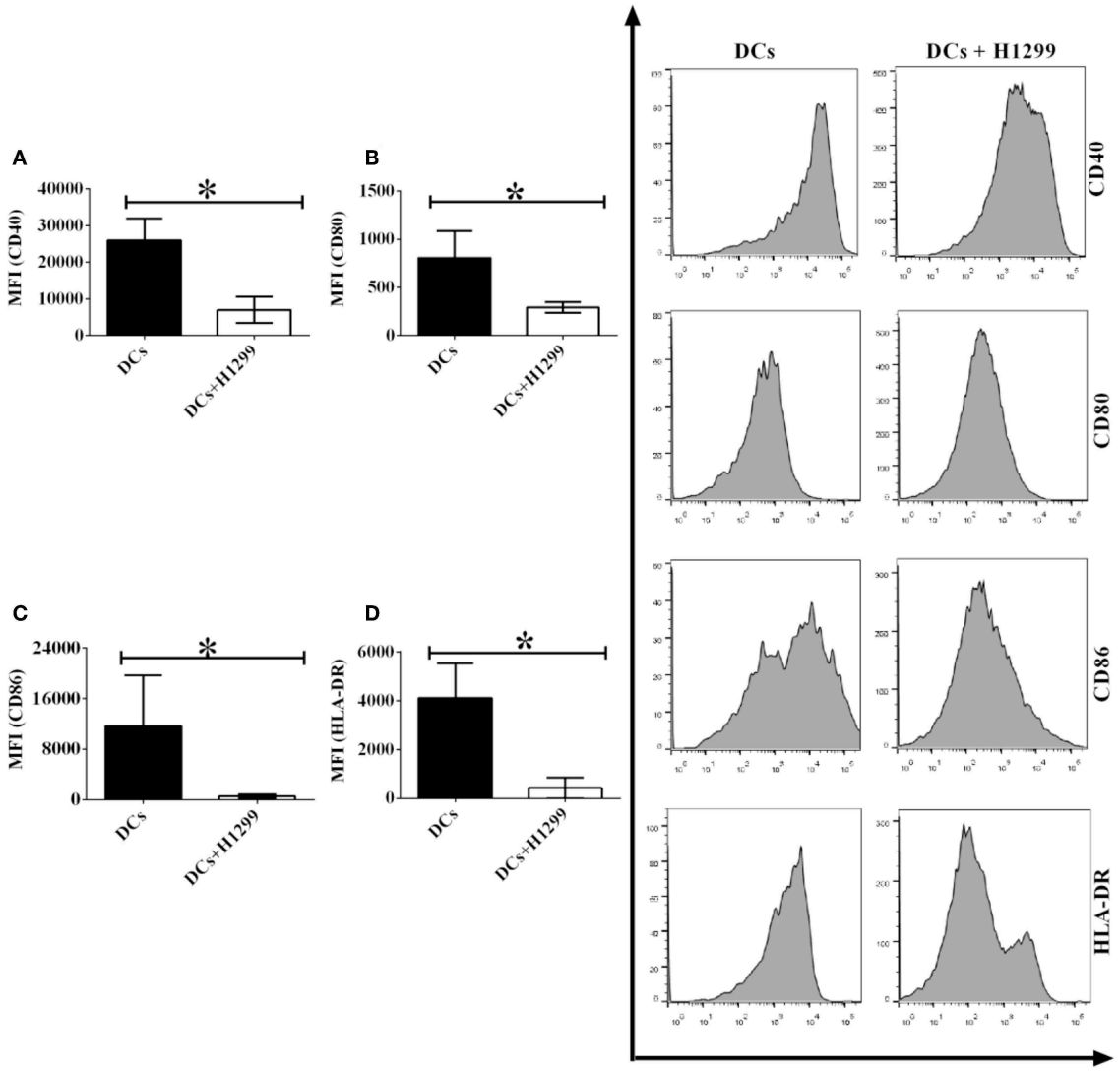

FIGURE 4 | H-1299 tumor cells suppress the expression of signal molecules on CD1c$^+$ DCs derived from NSCLC patients. PBMCs were isolated from three NSCLC patients and induced the development of DCs *in vitro*. DCs were incubated with H-1299 tumor cells for 24 h or were not cocultured with H-1299 cells as a control. DCs were stained with human CD1c, CD40, CD80, CD86, HLA-DR, and lineage antibodies. A flow cytometry assay was conducted, and Lin$^-$CD1c$^+$ cells were gated. Protein expression of CD40 **(A)**, CD80 **(B)**, CD86 **(C)**, and HLA-DR **(D)** on CD1c$^+$ DCs is shown. The error bars indicated in this figure represent the mean and SD of triplicate determinations of the mean fluorescence identities (MFI) in three independent experiments (*$P < 0.05$, $n = 3$, *t*-test).

not cocultured with primary tumor cells (**Figures 7A,C,D**). In contrast, the secretion of IL-10 and IL-27 by CD1c$^+$ DCs is enhanced after coculture with primary NSCLC cells compared with that by DCs that are not cocultured with primary NSCLC cells (**Figures 7B,E**). In addition, the experimental data demonstrate that the production of TGF-β by CD1c$^+$ DCs incubated with primary tumor cells is similar to that by CD1c$^+$ DCs that are not cocultured with primary tumor cells (**Figure 7F**). Since pro- and anti-inflammatory cytokines produced by DCs play an important role in regulating innate and adaptive immunity, our results suggest that primary NSCLC cells may affect DC-mediated immune function via modulating the production of pro- and anti-inflammatory cytokines *in vivo*. In addition, we also observed the expression of costimulatory molecules and production of pro-/anti-inflammatory cytokines by DCs derived from healthy donors and NSCLC patients (**Supplementary Figures 2**, **3**). The data of absolute numbers of DC subsets mediated by CD103 and CD205 were shown in **Supplementary Figure 7** (**Supplementary Results**).

DISCUSSION

We investigated the effect of NSCLC cells on development of CD1c$^+$ cDCs that are reported as one of three DC populations in human peripheral blood in Ziegler-Heitbrock et al. (14). Granot et al. reported that CD1c$^+$ DCs are the major typical DCs in lung-draining lymph nodes. CD1c$^+$ DCs play an important role

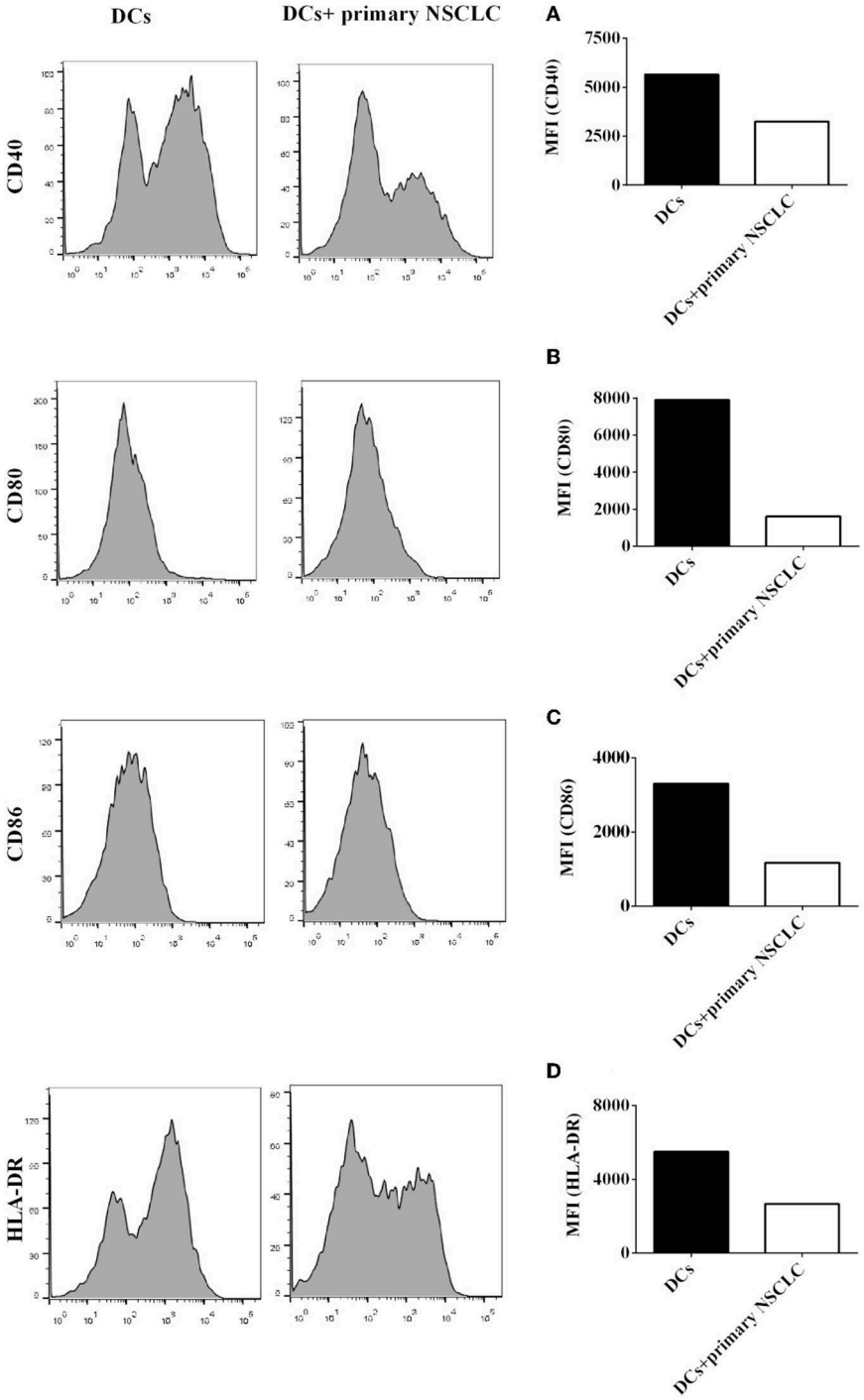

FIGURE 5 | Primary NSCLC cells suppress the expression of signal molecules on CD1c$^+$ DCs derived from NSCLC patients. Primary tumor cells were separated from tumor tissue of two NSCLC patients. PBMCs were also isolated from the same patients and induced the development of DCs *in vitro*. DCs were incubated with primary NSCLC cells for 24 h or were not cultured with primary tumor cells as a control. DCs were stained with human CD1c, CD40, CD80, CD86, HLA-DR, and lineage antibodies. A flow cytometry assay was conducted, and Lin$^-$CD1c$^+$ cells were gated. Protein expression of CD40 **(A)**, CD80 **(B)**, CD86 **(C)**, and HLA-DR **(D)** on CD1c$^+$ DCs is shown. The statistical figure shows the mean of duplicate determinations of the mean fluorescence identities in two independent experiments ($n = 2$).

in immune surveillance in local lung tissue (19). It is unclear whether NSCLC cells affect the immune function of CD1c$^+$ DCs *in vivo*. Recent research has indicated that there are multiple subsets of CD1c$^+$ DCs in humans. For example, Borriello et al. found that human CD14$^+$CD1c$^+$ DCs were induced by lipopolysaccharide (LPS) stimulation (20). De Monte et al. found

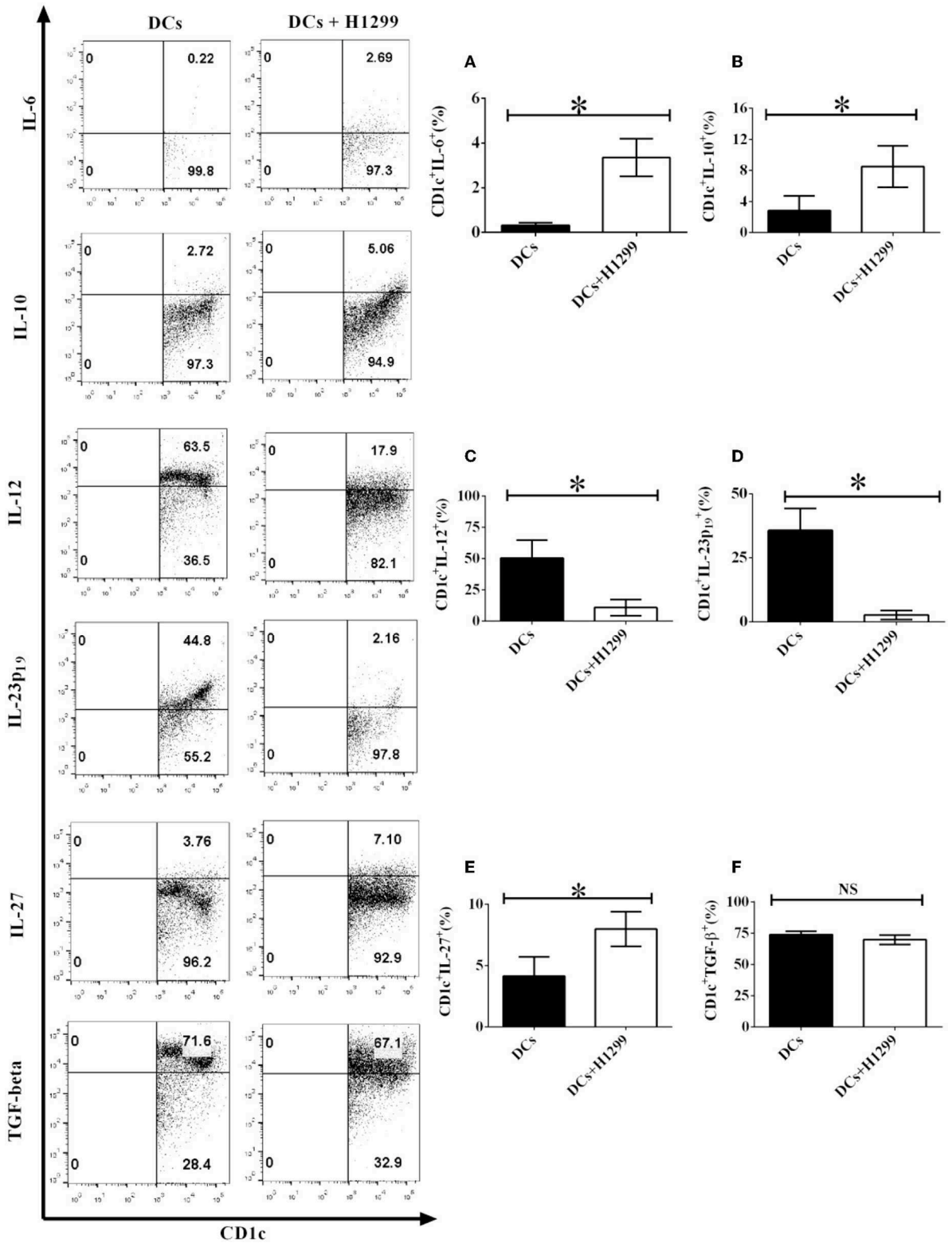

FIGURE 6 | H-1299 tumor cells modulate the production of pro- and anti-inflammatory cytokines by $CD1c^+$ DCs derived from NSCLC patients. PBMCs were obtained from three NSCLC patients. PBMCs were cultured in DC medium for 8 days to induce the development of DCs. DCs were stained with human CD1c, lineage, IL-6, IL-10, IL-12, IL-23 (p19), IL-27, and TGF-β antibodies. A flow cytometry assay was carried out and lin^-CD1c^+ cells were gated. Cytokine production, including IL-6 **(A)**, IL-10 **(B)**, IL-12 **(C)**, IL-23p19 **(D)**, IL-27 **(E)**, and TGF-β **(F)**, by $CD1c^+$ DCs was determined. The error bars shown in this figure represent the mean and SD of triplicate determinations of the frequency of cytokine production by $CD1c^+$ DCs in three independent experiments ($^*P < 0.05$, $n = 3$, *t*-test).

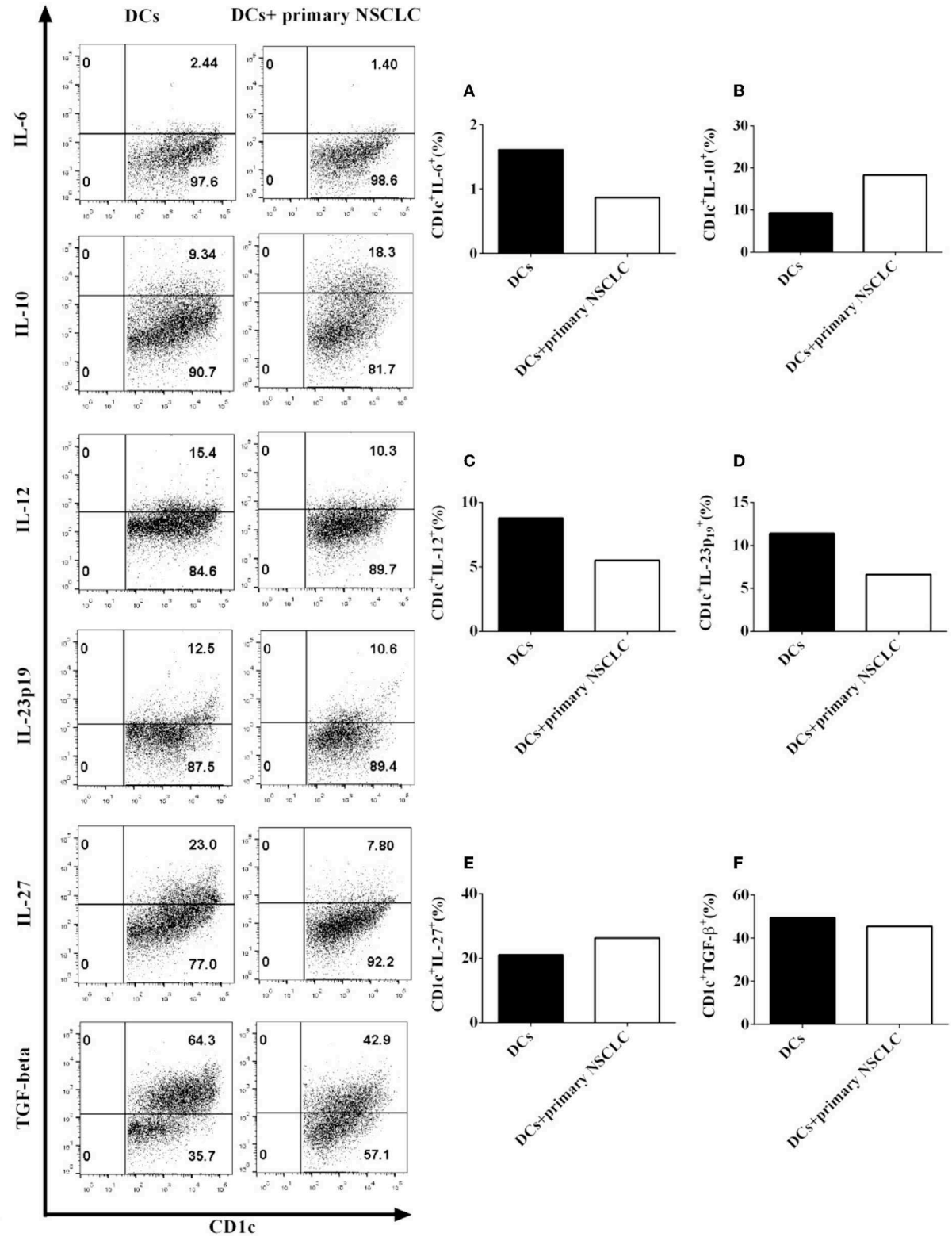

FIGURE 7 | Primary NSCLC cells modulate the production of pro- and anti-inflammatory cytokines by CD1c$^+$ DCs derived from two NSCLC patients. Primary tumor cells and PBMCs were isolated from the same NSCLC patients. PBMCs were cultured in DC medium for 8 days to induce the development of DCs. DCs were stained with human CD1c, lineage, IL-6, IL-10, IL-12, IL-23 (p19), IL-27, and TGF-β antibodies. A flow cytometry assay was carried out, and lin$^-$CD1c$^+$ cells were gated. The cytokine production of IL-6 **(A)**, IL-10 **(B)**, IL-12 **(C)**, IL-23 (p19) **(D)**, IL-27 **(E)**, and TGF-β **(F)** by CD1c$^+$ DCs was determined. The statistical figure shows the mean of duplicate determinations of cytokine production by CD1c$^+$ DCs in two independent experiments ($n = 2$).

that $CD1c^+$ $CD207^+$ DCs were present in human tonsils (21). Data from Zaba demonstrated that $CD11c^+$ $CD1c^+$ DCs were found in the upper dermis and could activate T cells (22). Since $CD1c^+$ DCs play an important role in innate and adaptive immunity (15, 23), it is necessary to reveal the effect of NSCLC on the differentiation of $CD1c^+$ DC subsets.

Two new $CD1c^+$ DC subsets ($lin^-CD1c^+CD205^+CD103^+$ DCs and $lin^-CD1c^+CD205^+CD103^-$ DCs) were identified in healthy donors and NSCLC patients (**Figures 1C,D**). Coculture with NSCLC cells led to the suppression of the development of the $lin^-CD1c^+CD205^+CD103^+$ DC subset (**Figure 2C**). At present, the immune function of $lin^-CD1c^+CD205^+CD103^+$ DCs is still unknown. CD205 is expressed on DCs and is a recognition receptor for necrotic and apoptotic cells (24). $CD205^+$ DCs engulf target cells through the CD205-mediated endocytosis pathway and present antigen epitopes to $CD4^+$ and $CD8^+$ T cells for recognition (25–27). The number of $lin^-CD1c^+CD205^+$ DCs in NSCLC patients was lower than that in healthy donors (**Figure 1A**). Our results imply that there are fewer $CD205^+$ DCs in NSCLC patients. This may decrease the efficiency of the endocytosis of apoptotic and necrotic tumor cells by DCs, which may reduce antigen presentation that induces $CD4^+$/$CD8^+$ T cell-mediated anti-tumor immunity.

Interestingly, Yamazaki et al. reported that $CD8^+CD205^+$ splenic DCs facilitate the development of regulatory T cells (T_{regs}) (28). Since there are more T_{regs} in NSCLC patients than in healthy people (29) and our data indicate that coculture with NSCLC cells elicits the development of $lin^-CD1c^+CD205^+$ DCs (**Figure 2A**), NSCLC cells may facilitate the differentiation of T_{regs} via modulating the development of $lin^-CD1c^+CD205^+$ DCs *in vivo*.

$CD103^+$ DCs play an important role in the induction of anti-tumor immunity. For instance, Mittal et al. found that $CD103^+$ DCs produce IL-12 via a basic leucine zipper ATF-like transcription factor 3 (BATF3)-mediated pathway to activate NK cells and inhibit tumor metastasis (30). Our results demonstrated that coculture with NSCLC cells blocks the development of $lin^-CD1c^+CD103^+$ DCs (**Figure 2B**). These results suggest that NSCLC cells may inhibit NK cell-mediated anti-tumor immunity through suppressing the immune function of $lin^-CD1c^+CD103^+$ DCs *in vivo*.

Interestingly, NSCLC cells elicit the development of $lin^-CD1c^+CD205^+CD103^+$ DCs derived from healthy donors (**Supplementary Figure 2C**) but inhibit the differentiation of $lin^-CD1c^+CD205^+CD103^+$ DCs derived from NSCLC patients (**Figures 2C**, **3C**). Our results imply that DCs in NSCLC patients may be different from those isolated from healthy donors. Their biological function may be blocked due to the NSCLC-induced immune suppressive microenvironment. Future work needs to be conducted to determine the reason why NSCLC cells have different effects on the development of the $CD1c^+$ DC subsets isolated from NSCLC patients and healthy donors.

DCs regulate immune function via the Signal 1, 2, and 3 transduction pathways. MHC I and II molecules on DCs bind to CTL epitopes and associate with T cell receptors (TCRs) for target cell recognition (Signal 1) (31). Furthermore, there are multiple costimulatory molecules, such as CD80 and CD86, expressed on DCs. These signal molecules bind to ligands expressed on T cells to modulate T cell activation (Signal 2) (32). For example, CD40 expressed on DCs binds to CD40L presented on T cells to initiate T cell-mediated immune responses. CD80 and CD86 expressed on DCs bind to CD28 and CD152 presented on T cells to induce T cell proliferation and are necessary for T cell survival (32, 33). In addition, DCs also produce cytokines to modulate the activation of immune cells (Signal 3) (34, 35). These are the molecular basis of the central role played by DCs in regulating the biological function of the immune system.

It is still unclear whether NSCLC cells can affect DC-mediated immune responses through regulating the protein expression of signal molecules expressed on DCs. We systemically investigated the effect of NSCLC cells on the expression of Signal 1-, 2-, and 3-associated molecules on $CD1c^+$ DCs (**Figures 4–7**). Coculture with NSCLC cells leads to the downregulation of the expression of CD40, CD80, CD86, and HLA-DR on human $CD1c^+$ DCs (**Figures 4**, **5**). The biological features of NSCLC-incubated DCs are similar to those of tolerogenic DCs, which have been previously used for DC-mediated immunotherapy to target autoimmune diseases (36–43). Our results suggest that NSCLC cells may be able to induce tolerogenic DCs with the low expression of costimulatory molecules and MHCs so that DC-mediated immune responses that are dependent on Signal 1-, 2-, and 3-associated molecules expressed on DCs are inhibited. NSCLC-induced tolerogenic DC subsets may function as part of the cellular mechanism involved in the NSCLC-mediated immune suppressive microenvironment *in vivo*.

DCs also produce multiple cytokines to modulate immune responses (44). For example, DCs secrete several pro-inflammatory cytokines, including IL-6, IL-12, and IL-23, to facilitate T cell-mediated immune responses (45). Nizzoli et al. reported that human $CD1c^+$ DCs activate cytotoxic T lymphocytes via IL-12 produced by $CD1c^+$ DCs (46). Aliahmadi et al. found that human Langerhans cells with activation of the Toll-like receptor 2-mediated signal transduction pathway facilitate the development of T helper 17 (Th17) cells through the IL-1-beta, IL-23, and TGF-beta-mediated signal transduction pathways (47). Since NSCLC cells downregulate the production of IL-12 and IL-23 in $CD1c^+$ DCs (**Figures 6C,D**), tolerogenic $CD1c^+$ DCs may block T cell-mediated anti-tumor immunity via suppressing the production of IL-12 and IL-23 by $CD1c^+$ DCs, which are necessary for T cell activation *in vivo*.

Both pro-inflammatory cytokines and anti-inflammatory cytokines can be produced by DCs (45). For example, DCs secrete IL-10, IL-27, and TGF-beta to modulate $CD8^+$ and $CD4^+$ T cell-mediated immune responses (34). Nizzoli et al. reported that $CD1c^+$ DCs shape naive $CD8^+$ T cell priming via IL-10-mediated signaling produced by $CD1c^+$ DCs (48). Tsoumakidou et al. found that tolerogenic $CD1c^+$ DCs derived from chronic obstructive pulmonary diseases (COPD) induce the generation of $CD4^+$ T_{regs} through IL-10- and IL-27-induced costimulatory ligands (49). It is known that there are more T_{regs} in NSCLC patients (29). Since NSCLC cells facilitate the production of IL-10 and IL-27 in $CD1c^+$ DCs (**Figures 6B,E**), NSCLC cells may block the activity of $CD8^+$ T cells and elicit the development of $CD4^+$ T_{regs} through IL-10 and IL-27 produced

by $CD1c^+$ DCs *in vivo*. This may be one aspect of the cellular and molecular mechanisms involved in the NSCLC-mediated immune suppressive microenvironment in NSCLC patients. We will conduct further studies of $CD1c^+$ DC subset-mediated T cell responses in the future.

It is still unclear how NSCLC cells modulate the development of $CD1c^+$ cDC subsets mediated by CD103 and CD205. It has been known that NSCLC cells can produce anti-inflammatory cytokines such as IL-10 and TGF-β, which may lead to tumor tolerance in NSCLC patients. In addition, NSCLC cells facilitate the production of TGF-β by DCs and elicit the development of T_{reg} in NSCLC patients so that the immune function of patients is inhibited. This probably is one of the mechanisms of immune suppressive microenvironment mediated by NSCLC *in vivo*. We will continue to investigate the molecular mechanisms of NSCLC-induced immune tolerogenic $CD1c^+$ DC subsets mediated by CD103 and CD205 in the future so that the cellular mechanisms of NSCLC-mediated immune suppressive microenvironment can be further elucidated.

In summary, we investigated the effect of NSCLC on the development of $CD1c^+$ DC subsets mediated by CD205 and CD103 in this project. We identified two new subpopulations of $CD1c^+$ DCs: $lin^-CD1c^+CD205^+CD103^+$ DCs and $lin^-CD1c^+CD205^+CD103^-$ DCs. NSCLC cells specifically suppress the development of $lin^-CD1c^+CD205^+CD103^+$ DCs. In addition, NSCLC cells downregulate the expression of costimulatory molecules (CD80 and CD86) and pro-inflammatory cytokines (IL-12 and IL-23); however, NSCLC cells facilitate the secretion of anti-inflammatory cytokines (IL-10) in $CD1c^+$ DCs. It can be concluded that NSCLC cells may induce the production of a tolerogenic $CD1c^+$ DC subset and thereby block anti-tumor immunity *in vivo*. Tolerogenic $CD1c^+$ DC subsets mediated by CD205 and CD103 may play an important role in the NSCLC-induced immune suppressive microenvironment.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Cas-lamvac Biotech Co., Ltd. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL, WX, YG, XChang, GW, and ZR conducted the experiments. LQ and XChen analyzed data and supervised project. FZ designed the experiments, supervised the research, and wrote 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.2019.02829/full#supplementary-material

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Long-Term Ibrutinib Therapy Reverses CD8$^+$ T Cell Exhaustion in B Cell Chronic Lymphocytic Leukaemia

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Chronic Lymphocytic Leukaemia (CLL) is associated with immune suppression and susceptibility to infection. CD8$^+$ T cell numbers are increased and demonstrate elevated expression of PD-1 and impaired function. The mechanisms driving these features of exhaustion are uncertain but are likely to include chronic immune recognition of tumor and/or infectious agents. We investigated the number, phenotype and function of total and virus-specific CD8+ T cells in 65 patients with CLL and 14 patients undergoing long-term ibrutinib therapy (median 21 months). Ibrutinib substantially reduced the number of both CD3+ T cells and CD8+ T cells. Importantly, this was associated with a reduction in PD-1 expression on CD8+ T cells (median 28 vs. 24%; $p = 0.042$) and 3.5 fold increase in cytokine production following mitogen stimulation. The influence of ibrutinib on antigen-specific CD8+ T cell function was assessed by HLA-peptide tetramers and revealed increased IFNγ and TNFα cytokine responses following stimulation with CMV or EBV peptides together with a 55% reduction in the frequency of "inflated" virus-specific CD8+ T cells. These findings reveal that long-term ibrutinib therapy is associated with substantial reversal of T cell exhaustion in B-CLL and is likely to contribute to the reduced infection risk seen in association with this agent.

Keywords: ibrutinib, chronic lymphocytic leukaemia (CLL), herpes viruses, exhaustion, EBV—epstein-barr virus, CD8 T cells, cytomegalovirus, immunotherapy

INTRODUCTION

Chronic lymphocytic leukaemia (CLL) is associated with marked perturbation of the immune system. A range of defects in T cell function are observed including impaired proliferation, cytotoxicity and cytokine production (1). Increased absolute numbers of CD8+ T cells, expanded populations of oligoclonal memory CD8+ T cells (2) and increased expression of immune checkpoint receptors including PD-1 also feature (3). The etiology of these abnormalities is unclear but may include chronic immune stimulation through infection and/or tumour engagement.

Ibrutinib inhibits Bruton Tyrosine Kinase (BTK) activity and has transformed CLL management (4). Ibrutinib has also been confirmed by molecular techniques to irreversibly inhibit interleukin-2 inducible kinase (ITK). ITK plays an important but not indispensable role in the CD4+ Th1 and CD8+ T cell activation signaling cascade, contributing to enhanced proliferation and activation following TCR ligation. In contrast, its role is pivotal and necessary for CD4+ Th2 polarization and function. As such, inhibition of ITK by ibrutinib encourages a skewing towards a Th1 phenotype and has been shown to advantage CD8+ and Th1 T cells, which rely on the redundant resting lymphocyte kinase (RLK) during ITK inhibition. RLK is a signaling kinase which is not inhibited by ibrutinib and provides additional activation of the TCR signaling cascade in the absence of functional ITK. Data on the impact of ibrutinib-induced ITK inhibition within CD8+ T cell populations is currently lacking (5). Understanding the impact of long-term ibrutinib therapy on immune function is an important question and analysis of antigen-specific responses is also currently lacking. Cytomegalovirus (CMV) and Epstein Barr Virus (EBV) are latent herpesvirus infections which infect the majority of the population. CMV and EBV are known to cause "memory inflation," a term used to describe the expansion of memory CD8+ T cells directed towards the virus and can arise in healthy individuals but also in patients with immune suppression. (2). Expanded populations of CMV-specific CD8+ T cells develop in patients with CLL that are latently infected with the virus.

T cells are known to be dysfunctional in patients with CLL. The term cell exhaustion is used to describe a state of T cell dysfunction that occurs through chronic antigen stimulation and can arise in the context of chronic viral infection or cancer. Exhausted T cells are characterized by the presence of multiple inhibitory receptors, poor proliferation, and cytotoxicity and impaired cytokine secretion (6). Patients with CLL are known to have features of T cell exhaustion with co-expression of CD244, PD-1, and CD160 at high frequencies (1).

Here we examine global and virus-specific T cell phenotype and function in patients with CLL including patients receiving ibrutinib therapy for up to 32 months. Decreased PD-1 expression and increased cytokine responses were observed within the global T cell repertoire following ibrutinib treatment whilst antigen-specific responses also showed increased functional activity and correction of the increased frequency of virus-specific cells.

METHODS

Seventy-nine patients with CLL were recruited [median age 70 (IQR: 63–79)], including 42 patients who had never been treated and 36 who had received chemo-immunotherapy. Of the 36 patients who had previously been treated, 23 were in remission, whilst 13 patients developed relapsed/refractory disease and subsequently were started on ibrutinib therapy, together with 1 patient who was treated with ibrutinib in a front line setting. The 14 patients being treated with ibrutinib, had received up to 32 months of therapy at the time of analysis. Samples were collected immediately prior to starting ibrutinib therapy and then during a subsequent clinic visit which occurred at least 6 months after starting ibrutinib. All patients included in this study were still taking the drug daily at the point the last sample was taken for analysis. Patients characteristics for the total cohort and patient subgroups can be found in (**Supplementary Tables 1–4**). Nineteen healthy donors were recruited for controls [median age 72 (IQR. 66–80)].

Following ficoll preparation, plasma and PBMCs were extracted, with CMV and EBV serostatus determined by ELISA and immunofluorescence, respectively (7, 8). DNA extraction was then performed on PBMC pellets using GenElute Mammalian Genomic DNA Miniprep kit (Sigma-Aldrich) and HLA typing was assigned using PCR methodology previously described (9).

Immunophenotypic Analysis of CMV and EBV-Specific CD8+ T Cells

Immunophenotyping was undertaken following APC-conjugated HLA class I tetramer staining of PBMCs at 37°C for 15 min. Details of the tetramers used can be found in **Supplementary Table 5**. Tetramers were conjugated to APC and a true tetramer response was verified through the lack of background staining by gating all CD3+ T cells, against CD8+ T cells and using a tetramer negative control. Surface staining with the following antibodies was then performed: live/dead blue dye (Invitrogen), anti-CD8 Amcyan (BD Biosciences), anti-CD3 APC-Cy7 (Biolegend), anti-PD-1 PercpCy5.5 (BD Biosciences), anti-CTLA4 PE-Cy7, anti-CD244 FITC, and anti-CD160 PE (Biolegend) before washing and flow cytometric analysis. Memory subset analysis utilized the same panel but included anti-CCR7 FITC (R&D systems) and anti-CD45RA AF700 (Biolegend) and omitted anti-CTLA4, anti-CD244, and anti-CD160. Example flow plots can be found in (**Figure S1**).

Peptide Stimulation Assays

Following identification of CMV positive and negative donors, 2×10^6 cells were incubated at 37°C for 5 h with either PMA and ionomycin, CMV peptide mix (10) or EBV peptide mix at 10 μg/ml (**Supplementary Table 6**), along with protein cocktail inhibitor mix (eBiosciences). Live/dead red dye (Invitrogen), anti-CD3 APC-Cy7 (Biolegend) anti-CD8 Amcyan (BD Biosciences), were then applied before fixation and permeabilisation and IFN-γ AF700 (Biolegend) and TNF-α PE-Cy7 staining (eBioscience). Example flow plots can be found in supplementary (**Figure S1**). For assessing immune cell activation and cytotoxic degranulation, 2×10^6 cells were stimulated with either the above peptide mixes overnight at 37°C or a cell stimulation cocktail (Invitrogen) for 5 h. At the time of stimulation, CD107a FITC (Biolegend), along with brefeldin A and monensin was incorporated into the stimulation panel (example staining of CD107a can be found in supplementary).

Statistical Analysis

Mann-Whitney or Kruskal-Wallis testing for comparisons and multiple regression models were performed.

RESULTS

Amongst untreated patients ($n = 42$), and those previously treated with chemo-immunotherapy only ($n = 23$), 18.3% of CD8+ T cells expressed PD-1, an increased frequency compared to healthy age-matched donors (10.8%; $p = 0.0001$) (**Figure 1A**). No association was observed in relation to previous treatment with chemo-immunotherapy (**Figure S2**).

Amongst patients treated with ibrutinib [median 21 months (range 6–32)] the CD3+ T cell count was substantially reduced [median 1,154 cells/μl to 216 cells/μl; ($p = 0.013$)] and the CD8+ T cell count also decreased markedly from median 515 cells/μl to 104 cells/μl; ($p = 0.011$). As expected, the total lymphocyte count fell from 25 to 3.4×10^9/l during the treatment period (**Figure 1A**).

Interestingly the use of ibrutinib was associated with a reduction in PD-1 expression on CD8+ T cells [28% pre-treatment vs. 24.6% ($p = 0.042$)]. In addition, patients who reached a complete response (CR) as defined by IWCLL criteria, had a greater delta change in their PD-1 expression compared to those obtaining a partial response (−0.25 vs. −0.03; $p = 0.043$) (11). Patients who achieved a CR with ibrutinib treatment, also tended to have a lower frequency of PD-1 CD8+ T cells prior to commencing therapy, although this did not reach statistical significance (24.05 vs. 35.3%; $p = 0.130$). Importantly the duration of ibrutinib therapy was not found to differ between patients who achieved PR compared to those reaching a CR (23.5 vs. 21 months, respectively; $p = 0.924$) and no difference was seen in expression of other inhibitory markers that are increased in patients with CLL (1) (CD244 (52 vs. 55%; $p = 0.426$), CD160 (25 vs. 23%; $p = 0.326$), or CTLA4 expression (3.05 vs. 2.55%; $p = 0.622$) (**Figure 1B**).

We next went on to examine the functional activity of T cells and initially stimulated PMBC with PMA and ionomycin mitogen. Serial samples from patients on ibrutinib exhibited a 3.5 fold increase during therapy in the proportion of CD8+T cells that produced TNFα and INFγ following mitogenic stimulation [TNFα 13.1–45.7% ($p = 0.013$); IFNγ 12.4–44.7% ($p = 0.0215$) $n = 13$] (**Figure 1C**). However, the absolute number of cytokine-positive CD8+ T cells remained unchanged [pre-treatment IFNγ producing CD8+ T cells: 144 vs. 311 cells/μL during ibrutinib ($p = 0.07$) and TNFα producing CD8+ T cells: 147 pre-treatment vs. 306 cells/μL during ibrutinib ($p = 0.09$)] suggesting that long-term ibrutinib therapy acts to reduce the frequency of hypofunctional T cells. To address the impact of ibrutinib on T cell cytotoxicity, PBMCS were incubated with a T cell stimulation cocktail and CD107a degranulation assessed. Although no statistical difference in the frequency of response was noted, a trend towards increased CD107a release was observed with therapy [pre-ibrutinib 11.1% vs. 24.2% during ibrutinib ($p = 0.485$; n = 4)]. To assess if ibrutinib therapy impacted on the memory status of CD8+ T cells, a comparison was made before and during therapy. However, no difference in the frequency of memory cell subsets of CD8+ T cells was found (2 way Anova of repeating measures $p = 0.998$; $n = 4$) (**Figure 1D**).

The impact of ibrutinib on antigen-specific immune responses was next investigated through the use of HLA-peptide tetramer staining and viral peptide stimulation. Donor CMV and EBV serostatus and HLA genotype was first determined. Following incubation overnight with pooled CMV or EBV peptide, CD8+ T cell release of CD107a was assessed and compared between samples taken before and during ibrutinib therapy. No difference was observed in the release of CD107a following antigen stimulation [21.34% before therapy vs. 22.16% during therapy ($p = 0.879$)].

Next, the appropriate HLA class I tetramer staining of PBMC was combined with surface membrane immunophenotyping. PD-1 expression on CMV-specific CD8+ T cells was not found to be modulated by ibrutinib therapy (12.6 vs. 11.1%; data not shown) suggesting that CMV-specific CD8+ T cells do not account for the reduced frequency of PD-1 positive cells observed in the total CD8+ T cell population. However, paired PBMC samples showed an increased frequency of cytokine production with BTKi treatment following CMV-peptide pool stimulation [IFNγ: 0.46–0.78% ($p = 0.048$) and TNFα: 0.69–1.05% ($p = 0.274$)] (**Figure 2A**). The frequency of cytokines produced by EBV-specific CD8+ T cells also increased [TNFα: 0.85–1.81% ($p = 0.047$) and IFNγ 0.63 vs. 2.34% ($p = 0.219$)] (**Figure 2A**). Despite this, no difference was found in their absolute number before and during ibrutinib therapy [CMV peptide stimulation: (IFNγ: 5.6 vs. 5.8 cells/μL ($p = 0.56$); TNFα 2.2 vs. 3.7 cells/μL ($p = 0.3$) and EBV peptide stimulation: IFNγ: 0.85 vs. 1.8 cells/μL ($p = 0.05$); TNFα 0.63 vs. 3.2 cells/μL ($p = 0.22$)]. This indicates that the frequency but not absolute number of hypofunctional EBV or CMV-specific CD8+ T cells is reduced during ibrutinib therapy.

We were further interested to see if this improved functional activity might lead to a reduction in the number of virus-specific T cells. Indeed, HLA-peptide tetramer staining showed that the median frequency of CMV-specific T cells fell from 1.7% of the CD8+ repertoire before ibrutinib to 1.1% during therapy ($p < 0.05$; $n = 7$). Similarly, EBV-specific responses were also reduced by over 50% (4.2% pre-ibrutinib vs. 1.9% during therapy; $n = 6$) (**Figures 2B,C**).

DISCUSSION

Ibrutinib has transformed the management of CLL and many patients have now been on continuous therapy for many years. However, despite proven efficacy in suppression of B cell lymphoproliferation little is known regarding the impact of ibrutinib on immune function. Our analysis determined the impact of ibrutinib on antigen-specific T cells for the first time and also assessed patients with the longest treatment duration to date (9).

The striking reduction in CD3+ and CD8+ T cell number during ibrutinib therapy has been observed previously (12). In contrast Long et al. noted that the total CD8+ T cell response actually increased during ibrutinib therapy but this was within the first 6 months of therapy, when the absolute lymphocyte count was almost double the initial pre-treatment lymphocyte

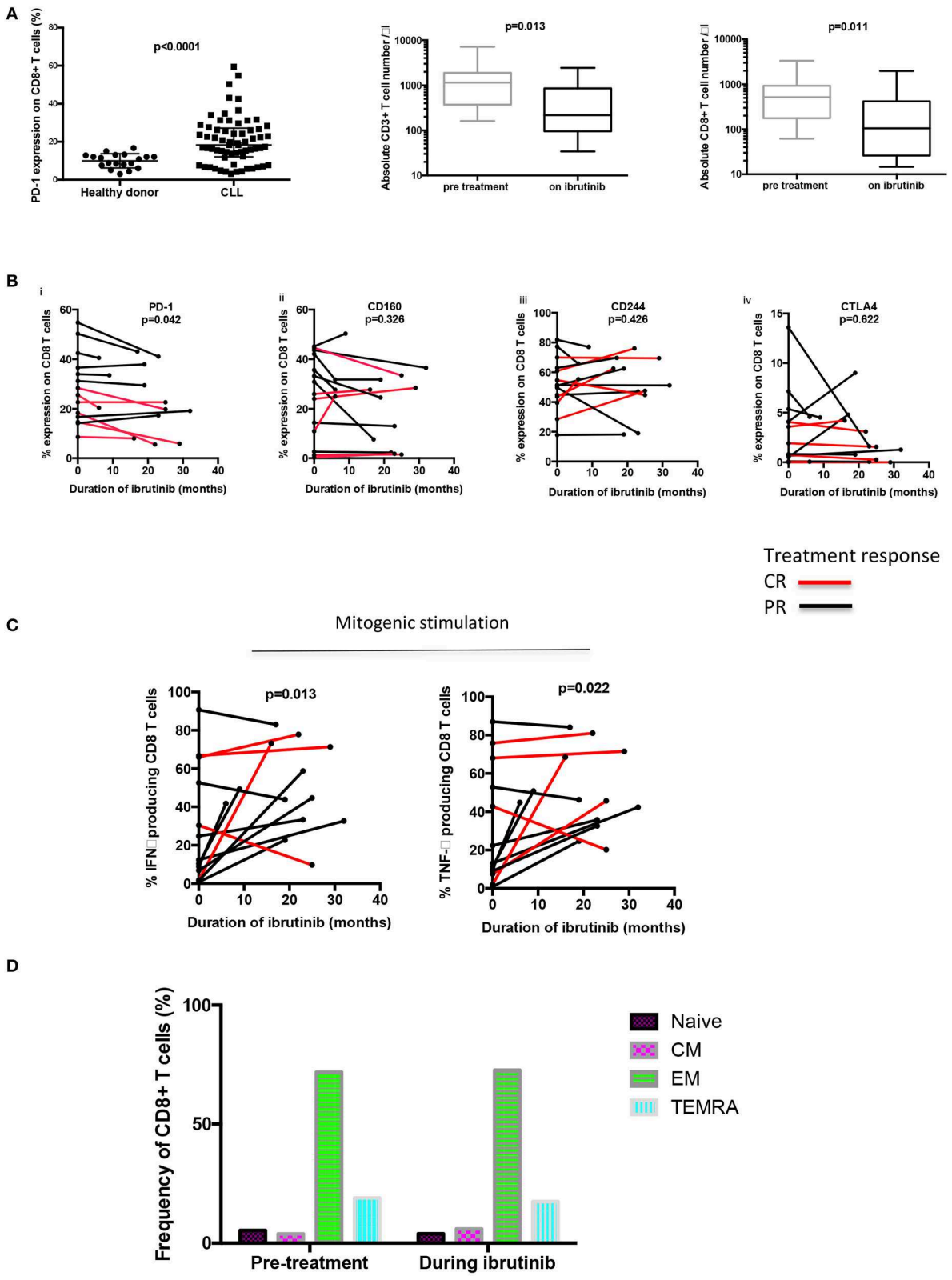

FIGURE 1 | Long term ibrutinib therapy decreases PD-1 expression on CD8+ T cells and increases the functional response to mitogen stimulation. **(A)** PD-1 expression on CD8+ T cells was ascertained using flow cytometry. An increased frequency of PD-1 expression was observed amongst untreated patients and those

(Continued)

FIGURE 1 | treated with chemo-immunotherapy only ($n = 65$), compared to healthy donors ($n = 19$). A reduction in the absolute number of both CD3+ and CD8+ T cells was observed during long term ibrutinib therapy. **(B)** The frequency of expression of checkpoint receptors on CD8+ T cells of 14 patients with relapsed refractory CLL treated with ibrutinib is shown over the treatment duration, including (i) PD-1, (ii) CD160, (iii) CD244, and (iv) CTLA4). A decreased percentage of PD-1 positive CD8+ T cells was observed in the patients with CLL during long-term ibrutinib therapy. **(C)** PBMCs from 13 patients with CLL were stimulated with PMA plus ionomycin, before and during ibrutinib therapy. The CD8+ T cells producing IFNγ and TNFα were identified through intracellular staining and flow cytometric analysis and an increased frequency of both cytokine-producing CD8+ T cells were found in B-CLL patients during ibrutinib therapy. **(D)** Memory subset analysis was performed using CCR7 and CD45RA to define naïve, central memory (CM), effector memory (EM), and T_{EMRA} CD8+ T cell populations. No difference in the frequency of the subsets of memory cells was found before or during ibrutinib therapy ($n = 4$).

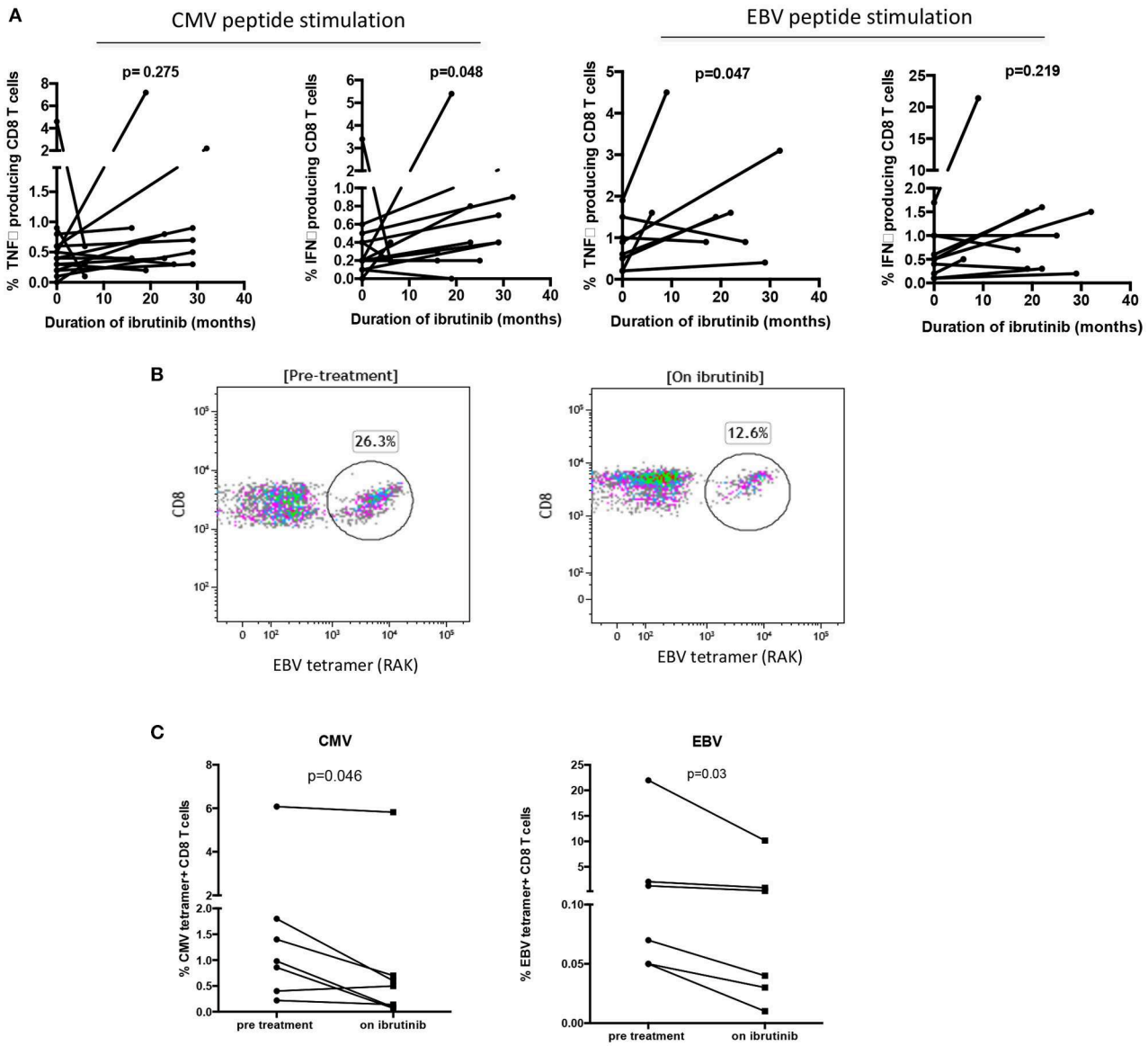

FIGURE 2 | Long term ibrutinib therapy decreases the frequency of virus-specific CD8+ T cells and improves the functional response to stimulation with viral peptides. PBMCs from patients with CLL were stimulated with pooled CMV and EBV peptides. The CD8+ T cells producing IFNγ and TNFα were identified through intracellular staining and flow cytometric analysis. The percentage of cytokine producing CD8+ T cells were compared between patients before and during ibrutinib therapy. **(A)** (i) With pooled CMV peptide stimulation, significantly increased frequencies of IFNγ producing CD8+ T cells were found in patients with CLL during ibrutinib therapy ($p = 0.048$). (ii) With pooled EBV peptide stimulation, significantly increased frequencies of TNFα producing CD8+ T cells were found in patients during ibrutinib therapy ($p = 0.047$). **(B)** An example of the flow cytometric plot of EBV tetramer staining is shown, demonstrating the reduced frequency of EBV specific CD8+ T cells during ibrutinib therapy. **(C)** The frequency of CMV specific CD8+ T cells and EBV-specific CD8+ T cells in B-CLL patients before and during ibrutinib therapy were compared. The frequencies of both virus-specific cells decreased during ibrutinib treatment in B-CLL patients ($p = 0.046$ for CMV and $p = 0.03$ for EBV).

count. The difference observed between this work and the work of Long et al., may reflect a difference in the duration of ibrutinib therapy as similarly to our findings, Niemann et al. reported a reduction in T cell numbers by week 48 of ibrutinib treatment (13). Increased expression of PD-1 on CD8+ T cells is a characteristic feature of patients with CLL and predicts progression risk (1, 3). Importantly, long-term ibrutinib therapy reduced PD-1 expression on CD8+ T cells and this effect was not observed following conventional chemotherapy. An intriguing observation was that the reduction in PD-1 expression was more pronounced in patients who went on to achieve a complete remission in response to ibrutinib therapy and this group also exhibited a trend towards a lower overall percentage of PD-1+ CD8+ T cells prior to therapy. It is currently unclear if this correlation reflects a secondary improvement in immune function within individuals who gain excellent clinical responses to ibrutinib or if reversal of T cell exhaustion may itself play a role in mediating the therapeutic response to ibrutinib treatment.

The expression of additional checkpoint proteins was not modulated by ibrutinib therapy. Expression of intracellular CTLA4 has previously been reported to decrease with ibrutinib therapy, whereas our analysis assessed surface expression staining (10). PD-1 is a defining phenotypic feature of T cell exhaustion and we observed increased cytokine responses within CD8+ cells following long-term ibrutinib therapy indicating that BTK inhibition also reverses features of functional exhaustion. This may be achieved partly through suppression of the CLL clone, which shares features with B regulatory cells and correction of a range of elevated cytokines is observed within the first 2 months of ibrutinib therapy (12, 14). Further mechanisms for reversal of T cell exhaustion may include a reduction in chronic antigenic stimulation both from the decrease in tumour load and improved immune competence against infective agents.

To evaluate the latter we focused on the immune response to latent herpesviruses, which drive expanded CD8+ T cell responses in CLL in a mechanism that is thought to reflect a response to increased endogenous viral replication. EBV infection is associated with accelerated time to disease progression in CLL (15) although CMV has no known deleterious effect (16). Of interest, the magnitude of CD8+T cell CMV-specific responses increased with advanced stage disease, in line with a previous study of CD4+ immunity (data not shown) (17). Importantly, the magnitude of the virus-specific immune responses reduced during ibrutinib therapy, with a comparable increase in peptide-specific cytokine responses. These findings are the first report of improvement in antigen-specific immune responses following ibrutinib therapy. The proportion of PD-1+ CMV-specific T cells was not influenced by ibrutinib therapy, despite a decrease in the global PD-1+ CD8+ pool, indicating that reversal of T cell exhaustion may be directed towards tumour specific T cell responses. This also suggests that PD-1 is not contributing to the improvement in antigen-immune response observed in virus-specific T cells. Indeed, previous published work found a normal cytokine response in CMV-specific CD8+ T cells in patients with CLL, when CMV peptide was presented via lymphoblastoid cell lines or healthy donor B cells and in a controlled B: T cell ratio. In contrast, cytokine responses were impaired when CMV peptide was presented via CLL cells. The improvement observed in herpes-virus specific T cells in this study may therefore relate to the reduction in the CLL clone, rather than the expression of PD-1 (18).

Inducible T cell kinase (ITK) plays an important role in the maintenance of Th2 CD4+ cells and memory CD8+ T cells and is known to be inhibited by ibrutinib (5). However, $Itk^{-/-}$ CD8+ memory T cells (in comparison to naïve CD8+ T cells) demonstrate normal recall responses to bacterial infection in terms of frequency and functionality and this is compatible with our findings (19). The impact of ibrutinib on the induction of primary immune responses mediated by naïve T cells deserves further investigation. Ibrutinib has previously been associated with improvements in T cell function including an increase the degree of diversity within the T cell repertoire (14) and enhanced outcome of CAR-T therapy in patients with CLL (20). Our findings now demonstrate a reversal in the degree of phenotypic and functional exhaustion and help to explain the encouraging clinical experience of BTKi therapy in relation to infection risk (21).

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethical approval was obtained from West Midlands regional ethics committee for patients (10/H1206/58) and for healthy donor controls (2002/073). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HP and HL designed the research. HP, NM, JB, NC, JZ, MK, and CO conducted the experiments. CH performed statistical analysis. SP, MK, and TM recruited patients and followed patients up. HP, GP, TS, and PM wrote 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.2019.02832/full#supplementary-material

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Characteristics of Tumor-Infiltrating Lymphocytes Prior to and During Immune Checkpoint Inhibitor Therapy

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The tumor immune contexture plays a major role for the clinical outcome of patients. High densities of CD45RO$^+$ T helper 1 cells and CD8$^+$ T cells are associated with improved survival of patients with various cancer entities. In contrast, a higher frequency of tumor-infiltrating M2 macrophages is correlated with poor prognosis. Recent studies provide evidence that the tumor immune architecture also essentially contributes to the clinical efficacy of immune checkpoint inhibitor (CPI) therapy in patients. Pretreatment melanoma samples from patients who experienced a clinical response to anti-programmed cell death protein 1 (PD-1) treatment show higher densities of infiltrating CD8$^+$ T cells compared to samples from patients that progressed during therapy. Anti-PD-1 therapy results in an increased density of tumor-infiltrating T lymphocytes in treatment responders. In addition, elevated frequencies of melanoma-infiltrating TCF7$^+$CD8$^+$ T cells are correlated with beneficial clinical outcome of anti-PD-1-treated patients. In contrast, a high density of tumor-infiltrating, dysfunctional PD-1$^+$CD38^{hi} CD8$^+$ cells in melanoma patients is associated with anti-PD-1 resistance. Such findings indicate that comprehensive tumor immune contexture profiling prior to and during CPI therapy may lead to the identification of underlying mechanisms for treatment response or resistance, and the design of improved immunotherapeutic strategies. Here, we focus on studies exploring the impact of intratumoral T and B cells at baseline on the clinical outcome of CPI-treated cancer patients. In addition, recent findings demonstrating the influence of CPIs on tumor-infiltrating lymphocytes are summarized.

Keywords: cancer immunotherapy, immune architecture, immune monitoring, immune checkpoint inhibition, cytotoxic T lymphocyte antigen 4, programmed cell death protein 1, programmed cell death 1 ligand 1

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INTRODUCTION

Accumulating evidence indicates that the tumor immune contexture plays a critical role for the clinical outcome of cancer patients (1–4). Major components of the tumor immune architecture are CD8$^+$ and CD4$^+$ T cells that can essentially contribute to tumor elimination. Activated CD8$^+$ T cells produce large amounts of proinflammatory cytokines such as tumor necrosis factor (TNF)-α

and interferon (IFN)-γ and exhibit a profound tumor-directed cytotoxicity. Stimulated $CD4^+$ T cells secrete various cytokines that promote the differentiation of B cells into antibody-producing plasma cells (5). They also enhance the capacity of dendritic cells (DCs) to induce $CD8^+$ T cell responses and can eliminate tumor cells directly (5). When analyzing the clinical relevance of tumor-infiltrating T cells, it has been demonstrated that high densities of $CD4^+$ memory T helper (T_H) 1 cells and $CD8^+$ T cells are associated with improved disease-free and overall survival (OS) of colorectal cancer patients (6, 7). Recently, a multi-center study has been initiated to assess the prognostic value of tumor-infiltrating T cell numbers in colon cancer patients (8). Patients with a so-called high Immunoscore, which is characterized by a high frequency of $CD3^+$ and $CD8^+$ T cells in the tumor center and the invasive margin, had the longest survival and the lowest risk of recurrence (8). These results suggest that the Immunoscore may represent a reliable estimate of the risk of disease recurrence and support its implementation in the classification of colon cancer. In addition to colorectal cancer patients, a correlation between high densities of T_H1 cells or $CD8^+$ T cells and good prognosis has also been reported for patients with other cancer entities (1, 3).

Macrophages and DCs are other key components of the tumor immune contexture that can profoundly influence tumor growth and spreading. Macrophages can be classified according to their phenotype and functional properties (9, 10). M1 macrophages, which express high levels of proinflammatory mediators such as TNF-α, interleukin (IL)-1β, reactive oxygen species, and nitric oxide, act in a tumoricidal manner. Based on their tumor-directed properties, M1 macrophages are generally associated with a favorable clinical outcome of cancer patients (1, 3). In contrast, M2 macrophages, which are characterized by the release of pro-angiogenic mediators such as vascular endothelial growth factor (VEGF) and immunosuppressive cytokines such as IL-10 and transforming growth factor-β, are generally correlated with poor prognosis among cancer patients (1, 3). DCs display an extraordinary capacity to induce and regulate T cell responses and efficiently enhance the immunomodulatory and cytotoxic potential of natural killer (NK) cells (11). Due to these functional capabilities, DCs play a major role in antitumor immunity. When investigating the clinical impact of blood DC subsets, it has been demonstrated that a higher expression of specific gene signatures for myeloid DC1 and DC2 as well as for plasmacytoid DCs are associated with a higher probability for disease-free survival of patients with luminal breast cancer (12). Furthermore, a higher DC1-specific gene signature was significantly associated with improved survival in patients with various cancer entities (13). However, tumor-infiltrating DCs can also be defective in their functional activity and can contribute to immune suppression (14). For example, we have shown that a higher density of 6-sulfo LacNAc monocytes (slanMo), representing a subset of human non-classical blood monocytes that can differentiate into DCs (15), is significantly associated with a poor prognosis of clear cell renal cell cancer (RCC) patients (16). The tumor-infiltrating slanMo displayed an immature phenotype and expressed IL-10, which may explain this correlation.

Recent studies revealed that the tumor immune contexture also essentially contributes to the clinical efficacy of immune checkpoint inhibitor (CPI) therapy that evolved as a very promising treatment modality for cancer patients (17). Antibody-mediated blockade of the immune checkpoint receptors cytotoxic T lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1) or programmed cell death 1 ligand 1 (PD-L1) resulted in objective clinical responses and enhanced survival of cancer patients (18–20). Here, the current knowledge about the impact of intratumoral T and B cells at baseline on the clinical outcome of CPI-treated patients and treatment-mediated effects on tumor-infiltrating lymphocytes is summarized.

CHARACTERISTICS OF INTRATUMORAL T CELLS PRIOR TO AND DURING ANTI-CTLA-4 THERAPY

Function and Therapeutic Targeting of CTLA-4

Cytotoxic T lymphocyte antigen 4 is a member of the immunoglobulin superfamily, which is induced on the surface of T cells by antigen binding to the T cell receptor (21–23). CTLA-4 competes with CD28 for binding to CD80 or CD86 on professional antigen-presenting cells (APCs). Thereby it binds CD80 and CD86 more tightly than CD28 and delivers a negative signal, which dampens the early T cell activation. CTLA-4 regulates the amplitude of $CD4^+$ T cell priming and also the $CD4^+$ T cell help for the induction of $CD8^+$ T cell responses in lymphoid tissues. CTLA-4 is constitutively expressed on regulatory T (T_{reg}) cells, enhancing their immunosuppressive activity (24). Accordingly, CTLA-4 blockade fosters the expansion, cytokine secretion, and cytotoxic potential of T effector cells and inhibits the immunosuppressive activity of T_{reg} cells, resulting in improved antitumor responses. Therefore, CTLA-4 blockade is an attractive immunotherapeutic strategy to significantly enhance effector T cell-mediated antitumor immunity (25). Two phase III clinical trials have been conducted to explore the therapeutic efficacy of the anti-CTLA-4 monoclonal antibody ipilimumab. Melanoma patients treated with ipilimumab with or without a glycoprotein 100 peptide vaccine showed significantly improved OS compared to patients receiving the peptide vaccine alone (26). Furthermore, the combination of the DNA-alkylating agent dacarbazin with ipilimumab led to improved OS in melanoma patients compared to dacarbazin alone (27). Based on these clinical trials, ipilimumab was approved by the United States Food and Drug Administration (FDA) for the treatment of patients with metastatic melanoma in 2011 (28).

Correlation Between Frequency and Phenotype of Intratumoral T Cells and Clinical Efficacy of CTLA-4 Blockade

Recently, the association between immunological parameters in tumor tissues at baseline and the clinical activity of anti-CTLA-4 therapy has been explored. Surprisingly, Hamid et al. found a positive correlation between clinical efficacy of CTLA-4 blockade and a high baseline expression of either the T_{reg} cell-associated transcription factor FoxP3 or the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) in melanoma patients (29). Whereas no correlation between the frequency of pre-existing tumor-infiltrating T cells and clinical activity was observed, an anti-CTLA-4 therapy-mediated increase of the intratumoral T cell density was associated with improved clinical outcome. Various studies further substantiate the influence of anti-CTLA-4 treatment on the frequency and phenotype of intratumoral T cells. Thus, CTLA-4 blockade resulted in a significant increase of $CD8^+$ T cells regardless of clinical responses in melanoma patients (30). Hodi et al. observed clinical responses in the majority of metastatic melanoma patients who received ipilimumab after vaccination with irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony-stimulating factor (GM-CSF) (31). Analysis of posttreatment biopsies from metastatic lesions revealed a relation between the extent of therapy-induced tumor necrosis and the natural logarithm of the ratio of tumor-infiltrating $CD8^+$ effector T cells to T_{reg} cells, suggesting that ipilimumab can alter the balance of effector T cells and T_{reg} cells (31). When investigating anti-CTLA-4 therapy-related effects on the density of tumor-infiltrating T_{reg} cells, Sharma et al. found that this treatment does not significantly modulate the frequency of T_{reg} cells in patients (32).

In further studies, the impact of anti-CTLA-4 therapy on the phenotype of intratumoral T cells has been explored. It has been reported that this therapeutic strategy enhances the density of tumor-infiltrating $CD4^+$ T cells expressing the costimulatory molecule inducible T cell costimulator (ICOS) (33). In addition, a subset of IFN-γ-producing T cells was detected within the $ICOS^+CD4^+$ T cell population, indicating that anti-CTLA4 therapy can induce a T_H1 polarization in $CD4^+$ effector cells (33). Wei et al. observed an expansion of tumor-infiltrating $ICOS^+$ T_H1-like $CD4^+$ T cells and exhausted-like $CD8^+$ T cells following anti-CTLA-4 blockade in melanoma patients (34). Moreover, an enhanced frequency of melanoma-infiltrating $ICOS^+$ $CD4^+$ T cells, sustained over 3 months of anti-CTLA-4 treatment, was associated with better OS (35). When evaluating tissue specimens from prostate cancer patients prior to and after anti-CTLA-4 blockade, Gao et al. detected a higher proportion of tumor-infiltrating $CD4^+$ T cells, $CD8^+$ T lymphocytes, and $CD68^+$ macrophages expressing PD-L1 or V-domain Ig suppressor of T cell activation (VISTA), representing another inhibitory immune checkpoint receptor (36), after treatment (37). PD-L1 and VISTA expression on these immune cell subsets may contribute to the poor responsiveness of prostate cancer patients to anti-CTLA-4 therapy. A summary of immune cell characteristics that may have an impact on the clinical efficacy of anti-CTLA-4 therapy is given in **Figure 1**.

CHARACTERISTICS OF TUMOR-INFILTRATING LYMPHOCYTES PRIOR TO AND DURING ANTI-PD-1/PD-L1 TREATMENT

Function and Therapeutic Targeting of the PD-1/PD-L1 Axis

Programmed cell death protein 1 is another immune checkpoint receptor of the immunoglobulin superfamily, which can be found on activated T effector cells, NK cells, and B cells (18, 38). PD-1 is also expressed by T_{reg} cells and fosters their proliferation after ligand binding (39). PD-L1 and PD-L2 represent the ligands for PD-1, the latter having a higher affinity to PD-1. PD-L1 can be widely detected on tumor cells as well as hematopoietic and non-hematopoietic cells and its expression is inducible by proinflammatory cytokines such as IFN-γ. PD-L2 is characterized by a more restricted expression pattern, being mainly detectable on APCs and induced mostly by IL-4 and GM-CSF (40–43). Besides PD-1, PD-L1 can also bind to CD80 on T cells, thereby delivering another inhibitory signal (44). The main role of PD-1 is to modulate important functional properties of antigen-experienced effector T cells within the peripheral tissues. Thus, expansion, cytokine release, and cytotoxic activity of stimulated T cells are inhibited upon interaction of PD-1 with its ligands, protecting the tissue from collateral damage during immune response (40, 45–47). This pathway is adopted by tumors leading to prevention from immune attack. Therefore, anti-PD-1 and anti-PD-L1 antibodies have been developed to enhance T cell-mediated antitumor immunity. The application of such antibodies induced objective clinical responses and improved survival in cancer patients (48–50). Consequently, the FDA approved anti-PD-1/PD-L1 therapy for various tumor entities (28, 51).

Correlation Between PD-L1 Expression by Tumor Cells and Tumor-Infiltrating Immune Cells and Clinical Efficacy of PD-1/PD-L1 Blockade

Various clinical trials clearly indicated that PD-L1 expression by tumor-infiltrating immune cells and tumor cells significantly influences the efficacy of anti-PD-1/PD-L1 treatment. Accordingly, an association between intratumoral PD-L1 expression in pretreatment tissue specimens and objective clinical responses in anti-PD-1/PD-L1-treated cancer patients has been reported (52). Herbst et al. demonstrated that a high level of intratumoral PD-L1, particularly when detected on tumor-infiltrating immune cells, was associated with clinical responses in anti-PD-L1 antibody-treated cancer patients (53). Topalian et al. observed that 9 of 25 patients with $PD\text{-}L1^+$ tumors experienced an objective clinical response, whereas none out of 17 patients with $PD\text{-}L1^-$ tumors achieved an objective response

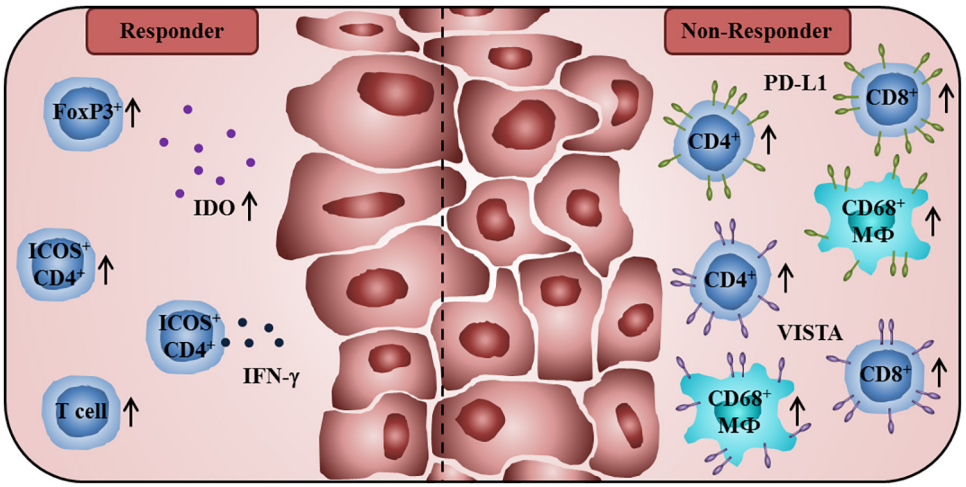

FIGURE 1 | Immunological characteristics of tumor patients receiving anti-CTLA-4 antibodies associated with improved clinical outcome or therapy resistance. A high baseline expression of T_{reg} cell-associated FoxP3 and IDO, and a treatment-induced increase of tumor-infiltrating T lymphocytes are associated with better clinical efficacy of CTLA-4 blockade. Anti-CTLA-4 therapy enhances the frequency of intratumoral $ICOS^+CD4^+$ T cells that is correlated with better OS. A proportion of these $ICOS^+CD4^+$ T cells is characterized by the production of IFN-γ. Non-responders to anti-CTLA-4 therapy show a higher percentage of PD-L1- or VISTA-expressing $CD4^+$ T cells, $CD8^+$ T lymphocytes, and $CD68^+$ macrophages in posttreatment tumor samples.

(54). In agreement with these findings, it has been reported that PD-L1 expression in at least 50% of tumor cells correlates with improved efficacy of anti-PD-1 therapy in non-small-cell lung cancer (NSCLC) patients (49). Further clinical trials yielded contradictory results (52). Motzer et al. investigated a large cohort of RCC patients undergoing anti-PD-1 therapy and found a reduced OS for patients with 1% or greater intratumoral PD-L1 expression compared to patients with less than 1% (50). Gettinger et al. did also not find a clear correlation between PD-L1 expression and clinical response or survival in anti-PD-1-treated NSCLC patients (55). However, the results are not always comparable since various assays, antibodies, cut-off values, and different scoring methods are utilized to determine $PD\text{-}L1^+$ cells by immunohistochemistry.

Association Between Frequency and Phenotype of Tumor-Infiltrating T Cells and Clinical Efficacy of PD-1/PD-L1 Blockade

Recent studies revealed that the density and phenotype of tumor-infiltrating T cells play an essential role for the clinical efficacy of anti-PD-1/PD-L1 therapy. Using melanoma tissue samples collected before and during treatment with anti-PD-1 antibodies, Tumeh et al. determined the frequency of tumor-infiltrating $CD8^+$ T cells (56). A higher density of melanoma-infiltrating $CD8^+$ T cells at baseline was indicative of responding patients, suggesting that pre-existing intratumoral $CD8^+$ T cells are predictors of a clinical response to anti-PD-1 therapy. This finding was further substantiated by another study, investigating RCC tissues from patients treated with anti-PD-L1 and anti-VEGF antibodies (57). McDermott et al. found a correlation between a high T effector gene signature expression at baseline and an improved overall response rate and progression-free survival (PFS) of the treated patients. In contrast, a high myeloid inflammation gene signature expression was associated with reduced PFS in patients receiving anti-PD-L1 alone or anti-PD-L1 and anti-VEGF antibodies. When performing an in-depth analysis of intratumoral $CD8^+$ T lymphocytes in NSCLC patients, Thommen et al. described three distinct $CD8^+$ T cell subsets based on PD-1 expression (58). In addition to $CD8^+$ T cell subpopulations with intermediate ($PD\text{-}1^N$) and no PD-1 expression, a subset with high PD-1 expression ($PD\text{-}1^T$) was identified that displayed a markedly different transcriptional and metabolic profile. The $PD\text{-}1^T$ $CD8^+$ T cells are characterized by the secretion of CXCL13 that can mediate recruitment of follicular T_H cells and B cells to the tumor microenvironment and may also foster the formation of intratumoral tertiary lymphoid structures (TLS). The presence of $PD\text{-}1^T$ T cells emerged as a strong predictor for the clinical outcome of anti-PD-1-treated NSCLC patients (58).

The impact of anti-PD-1 therapy on the phenotype and frequency of intratumoral T cells was also explored. Melanoma patients who responded to anti-PD-1 therapy showed an increased intratumoral $CD8^+$ T cell density that was associated with radiographic reduction of tumor size (56). In another study, two major intratumoral $CD8^+$ T cell states that were associated with clinical response have been identified in melanoma patients treated with PD-1- and/or CTLA-4 blockade (59). Single-cell RNA sequencing resulted in the identification of intratumoral $CD8^+$ T cells with increased expression of genes linked to memory, activation, and cell survival that were enriched in responding melanoma lesions. In contrast, $CD8^+$ T cells with increased expression of genes linked to exhaustion were enriched in non-responding lesions. Thus, the ratio of memory-like to exhausted $CD8^+$ T cells was linked with clinical outcome. In

addition, elevated levels of melanoma-infiltrating TCF7^{+}CD8^{+} T cells predicted clinical benefit in anti-PD-1-treated patients (59). By using a tumor mouse model, Siddiqui et al. showed that intratumoral TCF7^{+}PD-1^{+}CD8^{+} T cells with stem-like properties can mediate tumor control to CPI therapy (60). In addition, melanoma patients treated with anti-CTLA-4 and/or anti-PD-1-antibodies showed a higher proportion of intratumoral TCF7^{+}PD-1^{+}CD8^{+} T cells than untreated patients (60). Furthermore, an increased density of TCF7^{+}PD-1^{+}CD8^{+} T cells at baseline was associated with prolonged survival in melanoma patients treated with anti-CTLA-4 and anti-PD-1-antibodies (61). Moreover, Verma et al. reported that the status of CD8^{+} T cell priming essentially influences anti-PD-1 therapeutic resistance (62). Thus, administration of anti-PD-1 antibodies in unprimed or suboptimal primed CD8^{+} T cell conditions led to the generation of dysfunctional PD-1^{+}CD38^{hi}CD8^{+} cells that contribute to PD-1 blockade resistance and treatment failure. However, the induction of dysfunctional CD8^{+} cells was prevented and treatment resistance was reversed when anti-PD-1 therapy was applied to optimally primed CD8^{+} T lymphocytes. They also found that a high density of tumor-infiltrating PD-1^{+}CD38^{hi}CD8^{+} cells in melanoma patients can serve as a biomarker of anti-PD-1 resistance. Zappasodi et al. described an intratumoral accumulation of CD4^{+}FoxP3^{-}PD-1^{hi} T cells (4PD-1^{hi}) in immunotherapy-naïve melanoma and NSCLC patients (63). These T cells were shown to inhibit the proliferation and activation of T effector cells. In addition, the authors found that a lack of effective 4PD-1^{hi} reduction after PD-1 blockade correlates with poor prognosis (63).

Impact of the Frequency of Tumor-Infiltrating B Cells and TLS on Clinical Efficacy of Anti-PD-1 Therapy

Emerging evidence suggests that tumor-infiltrating B cells play an important role for the clinical outcome of anti-PD-1-treated cancer patients. Thus, a higher frequency of melanoma-infiltrating B cells with a plasmablast-like phenotype before therapy was associated with improved patient survival to anti-PD-1 treatment (64). More recently, Petitprez et al. observed that the sarcoma immune class E, which is characterized by TLS containing T cells, follicular DCs, and a high density of B cells, is correlated with an improved response rate and survival to PD-1 blockade (65). In addition, a higher density of tumor-infiltrating B cells and TLS has been detected in treatment responders in a cohort of melanoma patients receiving anti-PD-1-antibodies alone or combined with anti-CTLA-4 antibodies in a neoadjuvant setting (66). The importance of tumor-associated TLS for the clinical efficacy of anti-PD-1 treatment is further supported by another clinical trial, demonstrating that a higher TLS density at baseline was correlated with increased survival of melanoma patients (67). An overview about immune cell characteristics that may influence the clinical efficacy of anti-PD-1/PD-L1 therapy is given in **Figure 2**.

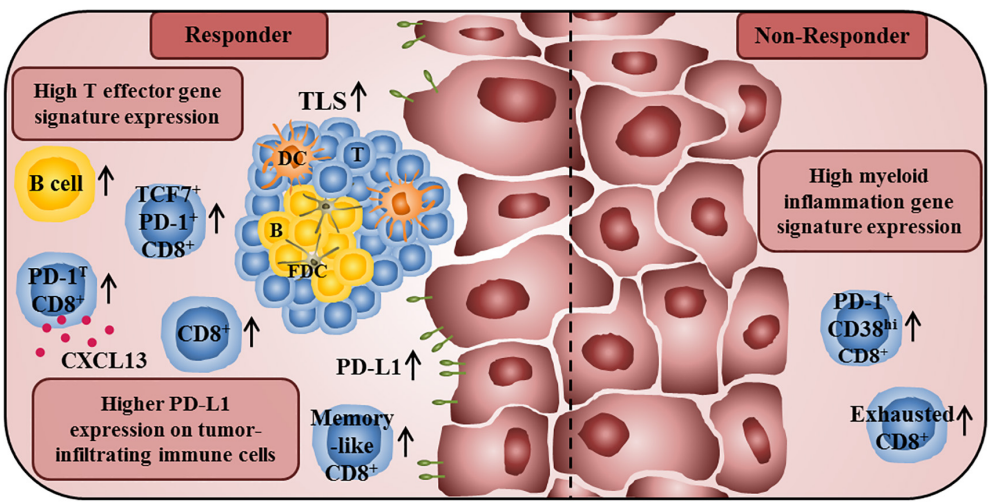

FIGURE 2 | Immune profile of anti-PD-1/PD-L1 antibody-treated tumor patients associated with improved clinical outcome or therapy resistance. A high T effector gene signature expression in pretherapy tumor samples is associated with improved survival of anti-PD-L1- and anti-VEGF-treated cancer patients. In addition, responders to anti-PD-1 treatment show a higher frequency of intratumoral CD8^{+} T cells at baseline and an increased frequency of tumor-infiltrating CD8^{+} T cells during therapy. Furthermore, they also have a higher proportion of intratumoral memory-like CD8^{+} T cells. The presence of PD-1^{T} CD8^{+} T cells, which are characterized by a high PD-1 expression and by the capability to secrete CXCL13, is also correlated with improved clinical outcome of anti-PD-1-treated cancer patients. Moreover, an increased frequency of TCF7^{+}PD-1^{+}CD8^{+} T cells in pretreatment tumor samples is associated with prolonged survival in patients treated with anti-CTLA-4 and anti-PD-1-antibodies. An increased density of B cells and TLS, consisting of a DC-containing T cell zone and a follicular DC-containing B cell zone, in pretreatment tumor samples is also correlated with an increased survival of anti-PD-1-treated patients. Furthermore, a higher PD-L1 expression on tumor cells and tumor-infiltrating immune cells is correlated with better clinical responses to anti-PD-1/PD-L1 therapy. In contrast, high frequencies of exhausted CD8^{+} T cells and PD-1^{+}CD38^{hi}CD8^{+} T cells in tumor tissues are associated with resistance to anti-PD-1 therapy. Non-responders to anti-PD-L1 and anti-VEGF therapy also show a high myeloid inflammation gene expression signature.

CONCLUSION

The location, density, and functional orientation of tumor-infiltrating immune cells play a critical role for the clinical outcome of cancer patients. Thus, high frequencies of $CD4^+$ T_H1 cells and $CD8^+$ T cells in the tumor center and the invasive margin were associated with improved OS of colorectal cancer patients. Whereas M1 macrophages were correlated with a favorable clinical outcome of cancer patients with various cancer entities, M2 macrophages were generally associated with poor prognosis. Such findings indicate that tumor-infiltrating immune cells can significantly influence tumor growth and spreading. Recent studies revealed that the tumor immune contexture also essentially contributes to the clinical efficacy of CTLA-4 or PD-1/PD-L1 blockade that induced objective clinical responses and improved survival in patients with various tumor types. However, a significant number of patients do not respond to CPI therapy. Therefore, deciphering the immunogenicity of the tumor cells and the tumor immune architecture prior to and during CPI therapy may lead to the discovery of novel modes of action or resistance and to the design of improved treatment modalities for cancer patients. For example, it has been demonstrated that a limited presentation of tumor-associated neoepitopes by tumor cells and the lack of pre-existing intratumoral T cells are associated with poor responsiveness of cancer patients to CPI therapy. Therefore, other treatment modalities that increase the expression of components of the antigen-processing and presentation machinery and the neoantigen load of tumor cells as well as promote T cell trafficking to tumor tissues are required to improve the clinical response rate to current CPI therapy. Promising treatment options comprise radiotherapy as well as the application of chemotherapeutic agents and epigenetic drugs that can efficiently increase tumor cell immunogenicity and stimulate antitumor immune responses. Vaccination strategies including neoantigens and the administration of non-modified or engineered T cells can increase the frequency of tumor-infiltrating and -reactive T lymphocytes. The intratumoral application of oncolytic viruses or adjuvants can also improve CPI-based therapies by direct tumor cell elimination, the recruitment of DCs and T cells to the tumor, and the activation of innate and adaptive antitumor immunity.

AUTHOR CONTRIBUTIONS

IP and AT drafted the manuscript. LM, RW, XL, M-OG, SR, MB, and MS reviewed and edited the manuscript.

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REVIEW
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Single-Cell Approaches to Profile the Response to Immune Checkpoint Inhibitors

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Novel treatments based upon the use of immune checkpoint inhibitors have an impressive efficacy in different types of cancer. Unfortunately, most patients do not derive benefit or lasting responses, and the reasons for the lack of therapeutic success are not known. Over the past two decades, a pressing need to deeply profile either the tumor microenvironment or cells responsible for the immune response has led investigators to integrate data obtained from traditional approaches with those obtained with new, more sophisticated, single-cell technologies, including high parameter flow cytometry, single-cell sequencing and high resolution imaging. The introduction and use of these technologies had, and still have a prominent impact in the field of cancer immunotherapy, allowing delving deeper into the molecular and cellular crosstalk between cancer and immune system, and fostering the identification of predictive biomarkers of response. In this review, besides the molecular and cellular cancer-immune system interactions, we are discussing how cutting-edge single-cell approaches are helping to point out the heterogeneity of immune cells in the tumor microenvironment and in blood.

Keywords: immunotherapy, immune checkpoint, single-cell technologies, cancer, immune system

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INTRODUCTION

Immune checkpoints are critical regulators of the immune system which modulate the duration and amplitude of immune responses to maintain self-tolerance and prevent autoimmunity. Among immune checkpoints, cytotoxic T lymphocyte antigen-4 (CTLA-4, or CD152), programmed death-1 (PD-1, or CD279) and programmed death ligand-1 (PD-L1, or CD274) have been intensively studied and antibodies against these molecules have been developed to successfully reinvigorate T cell functions and provide a durable immune response. Antibodies against immune checkpoints have demonstrated impressive efficacy, and now constitute the backbone of systemic therapy in different malignancies (1).

Despite considerable advancements in clinical care, epidemiologic data and ongoing clinical trials suggest that most patients receiving immune checkpoint inhibitors (ICI) do not derive benefit or stable and lasting responses. The mechanisms at the basis of this lack of responsiveness are multiple, and still not completely known. Over the past years, accumulating evidence suggested that the elevated neoantigen load (i.e., the number of antigens actually targeted by T cells) may have a

robust relationship with the response to ICI (2). In particular, the more is the neoantigen load, the better is the response to therapy. However, the intensity and efficacy of the immune response can vary upon neoantigens' clonality. It seems that neoantigens derived from clonal mutations, which appear early during tumorigenesis, may elicit more effective tumor responses than neoantigens derived from subclonal mutations, which are acquired later in tumorigenesis (3). This means that intratumoral heterogeneity may impact the response to ICI. Moreover, several biophysical matters occur in the binding and recognition between peptide-MHC complex and T cell receptor (TCR) (4), and current prediction algorithms are still unable to precisely define TCR binding capacity for specific neoantigens (2). An additional layer of complexity originates from the fact that T cells, which are the main mediator of anti-tumor immunity, are extremely heterogenous in the tumor microenvironment (TME), and that beyond T cells many other types of immune cells are present in the tumor tissue that could affect response to ICI (4). Furthermore, an anticancer immune response may be impaired also by a number of other factors, mainly immune cells polarized toward an immune suppressive phenotype (5).

Taken together, these observations suggest that most, if not all, of these components are involved in the clinical response to ICI, and that the identification of the mechanism(s) at the basis of such response is crucial, both to provide important insights into the molecular and cellular crosstalk between cancer and immune system, and possibly foster the identification of predictive biomarkers of response (6). In this scenario, recently several novel single-cell technologies have been used to draw an in-depth characterization of tumor and immune system ecosystems in different malignancies. Here, we first describe the interactions between tumor and immune cells and then give an overview of the cutting-edge single-cell approaches mainly used to interrogate cancer immunity both in the tumor microenvironment and in the blood. We also cover and discuss how single-cell analysis have revealed the vast heterogeneity characterizing intra-tumoral immune cells, mainly T cells, and how this knowledge is critical to understand the role of different cell states and phenotypes in the response to immune checkpoint inhibitors.

IMMUNE SYSTEM AND CANCER

Cancer Immunosurveillance and Immunoediting

The long-standing theory of immune surveillance suggests that cells and tissues are regularly monitored by the immune system, which is responsible for recognizing and eliminating the vast majority of nascent cancer cells. The interactions between cancer and the immune system are regulated by a complex network of biological pathways, and start during the early steps of carcinogenesis, when normal cells acquire biological capabilities which allow them to evolve progressively to a neoplastic state. Such capabilities are commonly known as hallmarks of cancer, and among them the ability to evade immune system is crucial to guarantee cancer cell survival and tumor progression (7).

Over the past years, accumulating evidences, both from murine models and clinical epidemiology, have validated the concept of cancer immunosurveillance, demonstrating that the immune response acts as a barrier to tumor development and progression, and is a critical determinant of susceptibility to tumors (8–13). This action is exerted through at least three distinct mechanisms: (i) protection of the host from viral infections, and thus suppression of tumors of viral etiology; (ii) prevention or resolution of inflammation, which facilitates tumorigenesis; (iii) identification and elimination of cancer cells, in certain tissues and on the basis of the expression of tumor-specific antigens (14). In particular, deficiencies in the number or functionality of CD8+ cytotoxic T lymphocytes (CTLs), CD4+ type-1 T helper (Th1) cells, natural killer (NK) cells, natural killer T (NKT) cells, B cells, or γδ T cells lead to increased susceptibility to carcinogen-induced tumors and spontaneous tumors development (9, 11, 15, 16). Similarly, unsensitivity to interferons (IFNs) or lack of perforin, interleukin (IL)-12, tumor necrosis factor (TNF)-α or IL-1β are associated with increased tumor susceptibility (10, 11, 17–20).

Reinforcing this notion, clinical epidemiology supports the existence of antitumoral immune response in some types of cancer. Firstly, evidence for immunosurveillance can be found in patients with acquired immunodeficiencies, like that caused by the human immunodeficiency virus (HIV), the cause of AIDS, who have an increased frequency of virus-associated malignancies, including Kaposi's sarcoma, lymphomas, urogenital cancers, and cervical cancers due to different strains of papillomavirus (21). Secondly, higher cancer prevalence has been observed in transplanted recipients treated with immunosuppressive drugs. Immunosuppression to prevent transplant rejection is associated with a 3- to 100-fold increased risk of developing certain types of cancer, mainly lymphomas (22). However, solid tumors with no viral etiology also occur with increased frequency. For example, patients receiving renal transplant have a 3-fold increase in the incidence of cancers respect to the general population, and a 200-fold increase of skin cancers (23). Patients with liver transplant also display a greater incidence of malignancies, including head and neck cancers, and skin cancers (24, 25).

Along with clinical epidemiology, other evidences support the theory of cancer immunosurveillance, including the identification of tumor antigens and of antibodies against those antigens. In cancer patients, humoral immune response has been detected against more than a hundred tumor-associated antigens, thus indicating that the immune system is well able to fight against cancer (26). However, whether or not the identification and quantification of these antibodies has a clear diagnostic and/or prognostic relevance is still unclear. Other spontaneous immune responses against cancer cells have been described in paraneoplastic autoimmune syndromes, caused by the activation of an immune response against self-antigens expressed on cancer cells. Paraneoplastic autoimmune syndromes are often caused by cross-reactivity between the anti-tumor immune response and antigens present in the nervous system, and the onset of neurologic symptoms typically precedes the diagnosis of a formerly undetected tumor (27).

Advancements made all over the past two decades have demonstrated that the immune system not only defends the host against tumor development, but also edits tumor immunogenicity, in a process referred to as cancer immunoediting. In its most complicated form, cancer immunoediting proceeds through three phases, termed elimination, equilibrium and escape. During the elimination phase, both innate and adaptive immune system collaborate to recognize and kill neoplastic cells. Cancer clones which survive the elimination phase can then progress through the equilibrium phase, in which tumor growth is limited, but cellular immunogenicity is edited by the adaptive immune system, mainly lymphocytes. During this phase, the pressure of the immune system together with the genetic instability of tumor cells can lead to the selection of neoplastic subclones with low immunogenicity, which can enter into the escape phase and evade the immune recognition. A large number of mechanisms operate to enable tumor immune escape by interfering with almost every step required to generate an effective immune response, i.e., the: (i) tumor capacity to downregulate antigens and/or MHC I; (ii) tumor expression and/or secretion of immunosuppressive molecules and/or antiphagocytosis signals; (iii) tumor modulation of lymphocytes' metabolism; (iv) recruitment of immune cells that actively mediate tolerance, or even promote tumor growth (28).

Since Virchow noted the presence of lymphocyte infiltration in solid tumors in 1863 (29), additional support for cancer immunosurveillance and immunoediting is evident in uncountable reports that describe the presence of immune cells infiltrating the tumors, and that correlate their frequency with patient prognosis. The presence of T cells inside tumors was observed, in late 1990's, in patients with melanoma, and then described for several other malignancies, including ovarian, colorectal (CRC) or lung cancer (30–36). From then on, a great attention has been paid to investigate the role of tumor-infiltrating lymphocytes (TILs). This effort resulted in the identification of TILs frequency as a *bona fide* indicator of improved prognosis and increased overall survival for several types of tumors.

Recent advancements in the characterization of the immune context within the tumor microenvironment have revealed that different classes of the so-called tumor immune environment (TIME) exist that are associated to tumor initiation and could affect the response to therapies (37). The TIME varies greatly across individuals and over distinct cancers. However, despite variability, two main classes can be described, which differ on the basis of composition, functional status and spatial distribution of immune cells. Infiltrated-excluded TIMEs are populated by immune cells mainly along the tumor margins, and are relatively poor of CTLs in the tumor core (37). Moreover, CTLs from this kind of TIME typically display low expression of activation or cytotoxicity markers, including granzyme(GZM)-B and IFN-γ (37). Conversely, infiltrated-inflamed TIMEs are characterized by large immune infiltration among neoplastic cells, with a high frequency of CTLs expressing GZM-B, IFN-γ, and PD-1. In some cases, infiltrated-inflamed TIMEs contain compartments which resemble tertiary lymphoid structures (TLSs), and act as sites of lymphoid recruitment and immune activation (38). Such compartments are generally located at the invasive tumor margin and in the stroma, and include naïve and activated T cells, regulatory T (Treg) cells, B cells and dendritic cells (DCs) (37). Over the past years, the immune network of the TME has become a focus of cancer research and therapeutics development, and the need to understand its great complexity and diversity in this context is now compelling.

Immune Checkpoints and Their Inhibitors

Immune checkpoints are molecules expressed on T cell plasma membrane able to inhibit or activate the development or execution of effector functions exerted by cytotoxic or pro-inflammatory T cells. Among immune checkpoints, CTLA-4 and PD-1 have been most actively studied in the field of clinical cancer immunotherapy.

CTLA-4 and CD28 are homologous molecules expressed by CD4+ and CD8+ T cells, which mediate antagonistic functions in T cell activation, and share two ligands, namely B7-1 (CD80) and B7-2 (CD86), expressed on antigen-presenting cells (APCs). CD28 interacts with the CD80 dimer with relatively high affinity and the CD86 monomer with lower affinity, to mediate T cell activation in conjunction with TCR signals. Conversely, CTLA-4 interacts with both ligands with higher affinity and avidity than CD28, to inhibit T cell activation. CTLA-4 is constitutively expressed on Treg cells or induced following T-cell activation via CD28 and TCR signaling (39). The humanized anti-CTLA-4 antibody ipilimumab was approved by the United States Food and Drug Administration (FDA) in 2011. It blocks the interaction between CTLA-4 and its ligands expressed by APCs, thereby preventing the transmission of inhibitory signals to CTLA-4-expressing T cells. Although the blocking of inhibitory signals is the main mechanistic contributor to ipilimumab functions, other still poorly known mechanisms are involved. For example, the effects of anti-CTLA-4 on Treg is still matter of debate. Indeed, the binding of CTLA-4 by ipilimumab on Treg within the tumor tissue would likely promote Treg depletion by antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis by NK cells and macrophages (40, 41). Recently it was found that both ipilimumab and tremelimumab, another anti-CTLA-4 drug, increase infiltration of intratumoral CD4+ and CD8+ T cells without significantly changing or depleting FOXP3+ cells within the TME (42). Nonetheless, regardless the mechanism of action, ipilimumab demonstrated impressive anti-tumor activity in several clinical settings in metastatic melanoma (43, 44).

Along with CTLA-4, the PD-1/PD-L1 system constitutes another immune checkpoint pathway mainly operating by controlling immune homeostasis. However, while transient expression of PD-1 is a feature of normal T lymphocyte activation, persistent antigen exposure leads to a sustained expression of PD-1 with a gradual loss of effector functions which are characteristic of exhausted T cell (45). PD-1 mediates an inhibitory signal in T cells after binding to its ligands, PD-L1 and PD-L2, which are expressed on APCs and cancer cells (46). The blockade of PD-1/PD-L1 pathway with anti-PD-1 or anti-PD-L1 antibodies, can successfully reinvigorate T cell functions and provide a durable response in different malignancies. There

are currently six inhibitors of the PD-1/PD-L1 pathway, namely nivolumab, pembrolizumab, cemiplimab (directed against PD-1), and atezolizumab, avelumab and durvalumab (directed against PD-L1), which have been approved by the FDA for the treatment of tumors like melanoma, lung cancer, renal-cell carcinoma (RCC), microsatellite instability-high CRC, classical Hodgkin lymphoma, head and neck squamous cell cancer (HNSCC), hepatocellular carcinoma (HCC), bladder cancer, gastro-oesophageal cancer, and unresectable or metastatic, microsatellite instability-high or mismatch repair deficient solid tumors (47).

The best examples of stable response are those observed in patients with advanced melanoma. In these patients, it was reported that 3-year overall survival was 34 and 52% for ipilimumab and nivolumab, respectively (48). The 3-year overall survival was 60, 55, 41% for nivolumab plus ipilimumab, nivolumab alone, or ipilimumab alone (49). In advanced RCC, the 2-year overall survival of patients treated with nivolumab plus ipilimumab was 28%, and complete responses were 11% (50–52). In other cancers, responses to immune checkpoint monotherapies were not as impressive as in melanoma. This means that despite considerable advancements in clinical care of some tumors, epidemiologic data and ongoing clinical trials suggest that most of the patients receiving ICI do not derive benefit or durable responses, and the mechanisms at the basis of this lack of responsiveness are multiple and still not completely known.

SINGLE-CELL APPROACHES TO IMMUNE PROFILE

Over the past two decades, a pressing need to deeply profile the TIME has led investigators to complement data obtained from traditional approaches, like immunohistochemistry, basic flow cytometry or measurements on bulk populations of cells, with data obtained with novel, more sophisticated, single-cell technologies. To date, a vast array of single-cell approaches, including high-parameter flow cytometry, deep sequencing, and high-resolution imaging are available to unmask cellular heterogeneity and to try to identify actionable hallmarks of efficient anticancer immunotherapy. In **Table 1** a general overview of single-cell technologies is provided together with their advantages and disadvantages.

High-Parameter Flow Cytometry

Last advances in proteomics and genomics are paving the way to comprehend the complexity and the heterogeneity of billions of specialized immune cells in cancer patients. For decades, immunologists relied mainly on flow cytometry, the first single-cell technique that now allows to study the expression and density of up to 30–40 antigens in a single-cell level. Flow cytometry is very popular technique used to measure physical and chemical characteristics of a population of cells/particles suspended in a fluid, and is routinely used both in basic research, and in clinical practice to perform cell count, determine cell phenotypes and functions allowing the monitoring of immune features in pathophysiological settings (64, 65). Flow cytometry is unmatched for its high throughput as several million cells can be analyzed in a few minutes. In addition, cells can be sorted achieving pure cell populations to perform further functional, metabolic and molecular analyses (66). Sample preparation for flow cytometry is relatively fast, but setting up a flow cytometry panel that includes 28–30 parameters takes a lot of time because of the need to optimize spectral overlap between fluorophores, and to choose best antibodies. These issues can be overcome by following precise rules applicable to panel design, and optimized panels published such as Optimized Multicolor Immunophenotyping Panels (OMIPs) (67–72). Together with fluorescent flow cytometry, mass cytometry (also called CyTOF—Cytometry by Time-Of-Flight) is a technology that allows simultaneous analysis of more than 40 different molecules, including cytokines and transcription factors, with minimal compensation (53, 54). This technique exploits the use of monoclonal antibodies conjugated with heavy-metal isotopes to stain cells and quadrupole time-of-flight mass spectrometer to perform the detection (73). Mass spectrometry is able to discriminate isotopes of different atomic weights with high accuracy, enabling more features to be assayed at the same time, so the quantity of reporter ions in a particular mass channel represents the marker expression with little signal overlap between parameters.

Among high-parameter single-cell technologies, at present flow cytometry is the gold standard. However, it reveals the different percentages of cell populations in different pathophysiological onsets barely identifying different clones (74). Flow cytometry perfectly captures the phenotype of cells, but fails to snap their biological complexity. The spectrum of phenotypic diversity of immune cells within the TME and in blood can better be appreciated by analyses at the single-cell level to explore cellular heterogeneity, in terms of gene expression and chromatin accessibility, that often confounds biomolecular variation from multi-omics approaches in bulk. Recently, the adaptation of high-parameter flow cytometry to imaging platforms took to the development of two promising technologies, known as Imaging Mass Cytometry (IMC), Co-detection by Indexing (CODEX) and multiepitope-ligand-cartography (MELC) (55–57, 75, 76). The former is used to process tissues, employs antibodies tagged with lanthanides and combines a high resolution laser ablation system with CyTOF (77). IMC enables the study of cell-cell interactions and of intercellular networks, thus providing information regarding the spatial distribution of cells within a tissue (57–59). CODEX employs oligonucleotide-conjugated antibodies. Although these technologies have not yet been applied to dissect immune responses in the field of cancer immunotherapy, the power will be used to investigate the role of immune cells in the TME.

Single-Cell RNA Sequencing

Single-cell RNA sequencing (scRNA-seq) technology provide a transformative view of cell-type-specific gene expression and allows to analyze hundreds of messenger RNAs (mRNAs) in a single experiment, enabling the reconstruction of a high-resolution map of cells according to their molecular signature

TABLE 1 | Advantages and disadvantages of the cutting-edge single-cell technologies to profile cancer immunity.

Methodology	Advantages	Disadvantages	References
Flow cytometry	• Evaluation of protein, RNA and DNA at a single-cell level simultaneously; • Easy and fast sample preparation; • Acquisition of sample is high-throughput and fast; • A plethora of unsupervised and supervised data analysis methods available (global data structure, cellular progression, cellular diversity, signaling network inference, correlative/predictive features of clinical outcome or sample type); • Possible to sort cells and perform further studies; • The cost is cheap (more or less, US $ 0.10 per cell).	• Limit to 30-parameters at time; • Spillover among different fluorescences; • Quality control of the data needed; • High level of expertise is needed to analyze data; • No information on tissue structure; • Acquisition of samples must occur in a few hours after staining due to photo bleaching.	(53)
Mass cytometry	• Evaluation of protein, RNA and DNA at a single cell level simultaneously (up to 40 parameters—theoretically around 100); • Sample preparation is fast; • Acquisition of sample is high-throughput; • Metal-tagged samples can be run up to 2 weeks after staining without notable loss of signal and can be cryopreserved up to 1 month without affecting the data quality or staining integrity of both surface and intracellular markers; • A plethora of unsupervised and supervised data analysis methods available (global data structure, cellular progression, cellular diversity, signaling network inference, correlative/predictive features of clinical outcome or sample type).	• Sample acquisition is not fast; • Difficult to measure molecules that are expressed at very low levels; • Quality control of the data needed; • Spillover between close isotopes; • High level of expertise needed to analyse data; • No information on tissue structure; • Impossible to recover living cells after analysis; • The cost is much higher than fluorescence-based flow cytometry (more or less, several US dollars per cell).	(54)
Image-flow cytometry	• Evaluation of protein, RNA and DNA at a single cell level simultaneously (up to 12 parameters); • Easy and fast sample preparation; • Up to 10 fluorescent images per cell; • Images up to 60x magnification; • Detailed localization of signal from fluorescent probes.	• Sample acquisition is not fast; • No information on tissue structure; • High expertise is needed to analyse data; • Only few software used to analyse data; • Not possible to perform unsupervised analysis.	(55)
Histo-cytometry	• Technology is based on multiplexed antibody staining, tiled high-resolution confocal microscopy, voxel gating, volumetric cell rendering, and quantitative analysis; • Gain positional and quantitative information on complex cellular subsets/phenotypes (defined by multiple markers) directly in tissue sections; • Very high-resolution imaging and accurate signal 3D allocation.	• 6–8 colors/parameters; • Spillover between fluorochromes; • Due to the lack of molecular level resolution, imaging does not spatially separate neighboring fluorescent molecules, instead colocalizing them to the same voxel (volumetric pixel); • Software dedicated to imaging; • Low- throughput.	(56)
Imaging mass cytometry	• Analytical platforms that successfully couple high-density analysis by mass cytometry to conventional histology; • Comprehensive exploration of individual cell phenotypes, cell–cell interactions, microenvironments, and morphological structures within intact tissues.	• 1 μm spot size • Sample preparation is slow and needs a lot of technical advices; • The rate of image acquisition by laser ablation is slow (1.5 mm^2 in 2 h), and sets a practical limit to the extent to which a slide can be scanned; • Many tissue markers of clinical importance show considerable intratumoral heterogeneity in their distribution patterns; • Data analysis remains challenging, and is performed by particular and dedicated software (like HistoCAT).	(57–59)
Single-cell RNA sequencing	• Different methods developed in recent years allow to investigate single-cell transcriptomics; • Two low-throughput plate-based methods (Smart-seq2 and CEL-Seq2) and five high-throughput methods (10x Chromium, Drop-seq, Seq-Well, inDrops, and sci-RNA-seq); • Standardized and optimized protocols; • Very high-throughput; • A plethora of data analysis methods available (global data structure, cellular progression, cellular diversity, signaling network inference, network reconstruction); • On the basis of the type of sequencing it is possible to identify cell clonality, allelic expression, alternative splicing, RNA editing; • 2,000–6,000 genes per cell for primary cells if SMART-seq2 is used; 1,000–3,000 genes if Drop-seq or InDrop is used;	• Long procedures to prepare cDNA libraries; • Sample preparation is long (2 days of protocol); • High cost of single cell sequencing (thousands of US $ per sample); • Data analysis requires the use of highly advanced bioinformatics methods; • Quality control, normalization and imputation needed; • Due to technical limitations and biological factors, scRNA-seq data have some background, and are more complex than bulk RNA-seq data. • The high variability of scRNA-seq data raises computational challenges in data analysis.	(60–62)

(Continued)

TABLE 1 | Continued

Methodology	Advantages	Disadvantages	References
	• Low cost of sample preparation: $3–6 per well (if SMART-seq2 protocol is used); $0.05 per cell (if DropSeq or InDrop protocol is applied).		
Single-cell ATAC sequencing	• It interrogates the genome for accessibility to DNA binding proteins in a single experiment; such challenge emphasizes the need for informative features to assess cell heterogeneity at the chromatin level; • scATAC-seq experiments sample DNA, compared to transcriptomic (scRNA-seq) data; • Single-cell ATAC libraries are created from single cells that have been exposed to the Tn5 transposase using one of the following protocols: Single cells are individually barcoded by a split-and-pool approach where unique barcodes added at each step can be used to identify reads originating from each cell, microfluidic droplet-based technologies are used to extract and label DNA from each cell, or each single cell is deposited into a multi-well plate for library preparation. • A plethora of data analysis methods available.	• Sample preparation is long (2 days of protocol); • Data analysis for expert requires the use of bioinformatics methods.	(61, 63)

(66). The first example of single-cell digital gene expression profiling was published in 2009, and since then on, a continuous effort has been made to improve experimental protocols and bioinformatics pipelines, which are essential to process data (60, 78, 79). A canonical scRNA-seq protocol involves several steps, including single cell isolation, cell lysis to obtain RNA, reverse transcription into the first-strand cDNA, second-strand synthesis, cDNA amplification, and sequencing (60–62).

Although single cells can be isolated by different techniques, the use of microdroplet-based microfluidics is now widely diffused among the majority of commercial platforms and allows the isolation of individual cells into aqueous droplets in a continuous oil phase. In every droplet, cells are lysed in a hypotonic buffer, and mRNAs are captured by poly-dT primers. When reverse transcription takes place, cDNA molecules originated from a given individual cells are identified by using short DNA barcode tags. Then, second strands are generated, and the small amount of synthesized double-strand cDNA is amplified by means of conventional polymerase chain reaction (PCR) or *in vitro* transcription, depending on the technology. Some protocols improved read coverage across transcripts, which significantly enhances detailed analyses of alternative transcript isoforms and identification of single nucleotide polymorphisms (SNPs) with high sensitivity and accuracy (80). Eventually, sequencing is performed, and once reads are obtained, data are pre-processed and analyzed through clustering of cells, classification and cell trajectory assignment (78, 79). Concerning T cells, during the last years, several algorithms have also been developed to utilize scRNA-seq data to reconstitute TCR information. TCR is a heterodimer composed of two chains, α and β, which result from genetic recombination of the V(D)J genes, and is responsible for the specificity of each T cell against cognate antigens. The diversity of TCRαβ repertoire is associated with efficient protection against several pathogens (81), and more recently, the clonality of both peripheral blood and tumor TCRαβ repertoire has also been associated with improved clinical outcome under anti-PD-1 or anti-CTLA-4 immunotherapy (82–84).

Despite its numerous pros and great potential, scRNA-seq suffers from the caveat that mRNA and protein expression do not always directly correlate. For this reason, recent technological advances have been made to capture new cell types with a better resolution, and to detect simultaneously mRNAs and proteins. For example, Cellular Indexing of Transcriptomes and Epitopes by Sequencing (CITE-seq), RNA Expression and Protein Sequencing (REAP-seq), Antibody sequencing (Ab-seq) enable the measurement of proteins and mRNAs in individual cells, by using antibodies labeled with DNA barcodes instead of fluorochromes, thus avoiding the limitations dictated by the possible spectral overlap of fluorescent signals (85–87). Quantifying proteins together with mRNAs allows to overcome the lack of correlation that sometimes exists between mRNA and protein levels, thus providing a more readout of cellular phenotype, at the single-cell level. Indeed, proteins, not mRNAs, are the real targets of drugs, and mRNA abundance cannot necessarily resemble protein abundance (85). Moreover, in certain settings, the measurement of protein abundance is more sensitive for markers with low levels of mRNA transcripts (85). Thus, CITE-seq, REAP-seq, and Abseq give an unbiased view of the mRNA and protein profile at the single-cell level, which is necessary to precisely identify cellular function, and provide important insights into the pathophysiology of multiple disorders. However, sample preparation requires more than 2 days and cells need to be fixed or lysed, therefore excluding the possibility to perform further analysis (85–87).

Another possibility to investigate both mRNA and proteins is the combination of scRNA-seq and high-parameter flow cytometry. The combinatorial use of scRNA-seq and high-parameter flow cytometry in the same sample would likely have a huge impact in the field of immunotherapy, as is associated with unique advantages to each method together with the advantage of using both methodologies. Whereas each technology uses

unsupervised clustering to identify different populations, scRNA-seq is totally unbiased as it analyses the expression of thousands of genes. Conversely, high-parameter flow-cytometry looks at 30–40 markers that are pre-selected based on *a priori* knowledge. Also, scRNA-seq allows transcriptomic analysis between individual cell subsets, including the use of Gene Set Enrichment Analysis (GSEA) and comparisons to human datasets. However, as already described, mRNA and protein do not always correlate, meaning that the information on protein expression delivered by high-parameter flow-cytometry is also central. However, to date, a few studies reported the combination of scRNA-seq and CyTOF to profile the tumor immune microenvironment (88, 89).

Single-Cell ATAC-Seq

The Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq) is a method for assessing genome-wide chromatin accessibility. ATAC-seq identifies accessible DNA regions by probing accessible chromatin with hyperactive mutant Tn5 transposase that inserts sequencing adapters into open regions of the genome (90). Single cell ATAC-seq (scATAC-seq) measures chromatin accessibility enabling marker-free identification of cell type-specific *cis*- and *trans*-regulatory elements and mapping of disease-associated enhancer activity and reconstruction of trajectories of cellular differentiation, and has been used to map gene regulation in cell-to-cell variability and rare cell phenotypes, including in healthy and malignant immune cells (61, 63).

The Analysis of Single-Cell Data

Single-cell technologies generate huge amount of information that allow the exploration of cellular diversity at unprecedented depth and throughput. For this reason, one of the major analytical challenge is how to visualize and understand this high-dimensional datasets originating from high dimensional flow cytometry, scRNA-seq and scATAC-seq. Data generated by high-dimensional flow cytometry (up to 30 parameters in several million cells) can no longer be analyzed by using classical manual analysis techniques involving the use of bidimensional dot plots (91). Manual gating analyses is hard to reproduce, as is subjective and biased, and for large data set is extremely time consuming. Large datasets are computationally demanding, and therefore require the development and the application of novel techniques.

Computational flow cytometry provides a set of packages to analyze and visualize large amount of cells in an unbiased manner (92). These tools are automated, meaning that the quality of data is fundamental to get rid of false positive. For this reason, before analyzing high-parameter flow cytometry datasets, files need to be perfectly compensated, cleaned from the presence of aggregates and turbulences during acquisition. Only after this step, data can be analyzed by unsupervised tools (93, 94).

scRNA-seq requires pre-processing of data based on quality control performance and alignment (78). Several efforts have been made from bioinformaticians to develop and optimize new software and packages able to provide insights on the complex biology and dynamics of cells (66). Most software provide information regarding identification and characterization of cell types and their spatial organization in time (78). A canonical pipeline of data analysis firstly requires data visualization. There are methods based on dimensionality reduction techniques, including principal component analysis (PCA), t-distributed stochastic neighbor embedding (t-SNE), One-Dimensional Soli-Expression by Nonlinear Stochastic Embedding (ONE-sense), Uniform Manifold Approximation and Projection (UMAP), that aim to preserve the main structure of data while reducing a high-dimensional data description to a lower-dimensional projection (95–97). An example of the analysis of the same data by using PCA, t-SNE, and UMAP is reported in **Figure 1**. In addition, clustering-based techniques are available that group cells into cell type clusters in the original, high-dimensional space and subsequently use visualization algorithms to represent these cell type clusters in a lower-dimensional space (93, 98, 99).

Secondly, differences in gene expression level between populations need to be analyzed. To this purpose, specialized methods have been designed for single-cell data that considers single cell features such as technical dropouts and shape of the distribution (100).

Thirdly, the software Monocle and Wanderlust independently introduced the concept of "pseudotemporal analysis," in which scRNA-seq data are collected from a population of cells undergoing a dynamic biological process and then computationally ordered into a trajectory that reflects the continuous changes in gene expression that occur from the beginning to the end of the process (101–103). Pseudotime trajectories allow to identify genes that exhibit differential expression over the course of the biological process and cluster them based on their expression dynamics. As of February 2020, more than seventy trajectory inference tools have already been developed (104).

THE WORKFLOW OF SINGLE-CELL EXPERIMENT

Regardless of the specific technology employed to generate a particular dataset, a common workflow can be formulated which involves multiple steps linking the initial study design to the final correlation to clinical data. A typical pipeline for single-cell experiments is reported in **Figure 2**. An accurate experimental planning is imperative to avoid technical issues and improve scientific reproducibility. Several professionals, including the statistician, the bioinformatician, the biologist and the clinician should be involved at this step to: (i) define the biological question; (ii) find patients; (iii) calculate the sample size; (iv) define the number of replicates; (v) decide the number of cells; (vi) define the sequencing depth (in the case of scRNA-seq or sc-ATACseq experiments); (vii) choose the appropriate equipment (105). At this stage, experimental protocols should be standardized, and appropriate positive and negative controls should be selected to ensure good quality results. Then, experiment is performed and raw data are generated. Alongside, data pre-processing is performed. Quality control involves the examination of data, their possible transformation and normalization, the check for technical issues, batch effects or

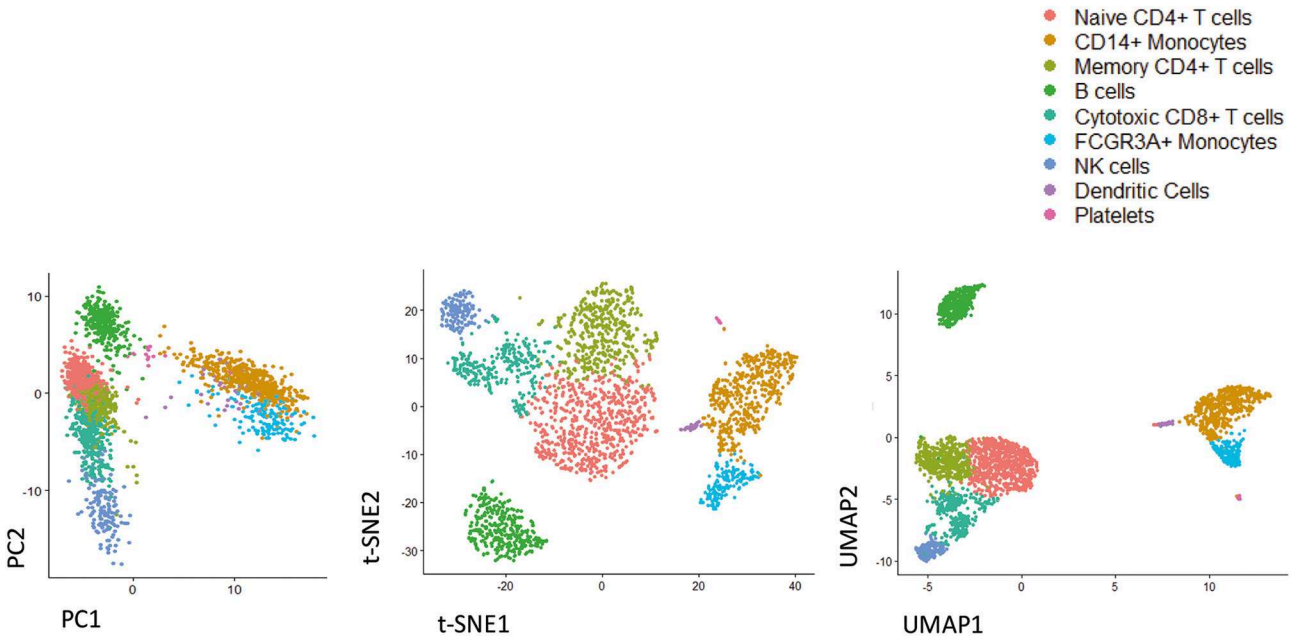

FIGURE 1 | Representative image of different dimensionality reduction techniques that are widely used in single cell studies. As shown by analyzing freely available scRNA-seq dataset (3k PBMC, from 10X Genomics), UMAP preserves much of the local and more of the global data structure, highlighting its ability to resolve even subtly differing cell population. From left to right PCA, t-SNE, UMAP.

unexpected results. At the end of the entire process, clean data need to be visualized and analyzed by computational approaches to identify clusters and trajectories, and potentially derive novel predictive biomarkers of response to ICI.

DATA FROM SINGLE-CELL STUDIES

Immune Cells in the Tumor Microenvironment

Tumors contain different cell populations in endless evolution. This diversity is commonly referred to as tumor heterogeneity, and is considered the main driver of resistance to therapy and metastasis (106). The full comprehension of this heterogeneity would be extremely important to optimize existing therapeutic intervention and find new strategies to break down relapses and mortality. The recent development of technologies based on sequencing individual cells has been crucial to address tumor heterogeneity and to elucidate how cells are organized into multicellular systems. Single cell profiles not only revealed that human tumors comprise subpopulations of genetically different diverse malignant cells, but also that a profusion of different cell types from the surrounding tissues and the immune system, each with a precise role in pathogenicity, is present within the TME (106, 107). The immune components of the tumor microenvironment in different kind of malignancies, including non-small cell lung cancer (NSCLC), clear cell RCC (ccRCC), breast cancer (BC), HCC, glioblastoma multiforme (GMB), microsatellite instability-stable CRC have been recently annotated and finely characterized (88, 108–111). In general, in the majority of these tumors, immune cells were mostly T cells, whereas myeloid cells, B cells and NK cells were found at lower frequencies (108–111). Only GMB had higher levels of CD68+ myeloid cells if compared to T cells (88).

In NSCLC tumor samples, different subsets of CD8+ T cells, conventional CD4+ T cells, and Treg cells have been found (109). Each subset is characterized by a precise gene expression signature, which reflects a specific functional status. Two main clusters were found at high frequency: (i) exhausted CD8+ T cells, characterized by increased expression of effector molecules, including GZM-A, granulysin (GNLY), perforin (PRF), GZM-B, NKG7, and inhibitory receptors, like lymphocyte activating (LAG)-3 (CD223), T cell immunoreceptor with Ig and ITIM domains (TIGIT), PD-1, and CTLA-4; (ii) suppressive CD4+ Treg cells, characterized by increased expression of costimulatory molecules, including CD28 and inducible T cell costimulatory (ICOS or CD278), and inhibitory receptors like CTLA-4 and TIGIT (109). Moreover, two CD8+ T cell subsets exhibited a functional state that precede exhaustion, and is indeed called pre-exhaustion state. These subsets do not express CTLA-4, and express mild levels of TIGIT, PD-1, and the transcription factor TOX, which is a critical driver of exhaustion (112–115). Whether or not pre-exhausted subsets could be more effectively reinvigorated by ICI than fully exhausted subsets is still not known. Furthermore, the expression level of PD-1 or other inhibitory receptors does not necessarily correlate with exhaustion-dependent dysfunction. It was indeed reported that tissue-resident memory T (T_{RM}) cells expressing PD-1, T-Cell Immunoglobulin And Mucin Domain-Containing Protein-3 (TIM-3) and negative for CD127 (the α chain of the IL-7 receptor), which are present in lung

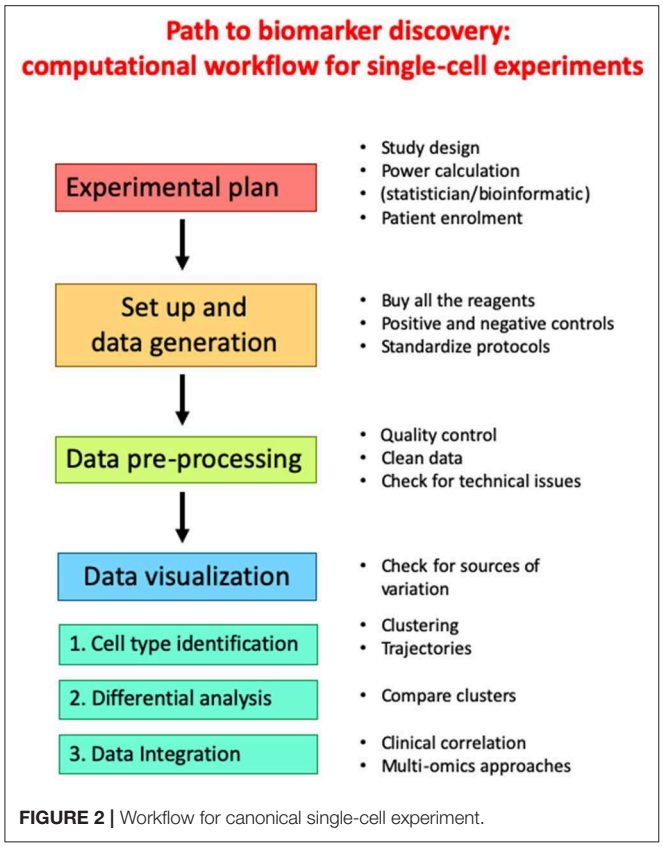

FIGURE 2 | Workflow for canonical single-cell experiment.

tumors, proliferate, can upregulate TCR activation–induced genes, exhibit a transcriptional signature indicative of effector, survival, and tissue-residency properties, and produce cytokines, like IL-2, IFN-γ and TNF-α (116). In early-stage triple-negative BC, among CD8+ T cells infiltrating the TME, T_{RM} cells display high levels of genes encoding for cytotoxic molecules, including *GZMB* and *PRF1*, high levels of genes encoding for inhibitory checkpoint, as well as high levels of genes associated with proliferation (117). This means that the expression of inhibitory receptors is not a unique feature of exhausted T cells as several highly functional effector cells also express those receptors. This also means that exhausted T cells are heterogenous, and that T cell exhaustion, as well as T cell dysfunctionality, is a gradual, rather than a discrete, state (118).

In ccRCC, in-depth immunophenotyping analysis identified the main immune cell types in both T cells and tumor-associated macrophages (TAMs) (108). Concerning T cells, eight CD4+ clusters, eleven CD8+ clusters, one CD4+/CD8+ double positive cluster, and one CD4–/CD8– double negative cluster were identified (108). Notably, whereas PD-1 had a broad expression both CD8+ and CD4+ T cell population, other inhibitory molecules, like TIM-3, CTLA-4, and 4-1BB (CD137) were expressed only by a few PD-1+ subsets, indicating that a pre-exhaustion status is also present in ccRCC (108). Interestingly, both in CD8+ and CD4+ T cells, PD-1 is co-expressed with CD38, which mediates immunosuppression by activating nitric oxide synthetase which in turn catalyzes the production of nitric oxide from arginine. Although CD38 has traditionally been linked to T cell activation, these data suggest that its expression is not restricted to activated cells, but rather can be extended to exhausted T cells, at least in ccRCC and BC (108, 110). In the latter, a higher frequency of PD-1^{high}CTLA-4+CD38+ T cells has been observed in tumor biopsies if compared to juxta-tumoral tissues, thus confirming that PD-1 and CD38 are both expressed in exhausted cells (110). Indeed, CD8+ T cells expressing high level of PD-1 also expressed the co-inhibitory molecules TIM-3 and CTLA-4, and the activation markers HLA-DR and CD38, which were not expressed by CD8+ T cells expressing intermediate levels of PD-1 (110). Similarly, HCC biopsies were mostly enriched by exhausted CD8+ T cells and Treg cells, and exhausted CD8+ T cells were increased in patients with late stage HCC if compared with early stage HCC (111).

Another cluster of special interest in HCC consisted of mucosal-associated invariant T (MAIT) cells, which are mainly involved in the protection against bacterial or viral mucosal infections (119). Although MAIT cells are considered as a first line defense in the liver, their role in liver cancer is still totally unexplored. Recent evidences revealed that tumor initiation and metastasis formation is reduced in mice knockout for MHC class I-related protein-1 (MR1), which is essential for MAIT development (120). A fraction of MAIT cells among tumor CD8+ T cells has been found also in NSCLC and CRC (109, 121). Interestingly, at least in chronic infections, MAIT cells can express inhibitory receptors, including PD-1, thus meaning that they could also be targets of ICI (122).

In uveal melanoma, single-cell analysis revealed that tumor-infiltrating immune cells, including CD8+ T cells and NK cells, mainly express LAG3, rather than PD-1 or CTLA-4 (123), thus partially explaining the limited efficacy of checkpoint inhibitor therapy in this type of tumors (124). This further confirms that PD-1 is not the exclusive determinant of CD8+ T exhaustion and that the expression of additional markers should be considered across different tumors. The situation is even further complicated by the fact that T cell exhaustion is associated with vast changes in chromatin accessibility (125). Emerging evidence revealed that exhausted CD8+ T cells are epigenetically distinct from functional memory CD8+ T cells, thus suggesting that exhausted T cells occupy a different differentiation state if compared to memory T cells (125).

ScRNA-seq analysis of NK cells obtained from human melanoma metastases indicated that seven clusters of tumor-infiltrating NK are present in these tissues, each with an individual functional specialization (126). NK cells were recently shown to recruit cross-presenting DCs to tumors that are critical for CD8+ T cell–mediated tumor immunity (126).

Although T cells have a dominant role in controlling cancer growth, there is growing interest for other subsets of immune elements that infiltrate the TME, including B and myeloid cells, and that could have a role in the response to therapy. Tumor-infiltrating myeloid cells (TIMs) consist of various subsets of granulocytes, monocytes, macrophages and DCs, at different stage of differentiation, that contribute to cancer progression and response to therapy (127, 128). Among

TIMs, the frequency of a specific subset of monocytes, i.e., CD14+, CD16–, HLA-DR^{high} monocytes, has been identified as predictor of progression-free and overall survival in patients with metastatic melanoma prior anti-PD-1 therapy (129). High-dimensional single-cell profiling of lung cancer revealed that an enrichment of macrophages expressing high levels of peroxisome proliferator-activated receptor (PPAR)-γ has been observed in lung adenocarcinoma at early stage (130). Macrophages in the TME have also been studied in breast cancer, renal cancer and HCC using scRNA-seq data (4, 108, 131). TAM-like macrophages in HCC highly express two genes, *SLC40A1* and *GPNMB*. The former encodes ferroportin, a transporter exporting iron from cells, and regulates pro-inflammatory cytokines, like IL-6, IL-23, and IL-1β, through a Toll like receptor (TLR)-dependent pathway (131).

Single-cell profiling of tumor biopsies also revealed that DCs can be present at the TME (4, 108, 130). Among TIMs, DCs are the best armed to prime and activate T cells (132), and among DCs, several subsets with a specific molecular signature have been found to be depleted or enriched in the TME. This was possible by combining CyTOF with single-cell transcriptomics. For example, CD141+ DCs express high levels of *CD207*, *CLEC9A*, and *XCR1* and preferentially interact with CD8+ T cells, whereas CD1c+ DCs express high levels of *CX3CR1*, *IRF4*, *CCL22*, and *CCL17,* which are involved in chemokine signaling, and are better equipped to interact with CD4+ T cells (130). Also LAMP3+ DCs have various interesting features (131). They indeed exhibit a higher migration capacity toward lymph node if compared to conventional DCs (131).

Checkpoint Inhibitor Therapy Effects on TILs and PBMCs

During the last years, single cell technologies have been used to interrogate a number of tumoral settings with the goal to understand both successful and ineffective immune responses after treatment with ICI, and identify accessible biomarkers that clinicians could use to discriminate between patients who most likely respond or not to therapy (2). The most important studies reporting the use of cutting-edge single-cell technologies to identify the effects of checkpoint inhibitor therapy on immune system are reported in **Table 2**. Concerning the type of neoplasia, the vast majority of studies regard patients with melanoma or NSCLC, treated with anti-CTLA-4 or anti-PD-1 or, in few cases, with both of them.

CD8+ T Cells

Overall, among immune cells, main differences have been found in T cell compartment, and among T lymphocytes, cytotoxic cells are often affected by checkpoint inhibitor therapy. Single-cell technologies have shown that cytotoxic T cells do not form a homogenous population but are a heterogenous mix of cells with different transcriptomes, phenotype and functional capacity. According to their differentiation state and on the basis of the expression levels of few proteins, CD8+ T cells have been typically classified in well-defined subsets of naïve, memory, and effector cells (148). During the last few years, high-dimensional single-cell profiling allowed immunologists to understand that a variety of other states with significant phenotypic and functional diversity is observed within the CD8+ T cell compartment (149). This heterogeneity becomes increasingly relevant at the level of the TME, both within and among patients, and could be at the basis of the mechanisms linking T cells states and response to checkpoint inhibitor therapy.

A study performed on freshly isolated metastatic melanoma samples from two cohorts of 20 patients used flow cytometry alone to show that an increased fraction of tumor-infiltrating CD8+ T cells expressing high level of PD-1 and CTLA-4 strongly correlated with response to therapy and progression-free survival (133). These cells were named as "partially exhausted," as they retained the capacity to produce IFN-γ but lose the ability to produce TNF-α and IL-2 (133). In another cohort of patients with melanoma treated with ICI, single-cell RNA profiling of immune cells from baseline, on-therapy and post-therapy tumor samples was performed (139). Exhausted cells were defined as those with increased expression of several genes encoding for inhibitory receptors, including *LAG3*, *FASLG*, *HAVCR2* (which encodes for TIM-3), *PDCD1* (which encodes for PD-1), *CD38* (139). It was also showed that TIM-3 and CD39 were markers for discriminating exhausted from memory CD8+ T cells, and that the elevated frequency of TCF7+, CD8+ T cells can predict with a positive outcome (139). Concerning CD39, it was also found that CD8+ TILs from lung cancer and CRC were not only specific for tumor antigens but also could recognize a broad range of epitopes unrelated to cancer, and that CD39 was critical to distinguish tumor-specific CD8+ TILs from bystander CD8+ T cells (150).

In other melanoma patients treated with anti-PD-1, the combination of scRNA-seq to TCR-seq allowed to identify a dysfunctional axis consisting of cells able to actively proliferate despite having an "exhausted" phenotype (144). The application of different single-cell technologies to three different cohorts of melanoma patients treated with anti-PD-1 allowed to understand that a noteworthy phenotypic heterogeneity is observed within CD8+ TILs that display characteristics of dysfunction, reflected by various combinations and expression levels of inhibitory receptor and activation markers, the proliferative capacity and the ability to produce cytokines and effector molecules. A resistance program that is associated with hallmarks of T cell exclusion and suppression has also been found in malignant cells prior to immunotherapy, likely indicating the presence of intrinsic resistance (137).

Other striking results of single-cell technologies have been obtained in blood samples from cancer patients treated with ICI. In those with melanoma, circulating Ki67+, CD8+ T cell response was correlated with tumor burden (134). Similar results were found in NSCLC treated with anti-PD-1. After therapy, an increase of Ki-67+, PD-1+, CD8+ T cells displaying an effector-like phenotype (HLA-DR+, CD38+, Bcl-2^{low}), costimulatory molecules (CD28+, CD27+, ICOS+), high levels of PD-1 and co-expression of CTLA-4 was observed in patients responding to therapy (135, 140). In the same patients, the expansion of CD39+, CD8+ T cells was observed a few days after a single dose of anti-PD-1 in a neoadjuvant setting (145). Tracking TCR clones and transcriptional phenotypes in basal cell carcinoma (BCC)

TABLE 2 | Main studies reporting the use of cutting-edge single-cell technologies to identify the effects of checkpoint inhibitor therapy on immune system.

Tumor type	Sample source	Technology	Main findings	References
Melanoma	TILs	Flow cytometry	• High level of CD8+, PD-1^{++}, CTLA-4^{++} TILs correlated with response to therapy and progression-free survival; • Functional analysis of these cells revealed a partially exhausted T cell phenotype; • Assessment of metastatic lesions during anti–PD-1 therapy demonstrated a release of T cell exhaustion, as measured by an accumulation of highly activated CD8+ T cells within tumors.	(133)
Melanoma	PBMCs TILs	Flow cytometry	• CD8+ T cells responding to therapy display an exhausted phenotype; • TIL clones in responding peripheral blood CD8+ T cell population and blood Ki67+, CD8+ T cell response correlates with tumor burden.	(134)
NSCLC	PBMCs	Flow cytometry	• Increase in Ki67+, PD-1+, CD8+ T cells following therapy in ∼70% of patients (after the first or second treatment cycle); • Effector-like phenotype (HLA-DR+, CD38+, Bcl-2^{low}), expressed costimulatory molecules (CD28, CD27, ICOS), and had high levels of PD-1 and coexpression of CTLA-4.	(135)
Melanoma	TILs	Mass cytometry; RNA-seq	• The CD8+ T cell population expanded in ICI-treated tumors displayed a CD45R0+, PD-1+, TBET+, EOMES+ phenotype; • CTLA-4 blockade induces expansion of ICOS+ Th1-like CD4+ T cells.	(136)
Melanoma	tumor	RNA-seq; scRNA-seq; *in situ* multiplex protein	• Resistance program expressed by malignant cells, associated with T cell exclusion and immune evasion. The program is expressed prior to immunotherapy, characterizes cold niches *in situ*, and predicts clinical responses therapy; • CDK4/6-inhibition represses this program in individual malignant cells, induces senescence, and reduces melanoma tumor outgrowth in mouse models *in vivo* when given in combination with immunotherapy.	(137)
NSCLC	TILs	Flow cytometry RNA-seq	• PD-1^{++} T cells showed a markedly different transcriptional and metabolic profile from PD-1^{+-} and PD-1^{-} lymphocytes, as well as an intrinsically high capacity for tumor recognition; • PD-1^{++} lymphocytes were impaired in classical effector cytokine production, they produced CXCL13, which mediates immune cell recruitment to tertiary lymphoid structures; • The presence of PD-1^{++} cells was strongly predictive for both response and survival.	(138)
Melanoma	tumor	scRNA-seq; ATAC-seq	• Two distinct states of CD8+ T cells were defined by clustering and associated with patient tumor regression or progression; • TCF7 was visualized within CD8+ T cells in fixed tumor samples and predicted positive clinical outcome.	(139)
Melanoma 1 patient, 90 years old	PBMCs TILs	Flow cytometry; TCR sequencing	• Proliferating CD8+ T cells exhibited an effector-like phenotype with expression of CD38, HLA-DR and Granzyme B, as well as expression of the positive costimulatory molecules CD28 and CD27; • TCR sequencing of peripheral blood CD8+ T cells revealed a highly oligoclonal repertoire at baseline with one clonotype accounting for 30%.	(140)
Melanoma	PBMCs	Mass cytometry	• Frequency of CD14+, CD16–, HLA-DR^{hi} monocytes before therapy is a strong predictor of progression-free and overall survival.	(129)
Melanoma, NSCLC	TILs PBMCs	Flow cytometry; RNA-seq	• CD4+, FoxP3-, PD-1^{hi} T cells (4PD1^{hi}, a TFH-like phenotype) negatively regulate T cell responses; • CTLA-4 and PD-1 blockade modulate 4PD1^{hi} frequency in opposing directions; • 4PD1^{hi} are a pharmacodynamic and negative prognostic factor of checkpoint blockade.	(141)
Melanoma, Prostate cancer, Bladder cancer	Tumor	IHC; CyTOF	• Both ipilimumab and tremelimumab increase the infiltration of CD4+ and CD8+ cells without significantly changing or depleting FOXP3 cells within the tumor microenvironment.	(42)

(Continued)

TABLE 2 | Continued

Tumor type	Sample source	Technology	Main findings	References
Melanoma	Tumor	RNA-seq; Multiplex IHC; CyTOF	• Tumors from non-responders to monotherapy often express other immune checkpoints and higher gene expression profile of EOMES+, CD69+, CD45RO+ T cells is associated with greater tumor shrinkage in both therapies.	(142)
Glioblastoma	Tumor TILs	Flow cytometry; RNA-seq	• Neoadjuvant nivolumab resulted in enhanced expression of chemokine transcripts, higher immune cell infiltration and augmented TCR clonal diversity among tumor-infiltrating T lymphocytes.	(143)
Melanoma	Tumor	MARS-seq; scTCR-seq	• scRNA-seq and TCR analysis in melanoma identifies a gradient of T cell dysfunction; • Cytotoxic T cells are unconnected to the dysfunctional gradient; • Proliferation in CD8+ T cells is most profound during early stages of dysfunction; • The abundance of dysfunctional CD8+ T cells is associated with tumor recognition.	(144)
Melanoma	TILs PBMCs	Flow cytometry; RNA-seq	• After a single dose of anti-PD-1, rapid pathologic and clinical responses associated with accumulation of exhausted CD8+ T cells in the tumor at 3 weeks, with reinvigoration in the blood observed as early as 1 week; • A pre-treatment immune signature (neoadjuvant response signature) associated with clinical benefit.	(145)
Melanoma	TILs	scRNA-seq; TCR sequencing	• Tracking TCR clones and transcriptional phenotypes revealed coupling of tumor recognition, clonal expansion and T cell dysfunction marked by clonal expansion of CD8+, CD39+ T cells; • The expanded clones consisted of novel clonotypes that had not previously been observed in the same tumor. Clonal replacement of T cells was preferentially observed in exhausted CD8+ T cells and evident in patients with basal or squamous cell carcinoma.	(146)
Basal cell carcinoma	PBMCs TILs	scATAC-seq	• Serial tumor biopsies before and after PD-1 blockade identifies chromatin regulators of therapy-responsive T cell subsets and reveals a shared regulatory program that governs intratumoral CD8+ T cell exhaustion and CD4+ T follicular helper cell development.	(63)
Melanoma, RCC	TILs	scRNA-seq; CyTOF	• B cells found in tumors of responders; • B cells localized in the TLSs; • CyTOF shows differential B cell phenotypes.	(147)

TILs, Tumor-infiltrating lymphocytes; PBMCs, peripheral blood mononuclear cells; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing; NSCLC, non-small cell lung cancer; TCR, T cell receptor; CyTOF, Cytometry by Time-Of-Flight; MARS-seq, massively parallel single-cell RNA-sequencing; scATAC-seq, single-cell Assay for Transposase-Accessible Chromatin using sequencing; ICI, immune checkpoint inhibitors; TLSs, tertiary lymphoid structures; IHC, immunohistochemistry.

also revealed clonal expansion of CD8+, CD39+ T cells, which co-expressed markers of chronic T cell activation and exhaustion. However, in this case, the expansion of T cell clones did not derive from pre-existing TILs, but from novel clonotypes that had not previously been observed in the same tumor (146). This suggests that the response to anti-PD-1 depends on the intrinsic capacity of tumors to recruit novel T cell clones, which replace pre-existing exhausted T cells that have insufficient capacity to reinvigorate in response to therapy (151).

In addition, data obtained from melanoma samples and peripheral blood from patients treated with anti-CTLA-4 and anti-PD-1 revealed that treatment-specific effects can be observed. Indeed, while anti-PD-1 mainly induced the expansion of specific tumor-infiltrating exhausted-like CD8+ T cell subsets, anti-CTLA-4 led to the expansion of an ICOS+ Th1-like CD4+ effector subsets other than engaging specific subsets of exhausted-like CD8 T cells (136). It was also reported that the population of CD8+, CD45RO+, PD-1+, TBET+, EOMES+ T cells increased after treatment only in TILs if compared to the peripheral blood (136), and that the gene expression signature of EOMES+, CD69+, CD45RO+ T cells was associated with greater tumor shrinkage in both therapies (142). Likewise, in a cohort of patients with NSCLC treated with anti-PD-1, the presence of PD-1^{++} T cells within the tumor was strongly predictive for both response and survival (138). PD-1^{++} T cells indeed produce C-X-C Motif Chemokine Ligand 13 (CXCL13), which mediates immune cell recruitment to TLSs (138). Similarly, in a cohort of patients with GMB treated with anti-PD-1 an enhanced expression of chemokine transcripts, higher immune cell infiltration and augmented TCR clonal

diversity among tumor-infiltrating TILs was reported (143). In summary, a large variability can be observed among different patients' cohorts concerning the abundance of different T cell functional states. An increase in CD8+ T cells with an effector-like phenotype expressing inhibitory/costimulatory molecules and proliferations markers has been described in several cancer settings after therapy with ICI. However, only in few cases this immune cellular response were correlated with a measurable clinical response.

CD4+ T Cells

The vast majority of recent studies based on single-cell technologies have been focused on CD8+ T cells, as their role in cancer surveillance, editing and control is compelling. However, a role in tumor control is also played by the CD4+ T cell compartment, as reflected by the observation that CD4+ T cells infiltrate the tumor, and by the prognostic value of several CD4 subsets in different malignancies (152–154). Distinct CD4+ T cells subsets have been described by means of single-cell technologies, including naïve cells, memory-like cells, Th1 cells, Treg, follicular helper T cells (T_{FH}), and even cytotoxic effector T cells (109, 111, 121, 141, 144, 146, 155, 156).

In NSCLC tumor and blood samples, scRNA-seq allowed to identify seven CD4+ T cell populations (109). Interestingly, among them an "exhausted" CD4+ T subset was present and displayed a gene signature comparable to that observed in exhausted CD8+ T cells. Two Treg clusters were also identified and one of them was defined as "suppressive Treg" as cells expressed high levels of *TNFRSF9* (encoding for 4-IBB), *TIGIT* and *CTLA-4* genes (109). A closer quantification of this cluster in blood and tumor samples revealed that a higher percentage of suppressive Treg cells was present in tumor if compared to blood (109).

A combination of scRNA-seq and TCR analysis allowed to identify a subset of "dysfunctional" CD4+ T cells in a cohort of melanoma patients, and again these dysfunctional cells expressed specific combinations of genes encoding for inhibitory checkpoints that partially overlapped with those observed in CD8+ T cells (144). The fact that in TME CD4+ T cell also express PD-1 and/or CTLA-4 suggests that most of the current immunotherapy strategies that use checkpoint inhibitor can potentially leverage on these cells. Although data dissecting the effects of these drugs on CD4+ T cells are still elusive, it was recently found that in melanoma patients treated with anti-PD-1/anti-CTLA4 the frequency of the T cell population characterized by a T_{FH}-like phenotype (CD4+, Foxp3-, PD-1^{high}) is modulated differently by the two drugs and is a negative prognostic factor of response to therapy (141).

Other Cells Than T Lymphocytes

Through mass cytometry and scRNA-seq, in GMB a unique subset of macrophages expressing high levels of CD73 able to persist after anti-PD-1 therapy was observed (88). Notably, a number of reports have shown that CD73 can induce immunosuppression in GMB (157, 158).

Tumor-infiltrating B cells exist and are mainly found in lymphoid aggregates, known as TLSs (147, 159). It was found that the density of CD20+ B cells and TLSs, together with the ratio of TLSs to tumor area were higher in responders than in non-responders (147). Moreover, a prognostic B-cell-related gene signature was found in patients with cutaneous melanoma or RCC. Several genes, including *FCRL5*, *IDO1*, *IFNG*, and *BTLA*, were indeed enriched in patients responding to therapy (147).

CONCLUSIONS

The interactions between tumor and immune system are ruled by several complex mechanisms, with several main players such as malignant cells, tumor infiltrate, tumor stroma and vasculature, and systemic factors. Among them, the heterogeneity of intra-tumor immune cells has been extensively studied by using traditional approaches, including basic flow cytometry and immunohistochemistry, which have the limitations described above. Recently, substantial advances in emerging techniques and bioinformatic pipelines have enabled researchers to investigate in detail the complexity of the TME, and to interrogate in depth previously unexplored cell types. Among single-cell approaches, scRNA-seq has been crucial for exploratory analysis, and the combination of scRNA-seq with mass cytometry has been even more helpful.

The application of single-cell technologies to tumor and blood samples has generated and will generate in the upcoming years, an explosion of new data with a clear impact in the translational clinical research, thanks to the identification of possible biomarkers. It is likely that the huge amount of information will also thoroughly revolutionize the field of basic research in immunology and cancer biology. A big effort should be posed to make all data, including the raw ones, available to the scientific community and to create rigs for data extraction. The information gathered from these technologies will add novel hallmarks of response to immune therapy that could be integrated in the routine clinical management.

Nonetheless, the route to the discovery of new biomarkers is still bumpy. Due to the high sensitivity of single-cell technologies, adequate attention must be put into experimental setup and execution. A very careful handling of cells during pre-processing and an adequate data analysis with potent bioinformatic tools are critical factors to preserve the native biological profile that will ensure meaningful conclusions.

Lastly but importantly, although a number of specific immune cell subsets have been identified that are associated with response or resistance to ICI, still additional studies should be planned to address the role and function of different types of immune cells in the TME. Investigating the role of T cell exhaustion and/or dysfunction in the TME and translating this knowledge to clinical practice can be considered main challenges in the battle against cancer.

AUTHOR CONTRIBUTIONS

CP, RD, GP, RS, and AC outlined the concept, wrote the manuscript, and overviewed the review. LG and SD wrote the manuscript. LG, SD, and DL designed and prepared the figures.

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Pro-inflammatory TNF-α and IFN-γ Promote Tumor Growth and Metastasis via Induction of MACC1

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Colorectal cancer (CRC) is one of the most common malignancies worldwide. Early stage CRC patients have a good prognosis. If distant metastasis occurs, the 5-year survival drops below 10%. Despite treatment success over the last decades, treatment options for metastatic disease are still limited. Therefore, novel targets are needed to foster therapy of advanced stage CRC patients and hinder progression of early stage patients into metastasis. A novel target is the crucial oncogene Metastasis-Associated in Colon Cancer 1 (MACC1) involved in molecular pathogenesis of CRC metastasis. MACC1 induces cell proliferation and motility, supports cellular survival and rewires metabolism resulting in increased metastasis *in vivo*. MACC1 is a prognostic biomarker not only for CRC but for more than 20 solid cancer entities. Inflammation plays a pivotal role in tumorigenesis, tumor progression and metastasis. For CRC, inflammatory bowel disease and ulcerative colitis are important inflammation associated risk factors. Certain cytokines, such as TNF-α and IFN-γ, are key factors in determining the contribution of the inflammatory process to CRC. Knowledge of the connection between inflammation and MACC1 driven tumors remains unclear. Gene expression analysis of CRC cells after cytokine stimulation was analyzed by qRT-PCR and Western blot. Cellular motility was assessed by Boyden chamber assays. MACC1 promoter activity after stimulation with pro-inflammatory cytokines was measured using promoter-luciferase constructs. To investigate signal transduction from receptor to effector molecules, blocking experiments using neutralizing antibodies and knockdown experiments were performed. Following TNF-α stimulation, MACC1 and c-Jun expression were significantly increased at the mRNA and protein level. Knockdown of c-Jun reduced MACC1 inducibility following TNF-α stimulation. TNF-α promoted MACC1-induced cell migration that was reverted following MACC1 knockdown. Moreover, MACC1 and c-Jun expression were downregulated by blocking TNFR1, but not TNFR2. Knock down of the NF-κB subunit, p65, reduced basal MACC1 and c-Jun mRNA expression levels. Adalimumab, a clinically approved monoclonal anti-TNF-α antibody, hindered MACC1 induction. The present

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study highlights that TNF-α regulates the induction of MACC1 via the NF-κB subunit p65 and the transcription factor c-Jun in CRC cells. This finding unravels a novel signaling pathway upstream of MACC1 and provides a potential therapeutic target for the treatment of CRC patients with an associated inflammation.

Keywords: MACC1, metastasis, TNF-α, pro-inflammatory cytokines, colorectal cancer

INTRODUCTION

Inflammation is a defense mechanism of the immune system of higher multicellular organisms (1). It is triggered by stimuli including pathogens, injuries, chemicals or radiation (2). The protective responses are essentially connected to the healing process after the trigger is removed (3). Inflammation is mediated and controlled by different cell types and secreted proteins including pro-inflammatory cytokines (4). The major pro-inflammatory cytokines in different diseases are TNF-α and IFN-γ (5–7). Both belong to the group of immune modulating molecules that act through specific cell-surface receptors and participate in autocrine, paracrine and endocrine signaling (8–11). They modulate the innate and adaptive immune system (4, 12). More importantly, they are also associated with chronic inflammation and represent crucial factors in tumor development (13–15). Chronic inflammation is known as causal risk factor for tumor development, but the intimate connection of inflammation and tumor development at the molecular level is still only partly understood.

Colorectal cancer (CRC) is a major cause of morbidity and mortality worldwide and especially in developed contries (16, 17). It contributes to more than 8% of all cancer incidences that affect both men and women, making it the third most common cancer globally (18). There are numerous risk factors for CRC like diet, "Western lifestyle," excessive alcohol and tobacco intake and, environmental exposure (19–22). Diseases like ulcerative colitis and Crohn's disease connect the formation of sporadic CRC and chronic inflammatory conditions (23–25). Sporadic CRC accounts for the majority of all CRC cases. A smaller fraction of about 10–15% of all CRC cases is based on hereditary risk factors like in familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) (26, 27). There is growing evidence that inflammation is not only connected to sporadic cases of CRC but that reduced inflammatory responses can equally reduce or delay the formation of hereditary CRCs (27, 28). Ulcerative colitis is responsible for 1% of all CRC cases due to chronic inflammation affecting the mucosa of the colon and rectum, and Crohn's disease and here in particular Crohn's colitis has also been shown to slightly increase the risk (16). Inflammation is causing differential gene expression for a broad spectrum of genes. Therefore, it is needed to understand, which of these genes are the most important drivers of CRC and might serve as biomarkers and as therapeutic targets in patient tailored treatments.

One such driver of tumor progression is the gene Metastasis-Associated in Colon Cancer 1 (MACC1). The importance of MACC1 has been first demonstrated in CRC as prognostic marker of metastasis formation and metastasis-free survival (29). Both MACC1 mRNA and protein are highly expressed in CRC tissues with metachronous metastases compared to tumors without metastases and to normal tissue. The expression of MACC1 is increased during the transition from adenomas to carcinomas (30, 31). This suggests that MACC1 represents an independent early prognostic marker for CRC metastasis (32, 33). Besides CRC, MACC1 is meanwhile also a prognostic marker for more than 20 solid tumor entities (34).

MACC1 is a causal driver of tumor progression and metastasis. The reason for the increased MACC1 gene expression is largely unknown. Here we analyzed the connection of inflammation and MACC1 expression in the context of pro-inflammatory cytokines.

MATERIALS AND METHODS

Immunhistochemical Staining of MACC1 in Patient Paraffin Tissue Sections

Written informed consent was obtained from all patients. All experiments were approved by the institutional review board of the Charité–Universitätsmedizin Berlin and conducted accordingly. The authors complied with all relevant ethical regulations for research involving human participants. MACC1 protein expression was assessed in 14 tissue samples (five male, nine female patients, median age 55.5 years) of ulcerative colitis and Crohn's disease patients.

For paraffin removal and antigen retrieval tissues were treated with Xylol, 2:1 vol/vol aceton/Tris and finally boiled in 10 mM citrate buffer pH 6.3. Specimens were blocked for 30 min at room temperature with horse serum and incubated with primary MACC1 antibody for 2 h (HPA020103, Sigma Aldrich, Munich, Germany). After washing, the slides were incubated with a biotinylated secondary anti rabbit antibody (30 min, room temperature) and streptavidin-peroxidase (VECTASTAIN Elite ABC HRP Kit, PK-6101 Vector Laboratories, Burlingame, CA, USA) for another 30 min at room temperature. Finally, staining was visualized with 3,3′-diaminobenzidin (DAB Peroxidase (HRP) Substrate Kit, SK-4100, Vector Laboratories) and nuclei were stained with haemalaun. The tissues were photographed using a magnification of 100 x for the overviews and 400 x for the insets.

Cell Culture

HCT116 (LGC Standards, Wesel, Germany) human CRC cells were cultured at 37°C, 100% atmospheric humidity and 5% CO_2 in RPMI (Thermo Fisher Scientific, Waltham, MA, USA) –emented with 10% fetal calf serum (Bio&Sell,

Feucht, Germany). Cells were harvested using trypsin/EDTA (Thermo Fisher Scientific) and counted in an automated cell counter (NanoEnTek, Seoul, Korea). Cells were regularly verified as mycoplasma-negative (Lonza, Basel, Switzerland). Authentication of cell lines was performed by short tandem repeat (STR) genotyping (Multiplexion, Heidelberg, Germany). STR genotypes were consistent with published genotypes.

Cytokine Treatment

Recombinant human TNF-α and IFN-γ (Peprotech, Hamburg, Germany) were stored at −20°C following reconstitution to 0.1 mg/ml in sterile, deionized water. To maintain the stability of the cytokines, small aliquots were created for single use. Briefly, 1×10^6 cells/well were seeded in 6-well plates and allowed to adhere for 24 h. Subsequently, cells were treated with increasing concentrations (1, 10, 100 ng/ml) of cytokines and harvested after 24 and 48 h. Each experiment was performed in triplicate.

siRNA Transfection

Preestablished siRNAs targeting c-Jun (Thermo Fisher Scientific), p65 (kind gift of Prof. Claus Scheidereit, Max-Delbrück-Center for Molecular Medicine, Berlin, Germany), as well as scrambled siRNA (Thermo Fisher Scientific) serving as a negative control, were used. 3×10^5 HCT116 cells were seeded in 6-well plates and cultured for 24 h. siRNAs were transfected using the RNAiMAX RNAiMAX transfection reagent following manufacturer's recommendations. Cells were harvested after incubation for 24 and 48 h. Experiments were performed in three biological replicates.

Plasmid Transfection

To analyze MACC1 promoter activity, pGL4.17-based (Promega, Fitchburg, WI, USA) promoter reporter constructs generated earlier were transfected prior to TNF-α treatment into HCT116 cells (35). Prior transfection using TransIT 2020 (Mirus, Madison, WI, USA) following manufacturer's recommendations, 7.5×10^4 cells were seeded into 24-well plates and allowed to adhere for 24 h. To normalize for transfection efficiency, the pGL4.74 (Promega) encoding for renilla luciferase plasmid was transfected in parallel. Following addition of the transfection complex the cells were grown for 24 h before TNF-α treatment started.

Dual Luciferase Reporter Gene Assay

The activities of the firefly and renilla luciferases were measured using the Dual-Luciferase reporter assay system (Promega). Cells transiently expressing the luciferase constructs were lysed in passive lysis buffer with gentle shaking for 15 min at room temperature. Equal amounts of lysate and luciferase substrate were added to 96-well luminescence plates (Corning, Corning, NY, USA). The firefly luminescence was quantified first using an Infinite M200 pro 96-well plate reader (Tecan, Männedorf, Switzerland). Following addition of the Stop&Glo reagent, the renilla luciferase luminescence was assessed. Firefly luciferase activities were normalized to renilla luciferase readings.

Cell Migration

For the evaluation of cell migration, the Boyden chamber assay was used. Membrane inserts (Sigma) with a pore size of 8 μm were used in 24-well plates. Cells were serum-starved overnight. The following day, 600 μl medium containing 10% FCS, without or with increasing amounts TNF-α (1, 10, 100 ng/ml), were added to each lower chamber. Then, 3×10^5 cells in 300 μl medium containing 1% FCS, without or with increasing amounts of TNF-α (1, 10, 100 ng/ml), were seeded into each transwell upper chamber. Cells were incubated for 24 h to allow migration. The cells that had migrated to the lower side of the membrane were harvested with trypsin/EDTA and pooled with the cells in the lower chamber prior to centrifugation (200x g, 5 min at room temperature). To analyze relative cell numbers the cell titer-glo reagent (Promega) was used. After incubation for 10 min in the dark, luminescence intensity was measured with an Infinite M200 pro 96-well plate reader. Each migration assay was performed three times in triplicate.

TNF-α and Adalimumab or TNFR Antibody Treatment

Adalimumab (HUMIRA®, Il, USA, 100 mg/ml) was stored at 4°C. For TNF-α treatment of HCT116 cells, 2×10^5 cells were plated in 6-well plates and allowed to adhere for 24 h. Then, TNF-α was diluted in RPMI media and added to fresh cell media. Sterile water served as control treatment. For co-treatment, TNF-α and Adalimumab were added to fresh RPMI 10% FBS media to achieve a final concentration of 10 ng/ml TNF-α and 1, 10, or 100 μg/ml Adalimumab. The cells were then incubated for 24 h at 37°C with 5% CO_2 before harvesting for RNA and protein isolation. For experiments blocking TNFR1 or TNFR2, cells were pretreated with the respective antibodies (TNFR1: MAB225-100 R&D; TNFR2: MAB726-100, R&D Systems, MN, USA) 1 h before adding 10 ng/ml TNF-α.

Total RNA Isolation, cDNA Synthesis and Quantitative Real-Time PCR

The total RNA was isolated using the GeneMatrix Universal RNA Purification Kit (Roboklon, Berlin, Germany), according to the manufacturer's instructions. Briefly, cells were harvested, lysed and applied to the columns. After washing the columns RNA was eluted with 50 μl nuclease-free H_2O. RNA concentration was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The samples were stored at −80°C until further use.

For reverse transcription 50 ng total RNA was used. Reverse transcription was performed with 2.5 μM random hexamers in 5 mM $MgCl_2$, 1x PCR buffer, 4 mM dNTPs pool, 1 U/μl RNAse inhibitor and 2.5 U/μl MuLV reverse transcriptase (all Thermo Fisher Scientific). The reaction was carried out at 42°C for 45 min, 99°C for 5 min and 5°C for 5 min. cDNA was stored at −20°C until use.

Quantitative PCR was performed using SYBR Green dye chemistry (GoTaq qPCR Master Mix, Promega) in a LightCycler 480 II system (Hoffmann—La Roche, Basel, Switzerland). The data were evaluated by the LightCycler 480 Software release 1.5.0 SP3. All primer sequences are summarized in **Table 1**.

TABLE 1 | Primer used for qRT-PCR.

Gene	Sequence
MACC1 F	5′– TTCTTTTGATTCCTCCGGTGA–3′
MACC1 R	5′– ACTCTGATGGGCATGTG TG–3′
c-Jun F	5′– CAGGTGGCACAGCTTAAACA–3′
c-Jun R	5′– GTTTGCAACTGCTGCGTTAG–3′
Sp1 F	5′– GCTCTGAACATCCAGCAAAA–3′
Sp1 R	5′– CAGAGTTTGGAACAGCCTGA–3′
p65 F	5′– ACAACCCCTTCCAAGTTCCT–3′
p65 R	5′– ATCTTGAGCTCGGCAGTGTT–3′
GAPDH F	5′– GAAGATGGTGATGGGATTTC–3′
GAPDH R	5′– GAAGGTGAAGGTCGGAGT–3′
G6PDH F	5′– ATCGACCACTACCTGGGCAA–3′
G6PDH R	5′– TTCTGCATCACGTCCCGGA–3′

F, forward; R, reverse.

Protein Extraction and Western Blotting

For total protein extraction, harvested and washed cells were lysed in RIPA buffer supplemented with cOmplete Protease Inhibitor Cocktail (Sigma) for 15 min on ice. Supernatants were collected following centrifugation at 20,000x g for 20 min at 4°C and stored at −80°C until further use.

The protein concentration of the supernatant was determined by a bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific) according to the manufacturer's instructions. The lysates were diluted in PBS, and quantified relative to a BSA standard curve. The absorbance was measured at 560 nm using the Tecan Infinite M200 pro.

For Western blotting, 20 μg total protein was mixed with 1x NUPAGE sample buffer (Thermo Fisher Scientific), supplemented with 10% DTT, and heated for 10 min at 95°C. Proteins were separated on 10% NuPAGE Bis-Tris gels (Thermo Fisher Scientific) in 500 ml MOPS buffer (Carl Roth, Karlsruhe, Germany) at 150 V for 1 h. The proteins were then transferred to a nitrocellulose membrane using a semi-dry turbo-blot (Bio-Rad, Hercules, CA, USA) electrotransfer apparatus. After blocking the membrane in 5% skimmed milk powder (Carl Roth) in TBST for 1 h at room temperature, the membrane was incubated with primary antibodies at 4°C overnight (rabbit anti-MACC1, HPA020081, Sigma; rabbit anti-c-Jun, 60A8, Cell Signaling; mouse anti-β-actin, A1978, Sigma). Protein bands were visualized with a suitable horseradish peroxidase conjugated secondary antibodies (anti-rabbit IgG-HRP, W4011, Promega; anti-mouse IgG-HRP, 31430, Thermo) and WesternBright ECL (Biozym, Hessisch Oldendorf, Germany) substrate. Light emission was documented using Fuji medical X-Ray films (Kisker Biotech, Schweinfurt, Germany).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 6 (GraphPad Software, San Diego, CA, USA). Comparisons of controls with multiple experimental groups were carried out using one-way analysis of variance (ANOVA) followed by a Dunnett *post-hoc* test. Statistical significance was defined for p-values below 0.05, with $^{*}p \leq 0.05$, $^{*}p \leq 0.01$ and $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

RESULTS

MACC1 Protein Level Is Increased in Inflamed Patient Tissue

We and other groups have shown that MACC1 expression levels are increased especially in tumor tissue of patients with poor outcome (34). For CRC it was shown that MACC1 occurs very early during the transition from adenoma to carcinoma. In order to provide insights of MACC1 gene expression in inflamed tissue before tumor development we stained tissues from ulcerative colitis and Crohn's disease patients for MACC1. A pathologist confirmed active inflammation and evaluated the microphotographs. Specimens of non-inflamed tissue showed weak MACC1 expression only (**Figure 1**). By contrast, inflamed tissues from ulcerative colitis and Crohn's disease patients revealed moderate to strong MACC1 expression mainly in the cytoplasm of the cells (**Figure 1**), indicating the association of chronic inflammation and increase in MACC1 expression. Tissues outside of inflamed areas of ulcerative colitis and Crohn's disease patients served as controls.

TNF-α and IFN-γ Regulate MACC1 mRNA and Protein Expression Levels

To evaluate the effect of inflammation on MACC1 in epithelial CRC cells, we assessed the impact of two major pro-inflammatory cytokines, TNF-α and IFN-γ on MACC1 expression. The CRC cell line HCT116 was treated with increasing concentrations of either TNF-α (**Figure 2A**) or IFN-γ (**Figure 2B**) for 24 and 48 h, respectively. The mRNA and protein expression levels of MACC1 were determined by qRT-PCR and Western blot.

Compared with the untreated control cells, MACC1 mRNA expression levels were significantly increased by 3-fold upon treatment with 1 ng/ml ($p < 0.05$), 10 ng/ml ($p < 0.01$), and 100 ng/ml ($p < 0.01$) TNF-α (**Figure 2A**, left panel). Following 48 h of treatment, the increase in mRNA expression levels of MACC1 declined but was still significantly elevated by 1.5- to 2-fold. Consistent with the increase in mRNA expression levels, MACC1 protein expression was also upregulated following 24 and 48 h TNF-α treatment in a dose-dependent manner. This finding was confirmed in three different established cell lines and three different primary cell models (**Supplementary Figure 1**).

Similarly, HCT116 cells were exposed to increasing concentrations of IFN-γ for 24 and 48 h (**Figure 2B**). MACC1 mRNA and protein expression levels were determined by qRT-PCR and Western blotting, respectively. For this cytokine, the increase in the MACC1 mRNA levels was still there but not as pronounced as for TN-α treatment. These experiments demonstrate that stimulation with pro-inflammatory cytokines was able to upregulate MACC1 mRNA and protein expression in a dose- and time-dependent manner.

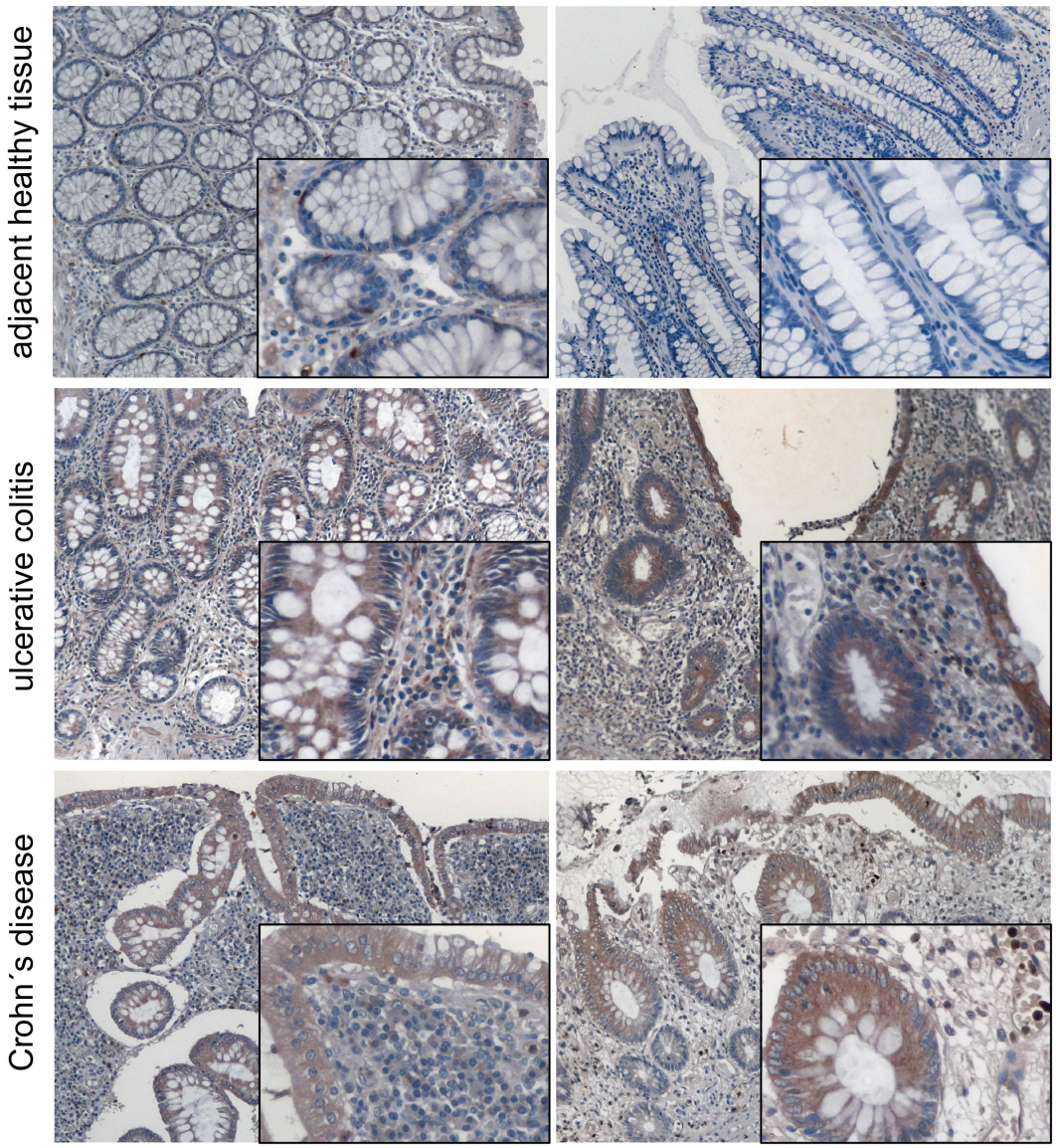

FIGURE 1 | MACC1 protein expression is increased in inflamed tissue. MACC1 protein expression was assessed in 14 tissue samples (five male, nine female patients, median age 55.5 years) of ulcerative colitis and Crohn's disease patients. Besides typical signs of extensive inflammation, areas of actively inflamed tissue show moderate to strong MACC1 staining especially in epithelial tissue compared to adjacent healthy tissue. The tissues were photographed using a magnification of 100 x for the overviews and 400 x for the insets.

TNF-α and IFN-γ Induce Cell Migration

As shown above, exposure to TNF-α induces MACC1. To explore, if this increased MACC1 expression results in increased migratory potential of cells, we tested HCT116 cells in the Boyden chamber assay. First, we confirmed MACC1-dependent changes in migration by either overexpressing MACC1 by stable transfection or specific downregulation of MACC1 by siRNA. Cell migration was increased with elevated MACC1 expression and decreased if MACC1 was knocked down by siRNA (**Figures 3A,B**). Treatment with increasing concentrations of TNF-α (1, 10, or 100 ng/ml) was performed for 24 h. TNF-α induced cell migration by more than 2-fold at a concentration of 1 ng/ml (**Figure 3A**), compared with unstimulated cells. Upon treatment with 10 ng/ml TNF-α, cell migration was even stronger induced by 3-fold in HCT116 cells, compared with control cells. Interestingly, at a concentration of 100 ng/ml TNF-α, cell migration was not as strongly induced as at lower TNF-α concentrations but still elevated above control levels. To confirm this we tested in addition to the Boyden chamber migration assay cellular motility in the wound healing (scratch) assay. TNF-α induced faster wound closure compared to control cells (**Supplementary Figure 2A**). The data clearly indicates that TNF-α was able to induce cell migration *in vitro* in a dose-dependent manner. To determine the role of the

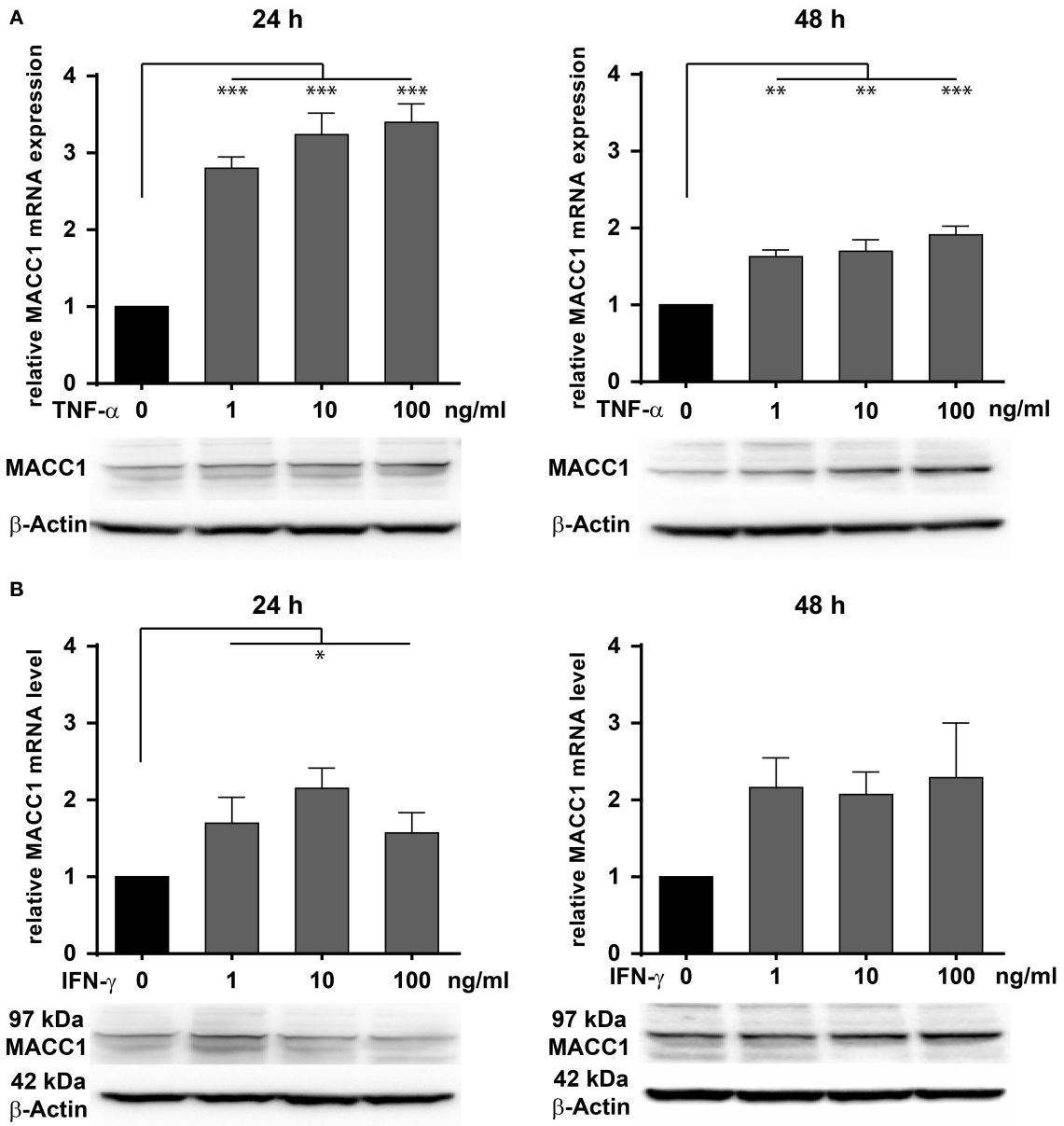

FIGURE 2 | Effects of TNF-α and IFN-γ stimulation on the MACC1 gene expression. HCT116 cells were treated with increasing concentrations of TNF-α (1, 10, 100 ng/ml) **(A)** and IFN-γ (1, 10, 100 ng/ml) **(B)** for 24 h (left side) and 48 h (right side). Cells without cytokine treatment served as controls. MACC1 mRNA expression levels were determined by qRT-PCR and normalized to GAPDH. Evaluation of MACC1 protein expression levels was performed by Western blot, and β-actin served as loading control. Both pro-inflammatory cytokines can upregulate MACC1 gene expression in a dose- and time-dependent manner. This effect was more pronounced for TNF-α. All experiments were performed as three biologically independent experiments. The data are presented as mean ± SEM with the statistical significance levels: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

pro-inflammatory cytokine IFN-γ on cell migration, HCT116 cells were treated with increasing concentrations of IFN-γ. This cytokine induced cell migration by 2-fold at concentrations of 1 as well as 10 ng/ml as compared with the unstimulated control cells (**Figure 3B**). However, the treatment with 100 ng/ml IFN-γ did not result in significant changes of cell migration. The data show that pro-inflammatory cytokines induce cell migration that is paralleled by an increased MACC1 expression. This was most efficient at lower concentrations of TNF-α and the effect of TNF-α was more pronounced than the effect of IFN-γ.

TNF-α Induces MACC1 via c-Jun

We have shown that MACC1 expression is regulated by the transcription factors AP-1 and SP1 (35). The transcription factor AP-1 is composed of two subunits with c-Jun being one of them. Here, the role of TNF-α on c-Jun activity driving MACC1

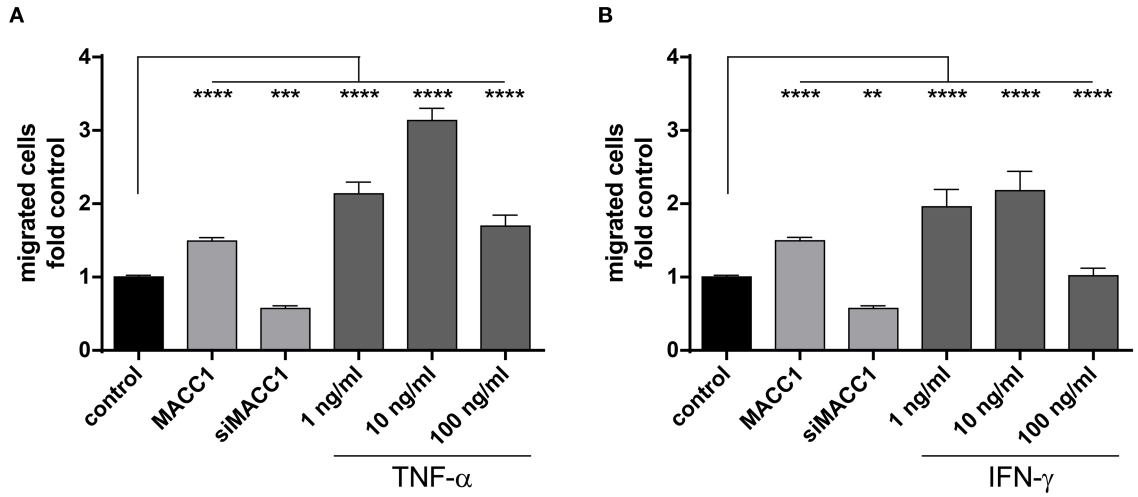

FIGURE 3 | MACC1 induced by pro-inflammatory cytokines increases migration. MACC1-dependent cell migration was confirmed by stable MACC1 overexpression or MACC1 siRNA-mediated MACC1 down-regulation. Cells were treated with either TNF-α **(A)** of IFN-γ **(B)** for 24 h before cell migration was measured. Results are representative of at least four independent experiments. The data are presented as mean ± SEM with the statistical significance levels: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

expression was investigated in CRC cells. The CRC cell line HCT116 was treated with increasing concentrations of TNF-α for 24 and 48 h. TNF-α potently stimulates c-Jun expression in a concentration-dependent manner at both the mRNA and protein level at 24 h (**Figure 4A**). The induction of c-Jun expression declined within 48 h after TNF-α application.

Besides c-Jun, the transcription factor Sp1 has been identified to bind and regulate the promoter of MACC1 (35). Like c-Jun, Sp1 activity can be regulated by TNF-α. HCT116 cells were treated with increasing concentrations of TNF-α for 24 and 48 h (**Figure 4**). Sp1 mRNA levels were subsequently determined by qRT-PCR. The mRNA levels of Sp1 were unchanged following TNF-α stimulation (**Figure 4B**). This suggests that TNF-α stimulation results in an increase of c-Jun transcription. In turn, elevated c-Jun protein levels led to increased MACC1 expression.

Since TNF-α treatment induced c-Jun, the role of the pro-inflammatory cytokine IFN-γ on the induction of c-Jun was also explored. HCT116 cells were treated with increasing concentrations of IFN-γ, and harvested after 24 or 48 h for analysis of c-Jun mRNA expression. No induction of c-Jun mRNA was seen in HCT116 cells (**Figure 4C**) following IFN-γ stimulation for 24 or 48 h. Similarly, the role of IFN-γ on the induction of Sp1 was also explored. Cells were treated with increasing concentrations of IFN-γ for 24 and 48 h (**Figure 4D**). Sp1 mRNA expression levels were subsequently analyzed by qRT-PCR. Similarly to TNF-α treatment, no induction of Sp1 mRNA expression was detected at any treatment concentration or time point. This indicates that IFN-γ has no effect on Sp1 expression. In conclusion, we demonstrated that TNF-α induces the expression of c-Jun, thereby impacting the control of MACC1 expression. Since TNF-α showed a stronger and more sustained effect on MACC1 gene expression and cell migration, this cytokine was further analyzed in more detail.

TNF-α Regulates MACC1 Promoter Activity Through c-Jun/AP-1 Interacting With a Functional AP-1 Transcription Factor Binding Site

We previously have cloned and described the MACC1 core promoter. We reported that MACC1 gene transcription relies on AP-1 and Sp1 protein activity and their respective promoter binding sites (35). As TNF-α induces c-Jun expression, a subunit of AP-1, we tested if this transcription factor has a direct role in MACC1 gene regulation after TNF-α stimulation. In parallel, Sp1 was also tested. We mutated the AP-1 and Sp1 transcription factor binding sites within the MACC1 promoter by site directed mutagenesis (35). HCT116 cells were transiently transfected with these AP-1 and Sp1 mutant promoter plasmids together with a renilla luciferase control plasmid for 24 h. Following TNF-α treatment for another 24 h, the luciferase activity as read out for the MACC1 promoter activity was analyzed using the Dual Luciferase reporter gene assay. Both the mutated AP-1 and Sp1 sites markedly reduced MACC1 promoter activity, accounting for the crucial role of the two binding sites for MACC1 promoter function (**Figure 5A**). TNF-α was able to induce the activity of the MACC1 promoter but failed to show this increase if the AP-1 or Sp1 binding site was mutated (**Figure 5A**). Since TNF-α was able to increase c-Jun but not Sp1 gene expression we tested, if siRNA mediated knock down of c-Jun impairs MACC1 gene expression and regulation by TNF-α (**Figures 5B,C**). Successful siRNA mediated c-Jun down regulation (**Figure 5B**) markedly reduced MACC1 gene expression (**Figure 5C**). Under these conditions, TNF-α treatment failed to increase c-Jun expression and subsequently MACC1 was not induced (**Figures 5B,C**). In summary, the AP-1 and Sp1 binding sites are indispensable elements for the transcriptional activation of the MACC1 gene.

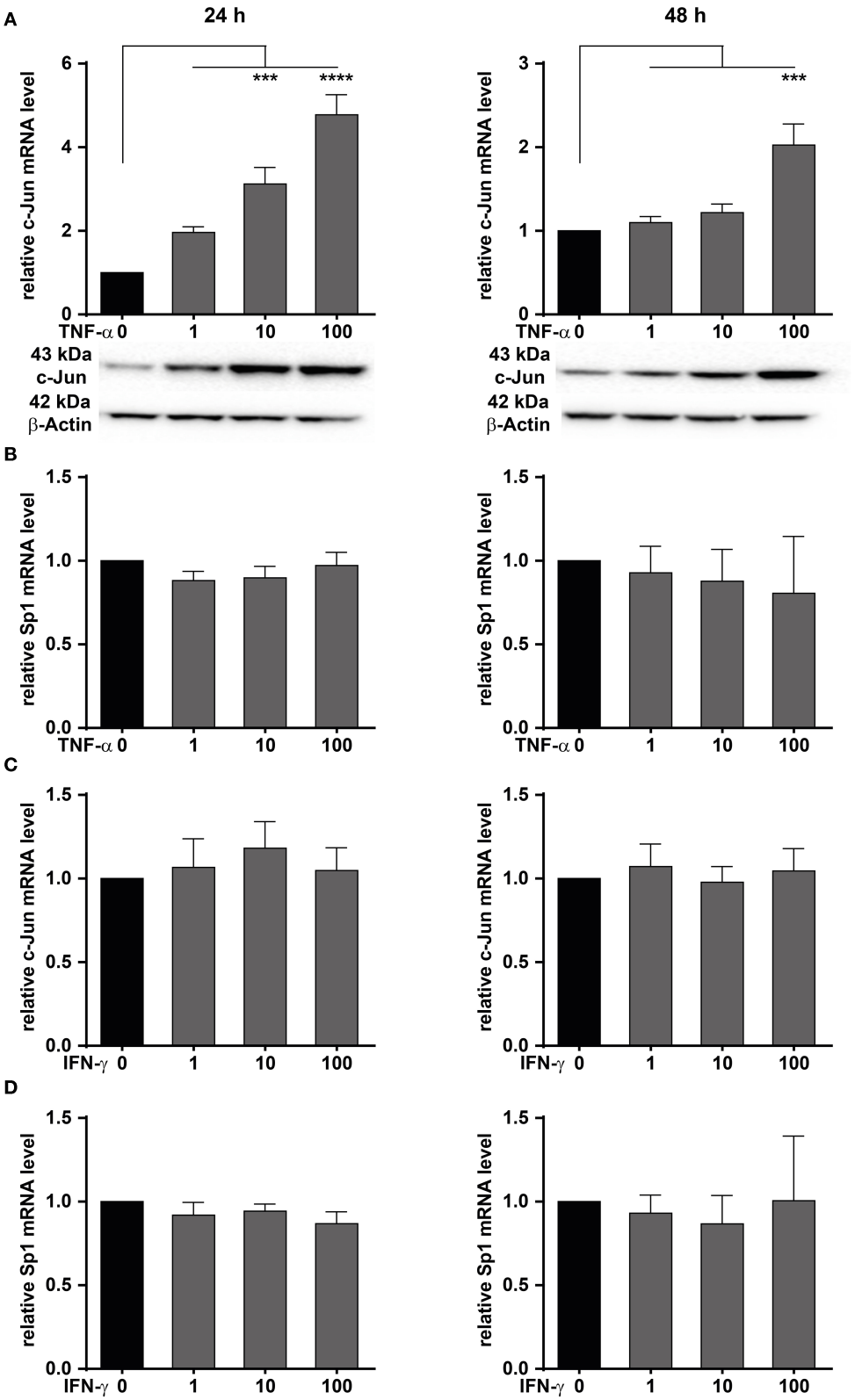

FIGURE 4 | TNF-α increases c-Jun mRNA and protein expression. HCT116 CRC cells were treated with increasing concentrations of TNF-α **(A,B)** or IFN-γ **(C,D)** for 24 (left panels) and 48 h (right panels). Cells without TNF-α treatment served as controls. The mRNA and protein expression levels of c-Jun and Sp1 were measured by qRT-PCR. Western blot was used to confirm the upregulation at the mRNA level. TNF-α treatment induces c-Jun expression at the mRNA and protein level. Results are representative of at least three independent experiments. The data are presented as mean ± SEM with the statistical significance levels: ***$p \leq 0.001$ and ****$p \leq 0.0001$.

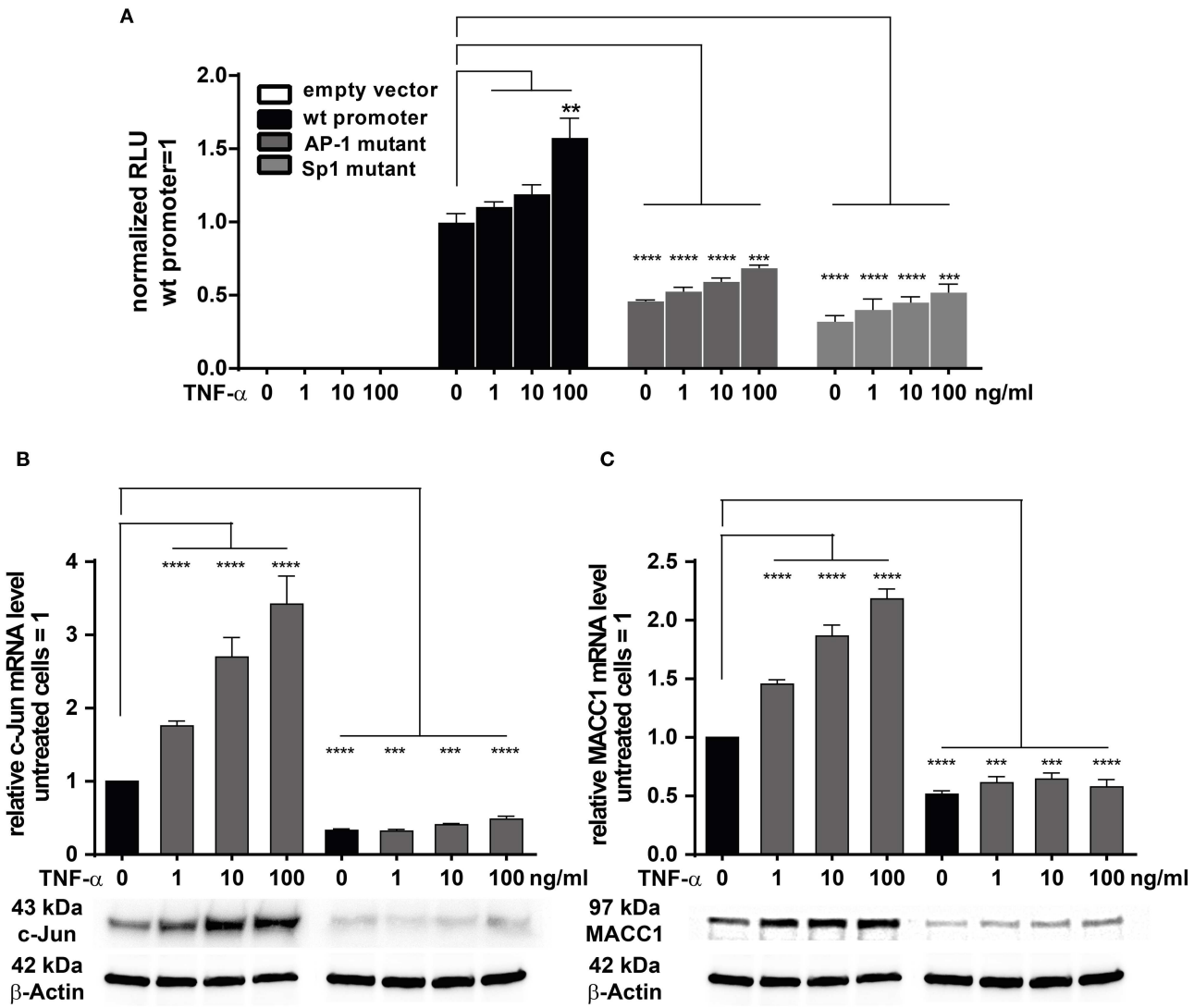

FIGURE 5 | TNF-α induces MACC1 via c-Jun/AP1. **(A)** HCT116 cells were transiently transfected with the MACC1 promoter reporter plasmids with either mutated AP-1 or Sp1 transcription factor binding sites along with a renilla luciferase control plasmid for 24 h. Cells were then treated with increasing concentrations of TNF-α. After 24 h of TNF-α treatment luciferase activity was measured and normalized to renilla luciferase activity. **(B,C)** HCT116 cells were transfected with the target-specific predesigned c-Jun siRNA or scrambled control siRNA for 24 h. Cells were then stimulated with increasing concentrations of TNF-α for another 24 h. Cells without TNF-α treatment served as controls. Cells were analyzed to assess the c-Jun and MACC1 mRNA and protein expression levels using qRT-PCR and Western blotting, respectively. The data is presented as mean ± SEM with the statistical significance levels: $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, and $^{****}p \leq 0.0001$.

In the context of TNF-α stimulation, the induction of MACC1 relies on the functional AP-1 transcription factor binding site.

Signaling Through TNFR1 and NF-κB Activates c-Jun for MACC1 Induction

TNF-α exerts its effects through binding to two membrane receptors, TNFR1 or TNFR2 (36–38). These receptors show different expression patterns: TNFR1 is extensively expressed in many cell types; but TNFR2 shows a limited expression range and is selectively found in immune and endothelial cells (38). Since TNF-α triggers MACC1 expression, we were interested in identifying the responsible receptor mediating this effect in cancer cells. To identify the responsible receptor in our model system, HCT116 cells were pre-incubated with specific blocking antibodies for either TNFR1 or TNFR2 for 1 h. Afterwards, the cells were stimulated with 10 ng/ml TNF-α. Following 24 h of TNF-α treatment, cells were harvested and analyzed for c-Jun and MACC1 expression both at the mRNA and protein levels. TNF-α stimulation upregulated both c-Jun and MACC1 expression in the control group. However, the upregulation of c-Jun (**Figure 6A**) disappeared at both the mRNA and protein level upon pretreatment with a TNFR1-specific blocking antibody. Contrary, TNF-α treatment successfully upregulated c-Jun expression, despite pretreatment with TNFR2-specific

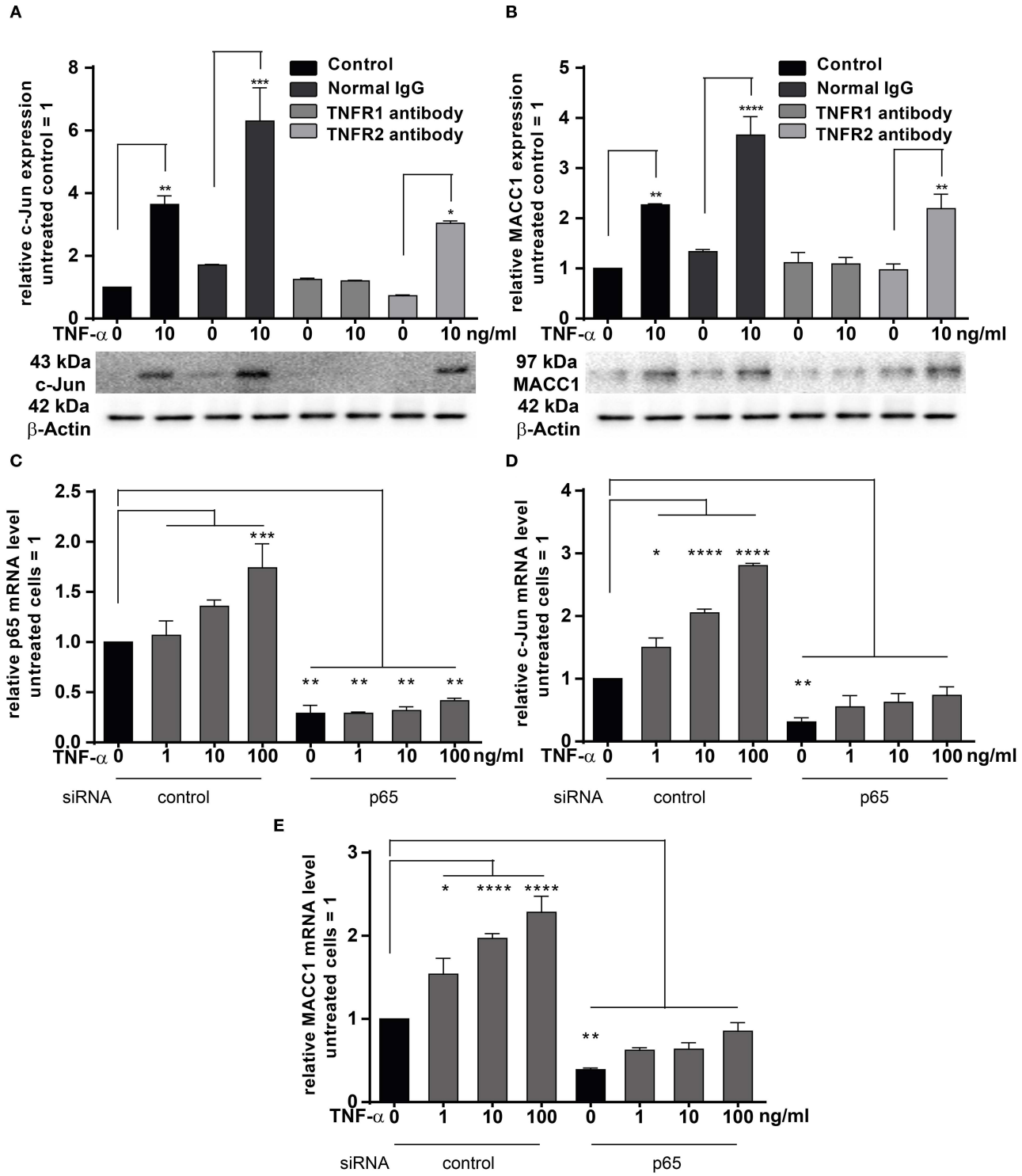

FIGURE 6 | TNFRI is the major receptor responsible for TNF-α mediated MACC1 induction. **(A,B)** One hour prior treatment with 10 ng/ml TNF-α HCT1116 cells were pre-incubated with specific blocking antibodies targeting TNFR1 or TNFR2 for 24 h. Cells were harvested and analyzed to assess the c-Jun **(A)** and MACC1 **(B)** mRNA and protein expression levels using qRT-PCR and Western blot, respectively. Isotype IgG antibodies not targeting the TNF receptors served as negative controls. **(C–E)** HCT116 cells were transfected with p65 siRNA or scrambled control for 24 h. Cells were then treated with increasing concentrations of TNF-α for another 24 h. Total RNA was extracted, reverse transcribed and the mRNA levels of p65 **(C)**, c-Jun **(D)**, and MACC1 **(E)** were quantified using qRT-PCR. The data are presented as mean ± SEM with the statistical significance levels: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$ and $^{****}p \leq 0.0001$.

blocking antibodies. This shows, that TNFR2 has only a minor role in regulating MACC1 expression after TNF-α stimulation. In accordance with the c-Jun expression pattern, the mRNA and protein expression levels of MACC1 (**Figure 6B**) showed no increase after TNF-α treatment upon pretreatment with a TNFR1-specific blocking antibody. As for c-Jun, MACC1 expression was still up-regulated upon pretreatment with a TNFR2-specific blocking antibody.

The pro-inflammatory NF-κB signaling is activated by at least three pathways (39). One of these pathways is the so-called "canonical" pathway triggered by TNF-α, which results in the activation of p65 that regulates the inflammatory responses (40). HCT116 cells were transfected with siRNA targeting p65 for 24 h. The cells were treated with increasing concentrations of TNF-α for another 24 h. Unstimulated cells served as controls. The mRNA expression levels of p65 (**Figure 6C**) were increased in a concentration-dependent manner by TNF-α treatment. Successful knock down of p65 abolished the induction of p65 by TNF-α stimulation. Next, the mRNA expression levels of c-Jun and MACC1 were examined. Again, both proteins were up-regulated by TNF-α treatment in a dose-dependent manner. Knock down of p65 abated basal mRNA expression levels of c-Jun (**Figure 6D**) and MACC1 (**Figure 6E**). The cells with p65 knock down showed only a marginal dose-dependent response to TNF-α treatment.

In conclusion, TNF-α executed c-Jun and MACC1 induction through TNFR1, but not TNFR2. Blocking TNFR1, but not TNFR2, inhibited both c-Jun and MACC1 induction by TNF-α at the mRNA and protein level. Additionally, c-Jun and MACC1 mRNA expression were inhibited by knock down of p65, indicating that the canonical NF-κB pathway is directly involved in the induction of c-Jun that regulates the MACC1 gene.

Adalimumab Can Reverse the TNF-α Induced MACC1 Expression

Adalimumab is a clinically approved TNF-α neutralizing monoclonal antibody applied widely in the treatment of chronic inflammatory diseases including Crohn's disease and ulcerative colitis. We therefore tested, if adalimumab can inhibit the TNF-α induced MACC1 induction. HCT116 cells were co-administered with 10 ng/ml TNF-α and increasing concentrations of adalimumab before MACC1 mRNA and protein expression was determined via qRT-PCR and Western blot, respectively. Compared to control cells, adalimumab treatment resulted in a significant decrease in MACC1 gene expression at all adalimumab concentrations tested (**Figure 7**). In addition we tested if adalimumab can revert the TNF-α effect in the wound healing (scratch) assay. Cellular motility is increased if cells are stimulated with TNF-α (**Supplementary Figure 2A**). If the cells are treated with adalimumab in parallel this effect is reverted to control levels (**Supplementary Figure 2B**).

These data confirm our previous findings that TNF-α increases MACC1 expression. More importantly, it demonstrates that adalimumab effectively inhibits TNF-α action and reduces its effect on MACC1 expression.

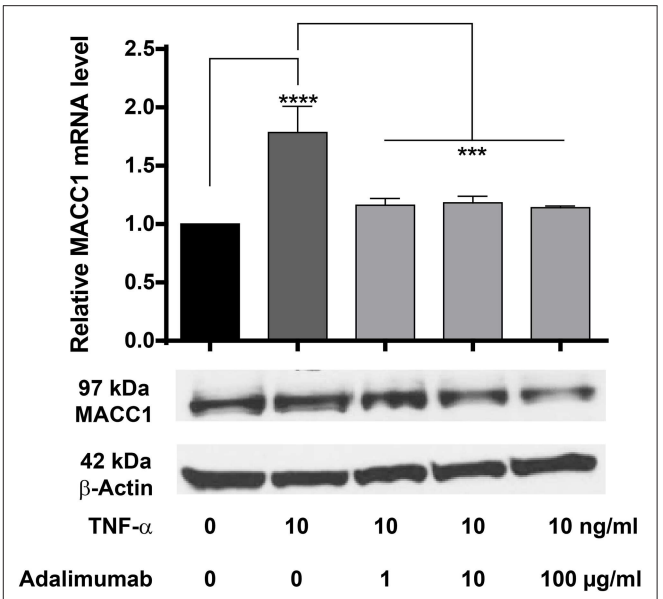

FIGURE 7 | Effect of TNF-α and the TNF-α neutralizing antibody adalimumab treatment on MACC1 mRNA and protein expression. HCT116 cells were treated with 10 ng/ml TNF-α and increasing concentrations of adalimumab (1, 10, 100 µg/ml) for 24 h before RNA isolation and qRT-PCR. TNF-α increased MACC1 gene expression at the mRNA and protein level that was abolished by blocking TNF-α with adalimumab. The data are presented as mean ± SEM with the statistical significance levels: ***$p \leq 0.001$ and **** $p \leq 0.0001$.

DISCUSSION

The close connection of inflammation and cancer is long known (41), but how inflammatory processes drive cancer development and progression is not thoroughly described. Here we report, that MACC1, a prognostic and predictive marker for numerous solid cancer entities, is increased in inflamed tissues. We analyzed in detail, how major pro-inflammatory cytokines mediate this elevated MACC1 gene expression leading to increased cellular motility. Most importantly, we show that the clinically approved TNF-α blocking antibody adalimumab can prevent the increase in MACC1 gene expression, offering a potential treatment option for patients.

The connection of inflammation and cancer, particularly CRC, involving pro-inflammatory cytokines was shown by numerous studies (42–44). Although the link of inflammation and cancer metastasis is already described, the cell specific and inflammation induced molecular mechanisms enabling cancer cells to metastasize are not thoroughly described (45–47). Expression of the MACC1 gene, particularly in CRC, can result in tumor invasion and metastasis. It is not known, why MACC1 expression increases during tumor development. It was demonstrated that MACC1 expression can be induced by IL-4 and lipopolysaccharide (LPS) in bone marrow-derived macrophages, suggesting that MACC1 might be involved in inflammatory processes (48). Therefore, an examination of the MACC1 gene regulation, particularly during inflammation,

can help to clarify the relationship between inflammation, carcinogenesis and metastasis in CRC.

We have shown that MACC1 expression is increased in inflamed tissue of ulcerative colitis and Crohn's disease patients. It is well accepted that TNF-α and IFN-γ are major players in the pathogenesis of these chronic diseases (49). Therefore, we hypothesized that these pro-inflammatory cytokines regulate MACC1 gene expression in CRC cells. We demonstrated here, that particularly TNF-α regulates MACC1 at both the transcriptional and translational level in a time- and dose-dependent manner. Thus, the chronic inflammatory microenvironment sustained by TNF-α might be an important condition of CRC progression. Inflammation regulates many aspects of cancer progression like proliferation, angiogenesis, invasion, and metastasis (50). For different tumor entities, not only time but cytokine concentration decides about molecular outcome (51, 52). We found that TNF-α concentrations affect levels of MACC1 mRNA and protein expression in a dose-dependent manner.

Increased MACC1 expression leads to cellular motility *in vitro* and metastasis *in vivo* (29). TNF-α was demonstrated as inducer of cell migration in cancer cells (53). TNF-α can contribute to migration of CRC cells through the epithelial-mesenchymal transition (EMT) (54). This process is further promoted by the combined activity of pro-inflammatory cytokines and MACC1. We found that low concentrations of TNF-α augment MACC1-induced cell migration, whereas high doses of TNF-α hinders cell migration in CRC cells overexpressing MACC1. In this setting cell death overrules the stimulating effects of TNF-α (55). Silencing of MACC1 mRNA abrogates the effects of TNF-α on cell migration and precludes cell responsiveness to TNF-α treatment. Hence, TNF-α increases cell migration by acting besides other factors, through the MACC1 gene, thereby augmenting the migratory potential of MACC1 in CRC.

The transcription factor c-Jun is stimulated by TNF-α through c-Jun N-terminal kinase (JNK) (56). This classical signaling pathway is known to be involved in inflammation and cancer (57, 58). We analyzed c-Jun mRNA and protein levels in response to TNF-α treatment and found that TNF-α induced transcription and translation of c-Jun in a dose-dependent manner in CRC cells. Hence, TNF-α can facilitate a variety of pathophysiological activities directly or indirectly by regulating c-Jun expression. This pathway is not only relevant for CRC, but for other tumor entities as well, like hepatocellular carcinoma, pancreatic cancer or nasopharyngeal carcinoma (59–61).

The c-Jun protein increased by TNF-α is part of the transcription factor AP-1 that was identified to drive MACC1 gene expression. The core promoter of MACC1 was identified between the nucleotides −992 to −18 relative to the transcriptional start site. This region drives transcription of the MACC1 gene with most of the regulatory features (35). The minimal essential core promoter region of MACC1 lies within nucleotides −426 to −18. It encompasses all sequences needed for MACC1 transcription, including initiation of transcription and basal activation of the MACC1 gene. The core promoter region contains functional binding sites for transcription factors, including AP-1, Sp1, and C/EBPs, which were shown to regulate MACC1 expression (35).

TNF-α mediates a variety of cell-signaling processes involved in the immune response and carcinogenesis, primarily via its interaction with TNFR1 and/or TNFR2 (62, 63). TNFR1 is a central regulator of signal transduction pathways whereas TNFR2 is expressed on a very narrow subset of immune cells (64–66). Based on our previous study on the effects of TNF-α on c-Jun/MACC1 signaling, we exposed CRC cells to blocking antibodies for TNFR1 or TNFR2, respectively. Blocking of TNFR1 did not change the basal MACC1 expression level but caused a loss of responsiveness of c-Jun and MACC1 mRNA and protein expression to TNF-α stimulation. In contrast, exposure to anti-TNFR2 antibodies did not preclude the stimulation of c-Jun and MACC1 by TNF-α. These results show that TNF-α induces c-Jun and MACC1 via TNFR1 signaling, but not TNFR2. Thus, these findings confirm a signaling axis comprising TNFR1 and c-Jun, leading to MACC1 expression that eventually mediates tumor progression and metastasis.

TNF-α induces NF-κB to activate signal transductions processes. NF-κB is a multifunctional transcription factor with essential roles in a variety of biological activities and cellular responses. NF-κB subunits form various homo- and heterodimers. In the canonical pathway, NF-κB is activated by pro-inflammatory cytokines, such as TNF-α (67).

Consistent with previous studies, we determined that TNF-α activates c-Jun to regulate the induction of MACC1 in CRC cells. We explored the effects of NF-κB signaling on c-Jun and MACC1 by knocking down p65. Our results showed that TNF-α increases the levels of p65 mRNA expression in a dose-dependent manner. In the context of p65 knockdown, the basal levels of c-Jun and MACC1 mRNA were lower and the TNF-α responsiveness was mainly lost. Therefore, the canonical NFκB pathway induces via p65—a subunit of NF-κB—directly or indirectly the transcription of c-Jun and controls the induction of MACC1 in CRC cells. Our findings indicate a notable signaling network involved in cancer development.

TNF-α activates NF-κB signaling, thereby contributing to inflammation, cell survival, proliferation, angiogenesis, tumor promotion, and metastasis (68, 69). The transcription factor NF-κB links inflammatory signaling and cancer. It is involved in nearly every stage of cancer development, including invasion and metastasis. NF-κB promotes tumor metastasis by regulating epithelial mesenchymal-transition (EMT) in CRC (70, 71). Furthermore, TNF-α, secreted by pro-inflammatory macrophages, enhances the metastatic potential of ovarian tumor cells via activation of the NF-κB signaling pathway (72).

With TNF-α/TNFR1, confidently established as an inducer of MACC1, we investigated whether a clinically approved TNF-α blocking antibody would prevent the induction of MACC1. The human TNF-α blocking monoclonal antibody adalimumab was used. Adalimumab is used in the treatment of a number of chronic inflammatory diseases, including rheumatoid arthritis, colitis ulcerosa or Crohn's disease. Adalimumab has been shown to induce apoptosis of human macrophages while down regulating levels of soluble TNF-α

as well as other pro-inflammatory cytokines (73–75). Here we show that adalimumab reduces TNF-α induced MACC1 over-expression. This indicates that a TNF-α specific antibody could be effective for treatment of MACC1 driven tumors. Interfering with MACC1 expression via TNF-α could prove to be a valuable additional therapeutic strategy against CRC metastasis.

Taken together, our findings support the hypothesis that the transcription factors c-Jun and NF-κB can be considered as a potential molecular target in CRC therapy for MACC1 driven tumors. Control of inflammation offers an effective approach for repressing or maybe even preventing tumor metastasis.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by institutional review board of the Charité–Universitätsmedizin Berlin. The patients/participants provided their written informed consent to participate in this study.

AUTHOR'S NOTE

Parts of this study were used in a dissertation thesis conducted at the Experimental and Clinical Research Center, Charité—Universitätsmedizin Berlin, and Max-Delbrück-Center for Molecular Medicine, Berlin-Buch (76).

AUTHOR CONTRIBUTIONS

DK, RG, BS, and US: study conception and design. CZ, DK, and IC-L: conducted experiments. DK, CZ, IC-L, and US: drafting the manuscript. All authors: analysis and interpretation of data and critical revision.

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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.2020.00980/full#supplementary-material

Supplementary Figure 1 | Effects of TNF-α on the MACC1 gene expression in different cell lines. Three different established cells and primary cell cultures were treated with increasing concentrations of TNF-α (1, 10, 100 ng/ml). Cells without cytokine treatment served as controls. MACC1 mRNA expression levels were determined by qRT-PCR. The pro-inflammatory cytokine TNF-α can upregulate MACC1 gene expression in a dose-dependent manner. The experiments were performed as three biologically independent experiments. The data are presented as mean ± SEM with the statistical significance levels: $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

Supplementary Figure 2 | TNF-α increases cellular motility in the wound healing (scratch) assay that is reverted by adalimumab. HCT116 cells were seeded at a density of 1.1×10^6 cells per ml in 96-well image lock plates. The cells were allowed to adhere for 6 h forming a confluent monolayer. Wounds (scratches) were applied using the wound maker tool. Directly after wounding the cells were treated with increasing amounts of TNF-α (1, 10, and 100 ng/ml) alone or in combination with 100 μg/ml adalimumab. The cells were monitored label-free every second hour in the IncuCyte live cell imaging system. TNF-α increased wound closure in a dose-dependent manner over time **(A)**. This phenotype could be reverted by adalimumab **(B)**.

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Targeting Autophagy Facilitates T Lymphocyte Migration by Inducing the Expression of CXCL10 in Gastric Cancer Cell Lines

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Autophagy is a type of cellular catabolic degradation process that occurs in response to nutrient starvation or metabolic stress, and is a valuable resource for highly proliferating cancer cells. Autophagy also facilitates the resistance of cancer cells to antitumor therapies. However, the involvement of autophagy in regulating CXCL10 expression in gastric cancer (GC) cells and T lymphocyte migration remains unclear. In this study, we aimed to investigate the effect of autophagy inhibition on CXCL10 expression and T lymphocyte infiltration in GC and elucidate the underlying mechanism. Analysis of public databases revealed a positive correlation between CXCL10 expression and both prognosis of patients with GC and the expression profile of T lymphocyte markers in the GCs. Chemotaxis and spheroid infiltration assays revealed that CXCL10 induced T lymphocyte migration and infiltration into GC spheroids, an *in vitro* three-dimensional cell culture model. In addition, *in vitro* autophagy inhibition in GC cells increased CXCL10 expression under both normal and hypoxic culture conditions. Further investigation on the underlying mechanism showed that *in vitro* autophagy inhibition suppressed the JNK signaling pathway and further enhanced CXCL10 expression in GC cells. Collectively, our results provide novel insights for understanding the role of autophagy in regulation of intra-tumor immunity.

Keywords: autophagy, CXCL10, gastric cancer cell lines, JNK, T lymphocyte migration, *in vitro*

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INTRODUCTION

A correlation between the presence of tumor-infiltrating lymphocytes (TILs) and overall patient survival has been reported in several tumor types (1–4) and the fundamental roles of TILs in tumor immunity have been investigated intensively (5–10). Therefore, immunomodulation using immune check-point inhibitors, one of the most rapidly growing cancer drug classes, is currently being explored as a cancer therapeutic approach. Some immune check-point blockade therapies, such as those involving monoclonal antibodies targeting cytotoxic T lymphocyte associated protein 4 (CTLA-4), programmed cell death protein 1 (PD-1), and PD-1 ligand (PD-L1), resulted in T lymphocyte-mediated tumor regression in various malignancies (11–17), including gastric carcinoma (18).

Gastric cancer (GC) is the fifth most common malignancy diagnosed worldwide, with 952,000 estimated new cases and 723,000 GC related-deaths in 2012 (19). Although immune

check-point inhibitors have shown promising results for GC treatment, the objective response rates remain low (18, 20). Thus, the effectiveness of this immunomodulatory strategy depends not only on the unleashing of pre-existing immunity but also on the presence of a baseline immune response (21). In fact, intra-tumor T lymphocyte recruitment is one of the potential rate-limiting steps in immunotherapy; therefore, many investigators have focused on the role of intra-tumoral chemokines in TIL recruitment into the tumor (22, 23).

It is well-known that T lymphocyte infiltration into the tumor is always insufficient when the chemokine receptors expressed on T lymphocytes do not match to the tumor-secreted chemokines (24). CXCR3, a predominant chemokine receptor expressed on TILs, is expressed in several solid tumors, including melanoma (25), colorectal cancer (26), and breast cancer (27). Moreover, TILs in lymphocyte-rich GCs predominantly express CXCR3 (28). Among the CXCR3 ligands, CXCL10 was reported to be associated with T lymphocyte infiltration into tumors. For example, CXCL10 expression was associated with T lymphocyte recruitment in melanoma metastases (25). In addition, intra-tumor induction of CXCL10 enhanced the infiltration of CXCR3+ cytotoxic T lymphocytes, thereby improving the antitumor effect of other therapies in some rodent solid tumor models (29, 30). However, the association between CXCL10 expression and T lymphocyte infiltration in GC remains poorly understood.

In recent years, autophagy in GC pathogenesis has been explored extensively, and autophagy inhibition is being considered as a potential strategy for GC treatment (31). Autophagy is critical for the digestion of intracellular contents and generation of energy to control cellular homeostasis (32). Autophagy was reported to play a pivotal role in GC cell survival and enhance tumor cell resistance to antitumor therapies (31). Therefore, autophagy inhibition may alter this tumor protective mechanism and potentiate anticancer drug-induced cell death in GC. In fact, an autophagy inhibitor chloroquine (CQ) was reported to improve the chemosensitivity of GC cells to platinum-based antitumor drugs (33, 34). Li et al. demonstrated that treatment with 3-MA, an alternative autophagy inhibitor, enhanced the curcumin-induced antitumor effect (35). Interestingly, a recent study showed that autophagy inhibition could induce CCL5 expression in melanoma cells, resulting in tumor regression facilitated by NK cell migration into the tumor bed (36).

In this study, we investigated the effect of autophagy inhibition on CXCL10 expression in GC cells and T lymphocyte migration toward GC cells. We also attempted to elucidate the mechanism underlying the observed effects of autophagy inhibition on CXCL10 expression in GC cells.

MATERIALS AND METHODS

Public Dataset Mining

Kaplan Meier-plotter (http://kmplot.com/analysis/) is an online database that enables evaluation of the effect of over 54,000 genes on survival in several cancer types, including GC, breast cancer, ovarian cancer, and lung cancer (37). This database was used to obtain prognostic information on CXCL10. Survival information and gene expression data were from Gene Expression Omnibus (GEO), European Genome-phenome Archive (EGA), and The Cancer Genome Atlas (TCGA) database.

Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/index.html) is a customizable online tool developed by Zhang lab of Peking University to analyze gene expression data in both tumor and normal tissues on the basis of TCGA and Genotype-Tissue Expression (GTEx) data (38). GEPIA was used for correlation analysis and for investigating the expression levels of autophagy-related genes (ATGs) between GCs and the normal tissues.

Cell Lines and Reagents

Human GC cell lines AGS, NCI-N87, BGC-823, HGC-27, KATO III, SGC-7901, SNU-1, SNU-5, and SNU-16 were purchased from American Type Culture Collection (ATCC). AGS, BGC-823, HGC-27, KATO III, and SNU-5 cells were cultured in DMEM-GlutaMAX medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), penicillin (100 U/ml), and streptomycin (100 μg/ml; Life technologies). NCI-N87, SGC-7901, SNU-1, and SNU-16 cells were cultured in RPMI 1640-GlutaMAX medium (Life Technologies) supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). All the cells were maintained in a 5% CO_2 humidified atmosphere at 37°C. The ATG5 and ATG7 siRNAs were purchased from Life Technologies. CQ, cobalt chloride ($CoCl_2$) and Sp600125 were purchased from Sigma. Anisomycin was purchased from Cell Signaling Technology. Recombinant CXCL10 protein, CXCL10 antibody and mouse IgG1 isotype control were purchased from R&D systems. The plasmid pIREShyg3 was purchased from GenScript and the coding sequence (CDS) of CXCL10 gene was cloned in pIREShyg3 using Nhel / BamHI to obtain the pIREShyg3-CXCL10 plasmid.

Cell Sorting and Activation of CD3+ T lymphocytes

CD3+ T lymphocytes were isolated from cryopreserved human peripheral blood mononuclear cells (PBMCs; StemExpress) using MACS microbeads (Miltenyi Biotec). After separation, T lymphocytes were stimulated using CD3/CD28 Dynabeads (Life Technologies) for 2 days, as described previously, and re-cultured without any external stimuli for another 2 days to induce the expression of CXCR3 (39). The primed T lymphocytes were used in the chemotaxis and spheroid infiltration assays.

Flow Cytometry Analysis

Cells were incubated with saturating amounts of various fluorescent-labeled antibody mix composed of PerCP-Cy5.5 labeled mouse anti-CD45 (Clone HI30, IgG1; BD Biosciences), PE labeled mouse anti-CD3 (Clone OKT3, IgG2a; Thermo Fisher Scientific), FITC labeled mouse anti-CXCR3 (Clone G025H7, IgG1; BioLegend) antibodies, and co-stained with Zombie Aqua^{TM} dye (BioLegend). Isotype and fluorochrome-matched mAbs were used for control staining. Stained cells were evaluated using the BD LSRFortessa X-200 flow cytometer (BD

Biosciences), and the data were analyzed using FlowJo software (Tree Star).

Chemotaxis Assay

The chemotaxis assay was performed in CytoSelect™ 24-well cell migration assay kit (5 μm pore size; Cell Biolabs) per the manufacturer's instructions (**Figure 2A**). Briefly, the primed T lymphocytes were prepared at density of 3×10^6 cells/ml in serum-free RPMI 1640 medium containing 0.5% bovine serum albumin (BSA), 2 mM $MgCl_2$, and 2 mM $CaCl_2$. For each well, the cells were placed in upper chamber (3×10^5 cells/100 μl) and the medium was loaded in the lower chamber. The plate was then incubated in a 37°C cell culture incubator for 5 h. The migrated cells were dissociated from the membrane, lysed, and detected using the patented CyQUANT® GR Dye (Life Technologies).

Tumor Spheroids and Spheroid Infiltration Assay

NCI-N87 spheroids were established using 96-well EZSPHERE SP micro-plates (Nacalai Tesque). The culture plate has a concave and ultra-low attachment bottom surface so that the cells adhere to each other, but not with the bottom surface of the plate. Therefore, the cells did not spread out on plastic, but formed spheroids. Here, the NCI-N87 cells were transfected with the pIREShyg3-CXCL10 plasmid; 1 day later, 8×10^4 CXCL10-transfected NCI-N87 cells were seeded with 200 μl medium in each well. The spheroids were formed 4 days after seeding. Then, 8×10^5 primed T lymphocytes were added into each well and incubated overnight (**Figure 2C**). The spheroids were then washed three times with PBS to remove the loosely attached T lymphocytes, fixed in 4% paraformaldehyde for 2 h, and embedded into paraffin for immunohistochemistry analysis.

Immunohistochemistry

Paraffin blocks were sectioned using a microtome to obtain 4 μm thick sections for immunostaining. The paraffin sections were dewaxed in xylene and hydrated in decreasing concentrations of ethanol. Sections were then incubate in 1 × DIVA Decloaker antigen retrieval solution (Biocare Medical) at 110°C for 15 min using the decloaking chamber (Biocare Medical). Following antigen retrieval, sections were incubated in peroxidazed 1 solution (Biocare Medical) at room temperature for 5 min to quench endogenous peroxidase activity. After blocked with background sniper at room temperature for 10 min, sections were incubated with a monoclonal rabbit anti-human CD3 antibody (0.3 μg/ml; Biocare Medical) in Dako REAL antibody diluent (Dako) at room temperature for 1 h. Sections were subsequently incubated with HRP-labeled goat anti-rabbit IgG polymer (Dako) at room temperature for 30 min. Finally, sections were exposed to liquid DAB+ substrate chromogen system (Dako) at room temperature for 5 min and counterstaining was performed using Gill's hematoxylin (Sigma).

Quantitative RT-PCR

Total RNA was extracted from the GC cell lines using RNeasy Plus Mini Kits (QIAGEN). Quantitative RT-PCR and data analysis were performed as described in our previous work (40, 41). Briefly, the SuperScript™ IV First-Strand Synthesis System (Life Technologies) was used to synthesize cDNA. PCR was performed and quantified using Power SYBR Green PCR Master Mix (Life Technologies). Primers used in the real-time quantitative PCR were as follows: CXCL10 (accession no. NM_001565), sense primer 5′- AAAAGAAGGGTGAGAAGAG-3′ and antisense primer 5′- AAGAACAATTATGGCTTGAC-3′; ATG5 (accession no. NM_004849), sense primer 5′-GCAACTCTGGATGGGATTGC-3′ and antisense primer 5′-AGGTCTTTCAGTCGTTGTCTGAT-3′; ATG7 (accession no. NM_ 006395), sense primer 5′-CATGGTGCTGGTTTCCTTGC-3′ and antisense primer 5′- GCTACTCCATCTGTGGGCTG-3′; GAPDH (accession no. NM_002046), sense primer 5′-CGGATTTGGTCGTATTGGG-3′ and antisense primer 5′-CTGGAAGATGGTGATGGGAT-3′.

The relative target gene mRNA level was calculated using the ΔCt method. The threshold cycle (Ct) values of the target gene mRNAs were initially normalized to the Ct values of the internal control GAPDH in the same samples: ΔCt = Ct (the target gene) – Ct (GAPDH). These values were further normalized to the control group: ΔΔCt = ΔCt (sample group) – ΔCt (control group). The fold change was then determined ($2^{-\Delta\Delta Ct}$). The relative target gene mRNA level represents an average fold calculated from separate experiments. PCR was performed at least three times, and similar results were observed.

Luminex Assay

The protein level of CXCL10 in the cell culture supernatant was assessed using the human Magnetic Luminex Assay (R&D Systems), which was performed per the manufacturer's instructions. Briefly, all the samples and standards were first mixed with the CXCL10 antibody coated magnetic microparticles and incubated for 2 h at room temperature on a horizontal orbital microplate shaker set at 800 rpm. After washing the microparticles, biotinylated detector antibodies were added and incubated for 1 h at room temperature on the shaker set at 800 rpm. Following a wash to remove any unbound biotinylated detector antibody, streptavidin-phycoerythrin conjugates were added and incubated for 30 min at room temperature on the shaker set at 800 rpm. Finally, the protein level of CXCL10 in the cell culture supernatant was analyzed using the Bio-Plex™ 200 system (Bio-Rad).

Western Blot

Cell lysis, protein extraction, and western blot analyses were performed as described in our previous work (40). Proteins were dissolved in a lysis buffer and separated using SDS/PAGE for western blot analyses. Primary antibodies included rabbit anti-Phospho-SAPK/JNK (Thr183/Tyr185), anti-SAPK/JNK, anti-Phospho-c-Jun (Ser73), anti-c-Jun, anti-ATG5, anti-LC3B and anti-GAPDH (Cell Signaling Technology). Secondary antibody was HRP-conjugated anti-rabbit IgGs (Life Technologies). The densitometric analyses of western blotting images

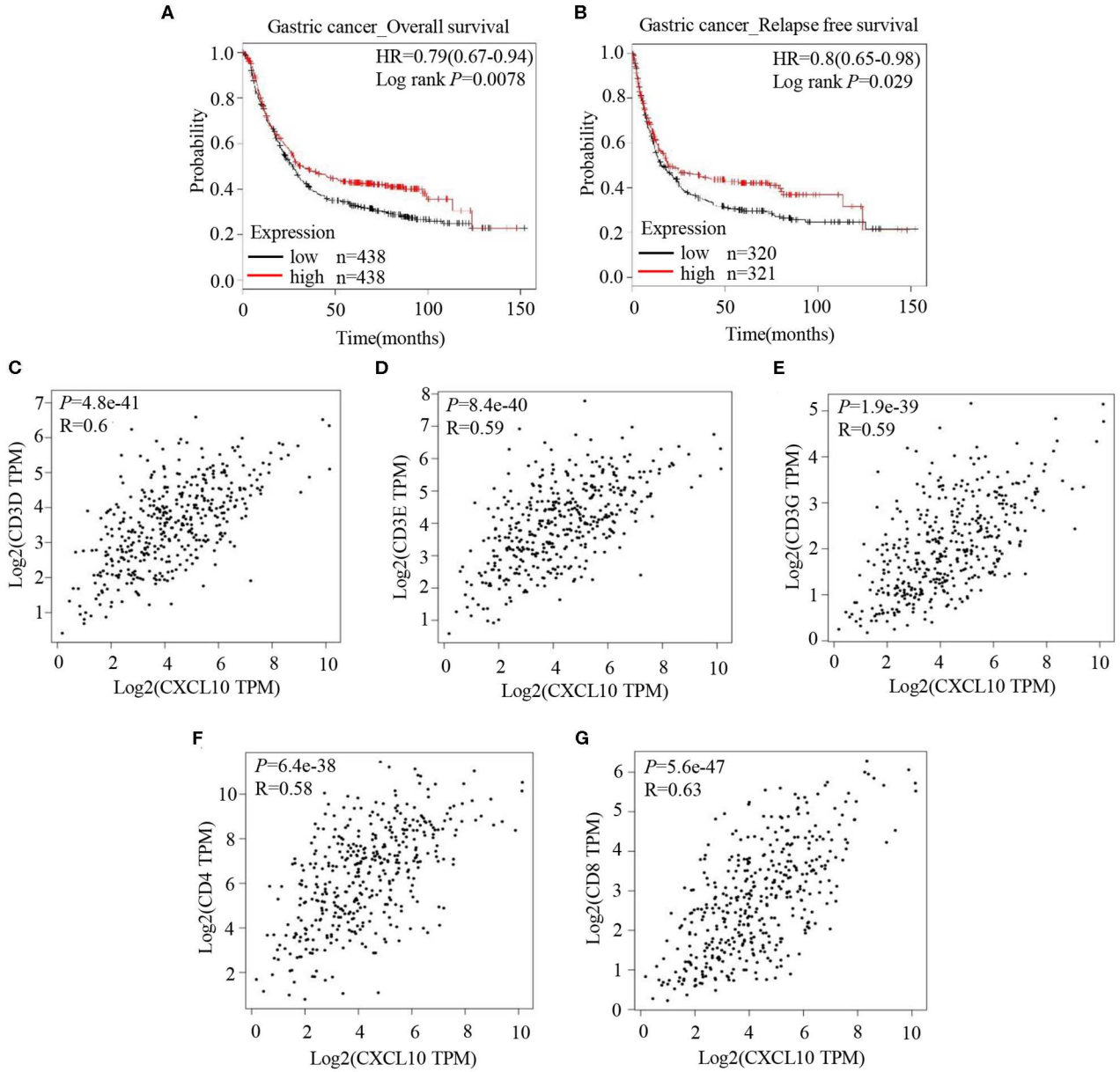

FIGURE 1 | CXCL10 expression is positively correlated with survival and expression of T lymphocyte markers in patients with GC. **(A)** Kaplan-Meier analysis of overall survival in GC patients with high CXCL10 expression and low CXCL10 expression ($P = 0.0078$, $n = 438$). **(B)** Kaplan-Meier analysis of relapse free survival in GC patients with high CXCL10 expression and low CXCL10 expression ($P = 0.029$, $n = 320$ and 321, respectively). **(C–G)** Scatter plots showing the correlation of CXCL10 with CD3D **(C)**, CD3E **(D)**, CD3G **(E)**, CD4 **(F)**, and CD8 **(G)** (Spearman's correlation test).

were performed using ImageJ software (National Institutes of Health).

Cell Viability Assay

Cell counting kit-8(CCK-8, Dojindo) was used to evaluate cell viability based on the dehydrogenase activity. AGS cell suspensions were first dispensed in a 96-well plate (1×10^4 in 100 μL/well) and cultured in DMEM with 10% FBS at 37°C for 24 h, and then were treated with vehicle, 10 and 20 μM CQ, respectively. After incubation for 0, 1, 2, and 3 days, 10 μl CCK-8 solution was added to each well and the plate was incubated at 37°C for 1 h. Finally, the absorbance at 450 nm was measured by using a SpectraMax M5 microplate reader (Molecular Devices).

Statistical Analysis

Data represent mean ± SE. Experimental data were subjected to statistical analyses using one-way ANOVA followed by Tukey *post-hoc* test or student's *t*-test with a significance level of $P < 0.05$.

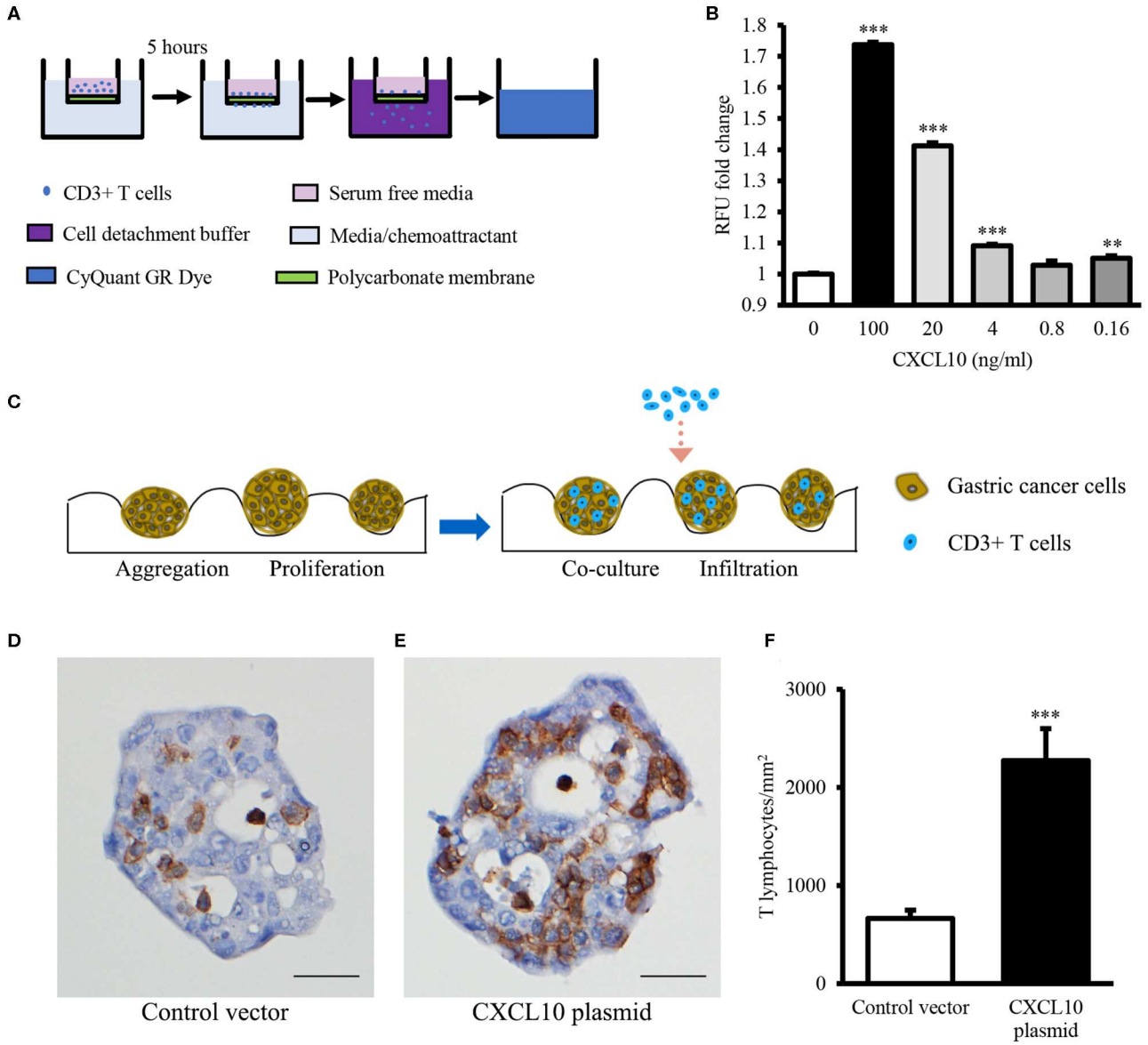

FIGURE 2 | CXCL10 recruits T lymphocytes in chemotaxis assay and GC spheroid infiltration assay. **(A)** Schematic representation of chemotaxis assay for T lymphocyte migration through the polycarbonate membrane toward different concentrations of recombinant CXCL10 protein. **(B)** Statistic analysis of fold change of migrated T lymphocytes. **(C)** Schematic representation of T lymphocyte infiltration into NCI-N87 spheroids. **(D,E)** Representative images of CD3 immunohistochemistry staining in NCI-N87 spheroids transfected with control vector **(D)** or CXCL10 plasmid **(E)**. **(F)** Histogram indicating the density of T lymphocytes in NCI-N87 spheroids. $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± SE. Scale bar: 25 μm.

RESULTS

CXCL10 Expression in GC Was Positively Correlated With Survival and Expression Profiles of Intra-tumor T lymphocyte Markers

Analysis of the prognostic information on CXCL10 in cancers (http://kmplot.com/analysis/) revealed a positive correlation of CXCL10 expression with both overall survival (**Figure 1A**, HR 0.79 [0.67–0.94], logrank $P = 0.0078$) and relapse free survival (**Figure 1B**, HR 0.8 [0.65–0.98], logrank $P = 0.029$) in patients with GC, but not in patients with breast cancer (**Figures S1A,D**), lung cancer (**Figures S1B,E**), or ovarian cancer (**Figures S1C,F**). In addition, correlation analysis in GEPIA showed strong positive correlation between CXCL10 expression and several T lymphocyte markers such as CD3D (**Figure 1C**, $P = 4.8e{-}41$, $R = 0.6$), CD3E (**Figure 1D**, $P = 8.4e{-}40$, $R = 0.59$), CD3G (**Figure 1E**, $P = 1.9e{-}39$, $R = 0.59$), CD4 (**Figure 1F**, $P = 6.4e{-}38$, $R = 0.58$), and CD8 (**Figure 1G**, $P = 5.6e{-}47$, $R = 0.63$). These results suggested that the CXCL10

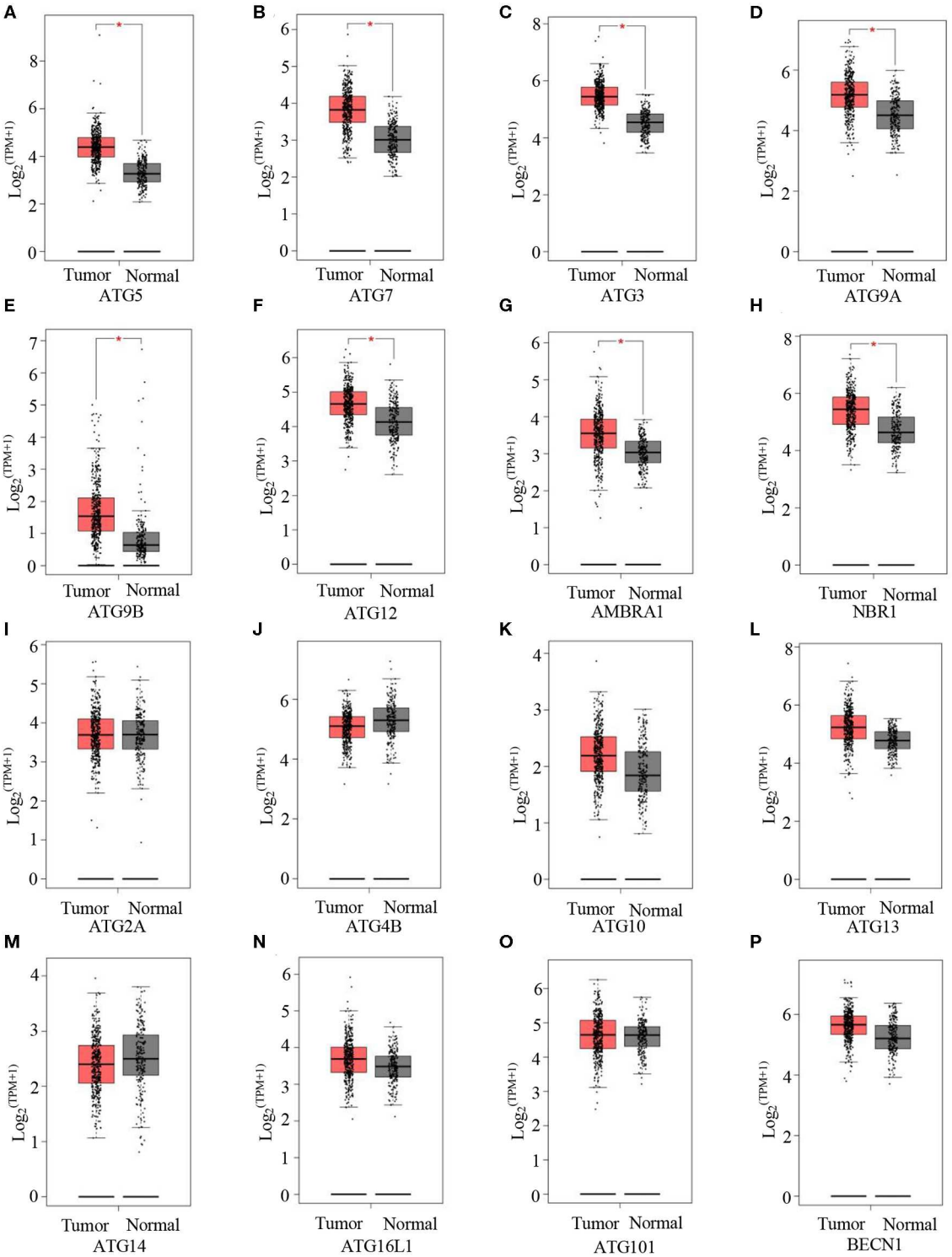

FIGURE 3 | Autophagy is activated in GC. **(A–P)** GEPIA analysis of the expression of ATG5 **(A)**, ATG7 **(B)**, ATG3 **(C)**, ATG9A **(D)**, ATG9B **(E)**, ATG12 **(F)**, ARBRA1 **(G)**, NBR1 **(H)**, ATG2A **(I)**, ATG4B **(J)**, ATG10 **(K)**, ATG13 **(L)**, ATG14 **(M)**, ATG16L1 **(N)**, ATG101 **(O)**, and BECN1 **(P)** in gastric tumors and normal tissues. $^*P < 0.05$.

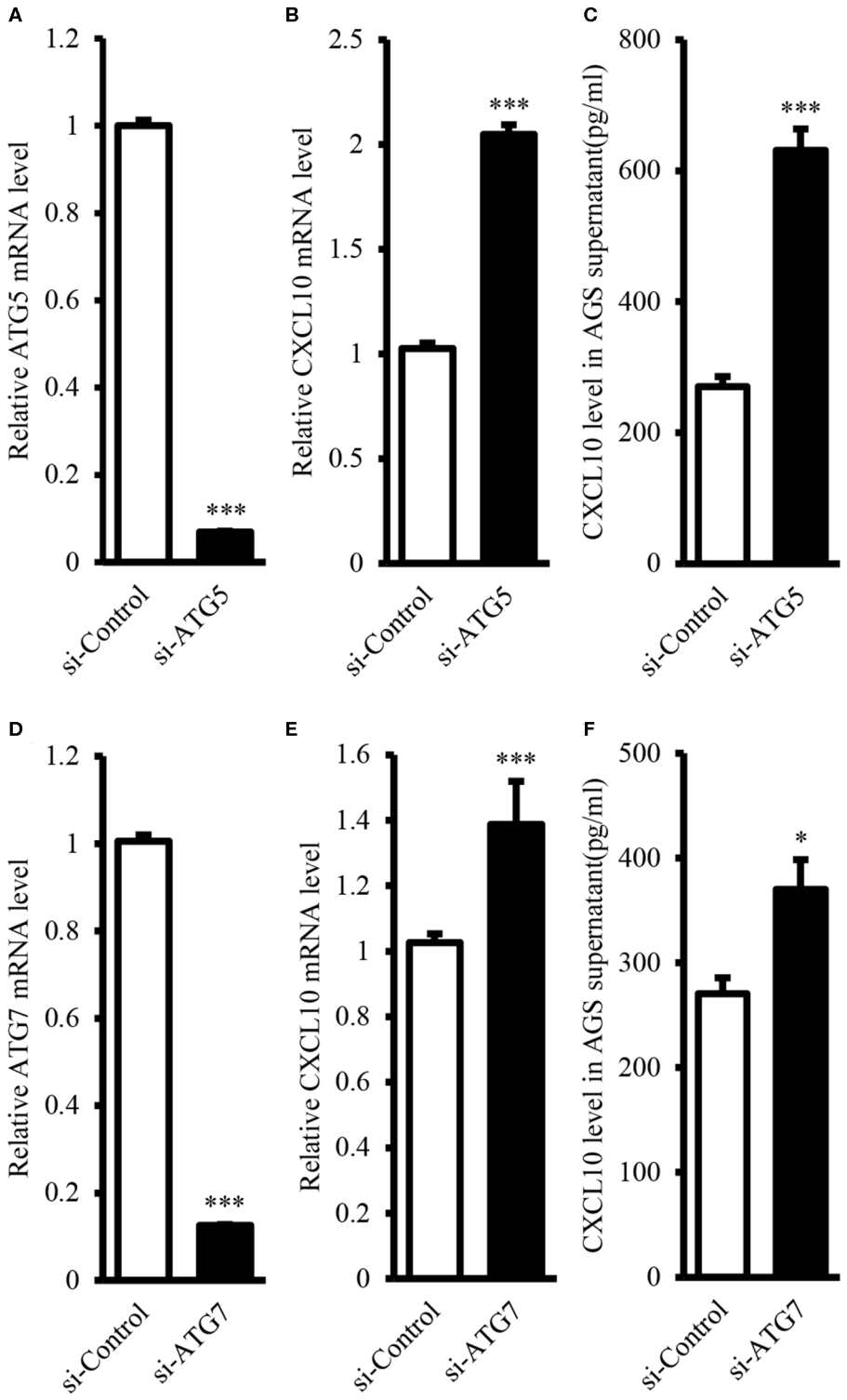

FIGURE 4 | ATG5 and ATG7 knockdown induced CXCL10 expression in AGS cells. **(A)** mRNA expression level of ATG5 in AGS cells transfected with ATG5 siRNA and control siRNA. **(B)** mRNA expression level of CXCL10 in AGS cells transfected with ATG5 siRNA and control siRNA. **(C)** CXCL10 protein level in the culture supernatant of AGS cells transfected with ATG5 siRNA and control siRNA. **(D)** mRNA expression level of ATG7 in AGS cells transfected with ATG7 siRNA and control siRNA. **(E)** mRNA expression level of CXCL10 in AGS cells transfected with ATG7 siRNA and control siRNA. **(F)** CXCL10 protein level in the culture supernatant of AGS cells transfected with ATG7 siRNA and control siRNA. $^{*}P < 0.05$, $^{***}P < 0.001$. Data represent mean ± SE.

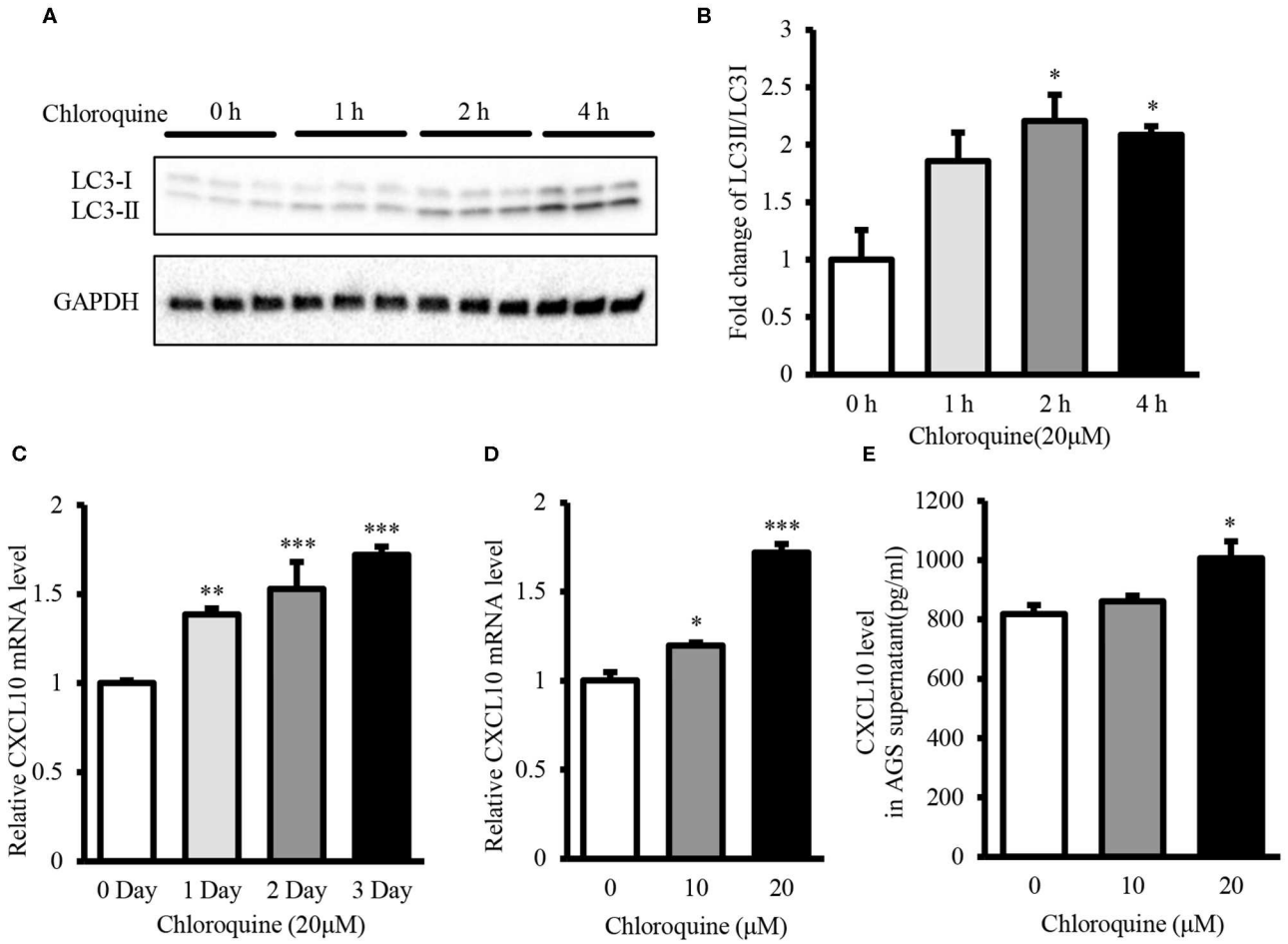

FIGURE 5 | CQ treatment induced CXCL10 expression in AGS cells. **(A)** Western blots showing LC3 expression in 20 μM CQ treated AGS cells. **(B)** Fold change of LC3II/LC3I ratio in 20 μM CQ treated AGS cells. **(C)** Time dependent CXCL10 mRNA expression in 20 μM CQ treated AGS cells. **(D)** CXCL10 mRNA expression in AGS cells treated with different doses of CQ for 3 days. **(E)** Protein level of CXCL10 in the culture supernatant of AGS cells treated with different doses of CQ. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± SE.

expression in GC might be positively correlated with intra-tumor T lymphocyte infiltration.

CXCL10 Recruited T lymphocytes in the Chemotaxis and GC Spheroid Infiltration Assay

Binding specificities of chemokines to their specific receptors are well-defined (42), and high expression of CXCR3 (the receptor of CXCL10) on effector T lymphocytes has been reported (43). Therefore, to confirm whether CXCL10 induces T lymphocyte infiltration, CXCR3+ T lymphocytes were required for the chemotaxis and spheroid infiltration assays. Because of the difficulties in detecting CXCR3 on most of the T lymphocytes freshly isolated from PBMCs of normal donors (**Figure S2**), CD3/CD28 Dynabeads were used to activate the T lymphocytes and induce the expression of CXCR3. After activation, over 90% of CD3/CD28 Dynabeads treated T lymphocytes were CXCR3+ (**Figure S2**). Chemotaxis assays revealed that CXCL10 recruited the primed T lymphocytes in a dose-dependent manner (**Figure 2B**).

In addition, to further confirm whether CXCL10 facilitates T lymphocyte infiltration in GC, GC spheroids were established using NCI-N87 cells transfected with CXCL10 or control plasmid (**Figures S3, S4**). Compared with the control vector-transfected spheroids, the CXCL10-overexpressing GC spheroids showed significantly high infiltration of T lymphocytes (**Figures 2D–F**).

Autophagy Was Activated in GC as Determined by GEPIA Analysis

Next, we evaluated autophagy activation in GC. Here, GEPIA was used to detect the expression levels of a few ATGs between GCs and normal tissues. Compared with normal tissues, tumor tissues showed significantly higher mRNA levels of the following key autophagy genes: ATG5 (**Figure 3A**), ATG7 (**Figure 3B**), ATG3 (**Figure 3C**), ATG9A (**Figure 3D**), ATG9B (**Figure 3E**), ATG12

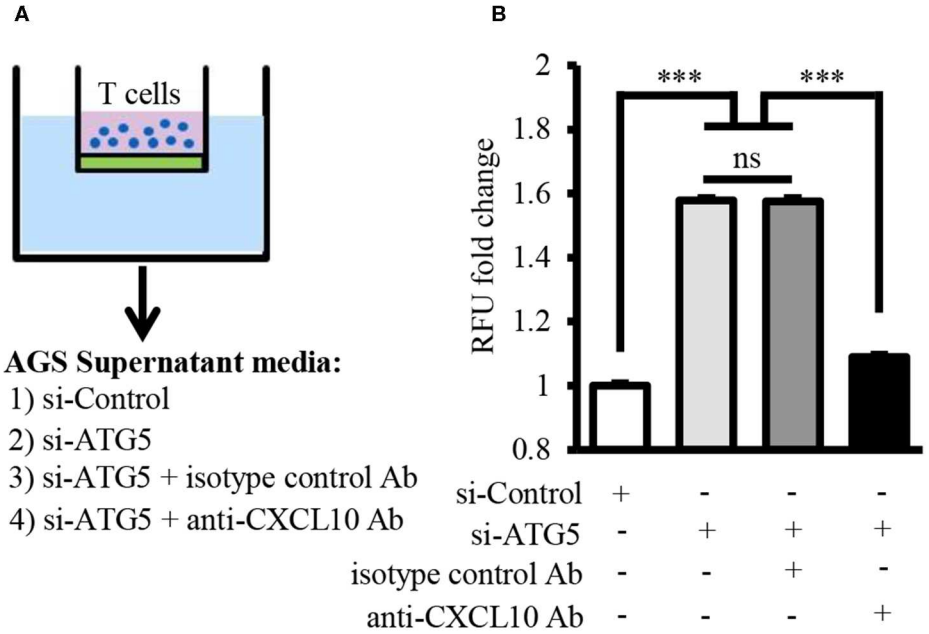

FIGURE 6 | Autophagy inhibition facilitated T lymphocyte migration by inducing CXCL10 secretion. **(A)** Schematic representation of chemotaxis assay for T lymphocyte migration through polycarbonate membrane toward three different mediums. **(B)** Fold change of migrated T lymphocytes. ***$P < 0.001$. Data represent mean ± SE.

(**Figure 3F**), AMBRA1 (**Figure 3G**), and NBR1 (**Figure 3H**). These data indicate increased autophagy in GCs.

Autophagy Inhibition Enhanced CXCL10 Expression in AGS Cells

It is well-known that ATG proteins are critical for the formation of autophagosome and the activity of autophagy (44, 45). ATG5 and ATG7 are two of the most important components of the ATG family; therefore, ATG5 or ATG7 ablation is sufficient to impair autophagic functions (46–52). In this study, we aimed to induce ablation of ATG5 or ATG7 in AGS cells, as AGS cells showed the highest endogenous CXCL10 expression level among the available GC cell lines (**Figure S3**). ATG5 siRNA transfection in AGS cells significantly suppressed ATG5 expression at both mRNA (**Figure 4A**) and protein levels (**Figures 7A,F**). Such ATG5 knockdown inhibited autophagy, as demonstrated by decreased LC3II/LC3I ratio (**Figures 7A,E**). In addition, ATG5 knockdown significantly induced CXCL10 mRNA expression in AGS cells (**Figure 4B**) and significantly increased CXCL10 secretion by AGS cells (**Figure 4C**). Similarly, ATG7 knockdown significantly induced CXCL10 expression at both mRNA and protein levels (**Figures 4E,F**).

CQ inhibits autophagic flux by decreasing the fusion of autophagosome-lysosome (53). Therefore, we used CQ to further confirm whether autophagy inhibition could induce CXCL10 expression in AGS cells. Treatment with 20 μM CQ significantly induced the accumulation of LC3-II in a time-dependent manner (**Figures 5A,B**), as reported previously (53–55). Furthermore, 20 μM CQ significantly induced CXCL10 mRNA expression in a time-dependent manner in AGS cells without affecting the cellular viability (**Figure 5C**, **Figure S5**). The maximal induction effect was observed at day 3. When incubation time was fixed for 3 days, Treatment with 10 and 20 μM CQ significantly induced CXCL10 mRNA in AGS cells (**Figure 5D**). In addition, CXCL10 secretion by AGS cells treated with 20 μM CQ was significantly higher than that by control cells (**Figure 5E**).

Autophagy Inhibition Facilitated T lymphocyte Migration by Inducing CXCL10 Secretion

Chemotaxis assay revealed that T lymphocyte recruitment by culture supernatant of ATG5-knockdown AGS cells was significantly higher than that by culture supernatant of control cells (**Figures 6A,B**). This T lymphocyte recruitment was effectively blocked in the presence of neutralizing anti-CXCL10 antibody (**Figure 6B**).

Autophagy Inhibition Enhanced CXCL10 Expression by Suppressing the Inhibitory Effect of JNK Signaling

Next, we investigated the mechanism underlying the induction of CXCL10 expression via autophagy inhibition. Here, we demonstrated that ATG5 knockdown was sufficient to inhibit autophagy (**Figures 7A,E,F**) and investigated the levels of components of the JNK signaling pathway in AGS cells. ATG5 knockdown significantly decreased the levels of phospho-JNK (**Figures 7A,B**), phospho-c-Jun (**Figures 7A,C**), and c-Jun (**Figures 7A,D**), thereby suppressing JNK signaling.

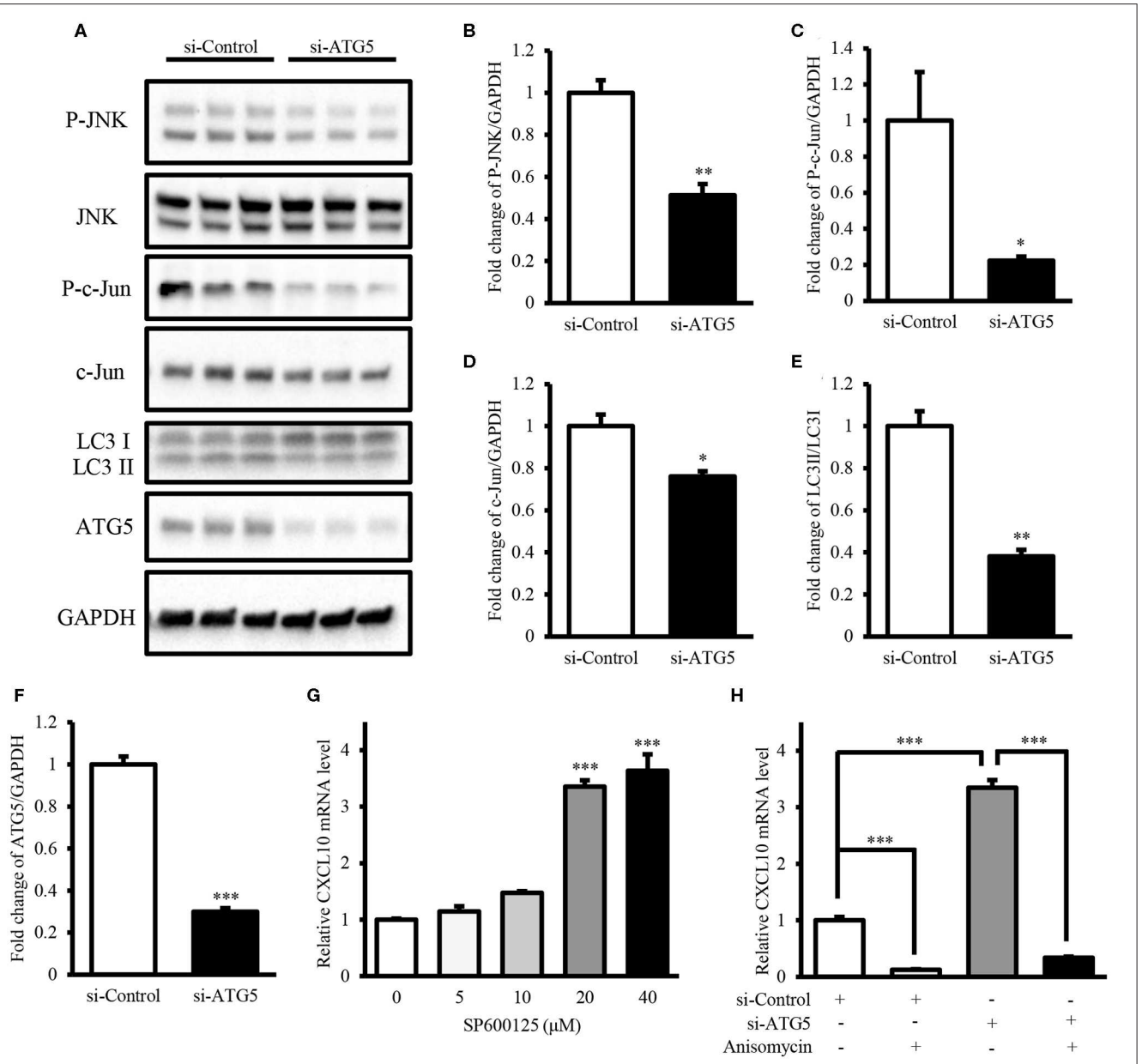

FIGURE 7 | Autophagy inhibition induced CXCL10 expression by suppressing the inhibitory effects of JNK signaling. **(A)** Western blots for phospho-JNK, JNK, phospho-c-Jun, c-Jun, LC3, ATG5, and GAPDH levels in AGS cells transfected with ATG5 siRNA or control siRNA. **(B–F)** Relative protein levels of P-JNK **(B)**, P-c-Jun **(C)**, c-Jun **(D)**, LC3II/LC3I **(E)**, and ATG5 **(F)**. **(G)** CXCL10 mRNA levels in AGS cells treated with different doses of SP600125. **(H)** CXCL10 mRNA levels in ATG5 siRNA transfected AGS cells treated with or without 100 ng/ml anisomycin. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± SE.

Treatment with the JNK inhibitor SP600125 resulted in a dose-dependent increase in CXCL10 mRNA expression in AGS cells, and 20 and 40 μM SP600125 showed a significant increase in CXCL10 mRNA levels (**Figure 7G**). In addition, treatment with 100 ng/ml anisomycin, a JNK activator, significantly inhibited CXCL10 mRNA expression in control-vector transfected AGS cells and significantly suppressed the ATG5 knockdown-induced increase in CXCL10 mRNA expression (**Figure 7H**). Collectively, these data suggest that autophagy inhibition induced CXCL10 expression via suppression of the inhibitory effects of JNK signaling.

Autophagy Inhibition Induced CXCL10 Expression in $CoCl_2$-Treated AGS Cells

Intra-tumor hypoxia is an important characteristic of 50–60% malignant tumors (56). Moreover, GEPIA showed that mRNA level of HIF1α, the hypoxia marker, in GCs was significantly higher than that in normal gastric tissues (**Figure 8A**). Therefore, we investigated the effect of autophagy inhibition on CXCL10

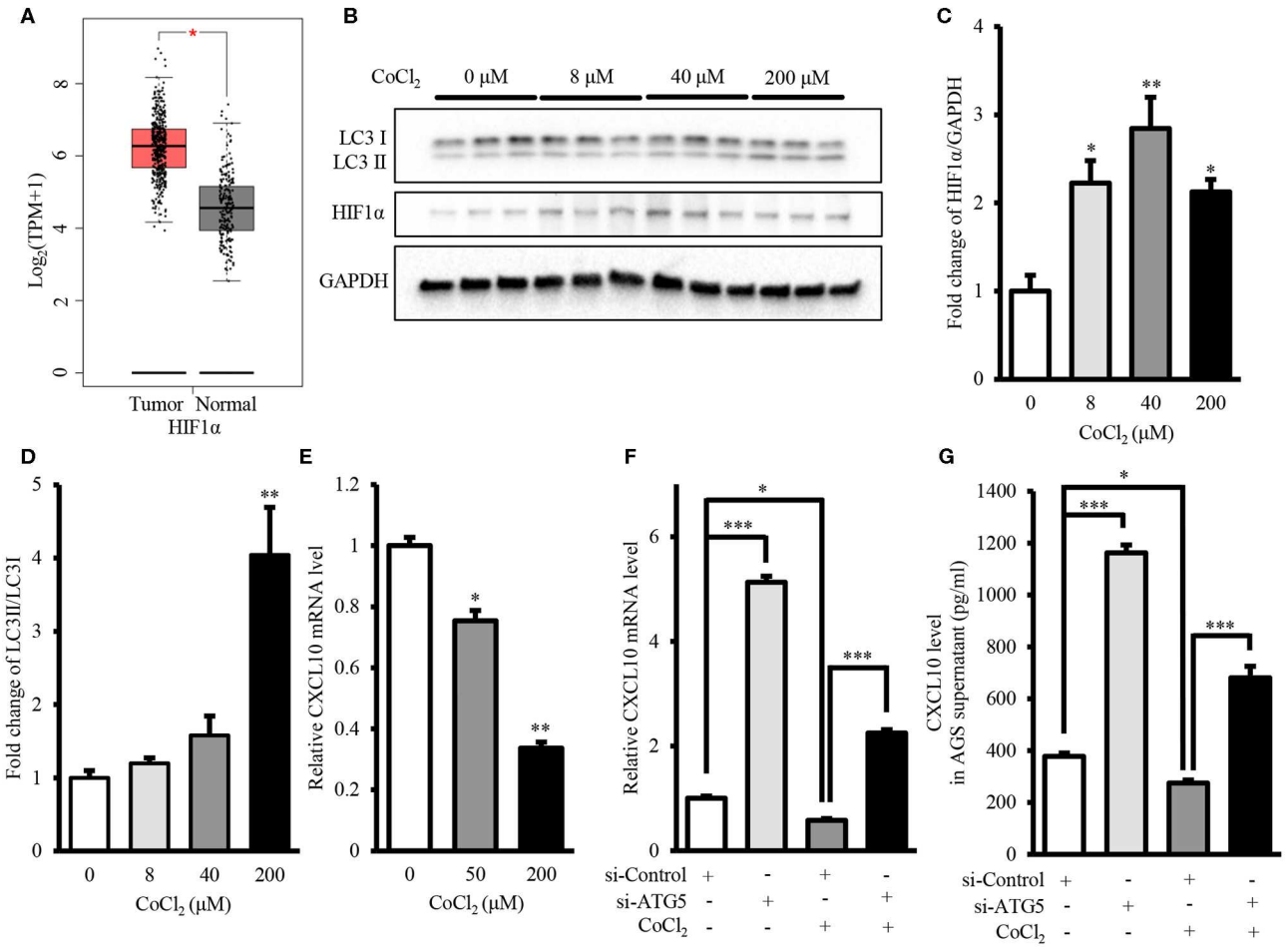

FIGURE 8 | Autophagy inhibition induced CXCL10 expression in $CoCl_2$-treated AGS cells. **(A)** GEPIA analysis of HIF1α expression in gastric tumors and normal tissues. **(B)** Western blots for HIF1α and LC3 in AGS cells treated with different concentrations of $CoCl_2$. **(C,D)** Relative protein levels of HIF1α **(C)** and LC3II/LC3I ratio **(D)** in AGS cells treated with different concentrations of $CoCl_2$. **(E)** CXCL10 mRNA levels of in $CoCl_2$-treated AGS cells. **(F)** CXCL10 mRNA levels in ATG5 siRNA transfected AGS cells treated with or without $CoCl_2$. **(G)** CXCL10 protein levels in the culture supernatant of ATG5 siRNA transfected AGS cells treated with or without $CoCl_2$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. Data represent mean ± SE.

expression under hypoxia mimetic conditions. Treatment with $CoCl_2$, a hypoxia mimetic reagent, significantly increased HIF1α protein level in AGS cells (**Figures 8B,C**). Treatment with 200 μM $CoCl_2$ significantly increased the LC3II/LC3I ratio, indicating increased autophagic activity in AGS cells (**Figures 8B,D**). Furthermore, $CoCl_2$ decreased CXCL10 expression in a dose-dependent manner, and both 50 and 200 μM $CoCl_2$ significantly decreased CXCL10 mRNA levels in AGS cells (**Figure 8E**). ATG5 knockdown significantly increased CXCL10 expression in $CoCl_2$ treated AGS cells at both mRNA and protein levels (**Figures 8F,G**).

DISCUSSION

In this study, we demonstrated that intra-tumor CXCL10 is an important chemokine that contributes to intra-tumor infiltration of T lymphocytes in GC. We also showed that autophagy inhibition could effectively facilitate T lymphocyte migration into the tumor microenvironment by inhibiting the JNK pathway and further inducing the expression of CXCL10 (**Figure 9**). This might represent a novel therapeutic strategy to enhance the effectiveness of solid tumor immunotherapies such as immune check-point blockade.

It is well-known that the levels of T lymphocyte infiltration into the tumor determine the efficacy of immunotherapy. Primed T lymphocytes gain the expression of certain homing molecules (such as CXCR3) on their surface and thus obtain the capability to migrate toward the tumor site (24). In our study, CXCL10, the well-accepted CXCR3 ligand, functioned as a chemoattractant for T lymphocytes (**Figures 2A,B**) and recruited T lymphocytes to GC spheroids (**Figures 2C–F**). Moreover, CXCL10 expression was positively correlated with overall survival (**Figure 1A**) and relapse-free survival (**Figure 1B**) in patients with GC. Consistent with our observations, Barash et al. indicated that CXCL10 administration not only induced the infiltration of T cells

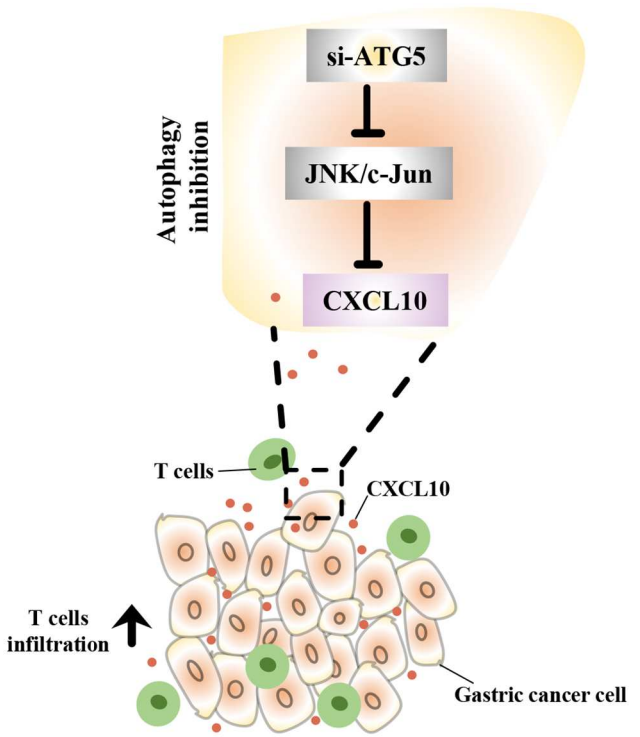

FIGURE 9 | Schematic representation of the mechanism underlying the increased CXCL10 expression in autophagy inhibited GC cells. In GC cells, autophagy inhibition suppressed JNK signaling and subsequently induced CXCL10 expression. As a result, increased CXCL10 recruited more T lymphocytes into gastric tumors.

and NK cells into myeloma tumors but also reduced the accumulation of Treg cells at the tumor site, thereby suppressing tumor progression (57). In addition, CD26 inhibition was reported to enhance T lymphocyte trafficking into melanoma tumor by inducing the intra-tumor expression of CXCL10, further improving the efficacy of immunotherapy (58). In addition to being a potent chemoattractant for T lymphocytes, CXCL10 also inhibits tumor growth via suppressing angiogenesis (59–63). Furthermore, CXCL10 overexpression improved the radiosensitivity of tumors in a rodent cervical cancer model (64). In total, the evidence suggests that CXCL10 could be a potential novel candidate for the GC targeted therapy.

Considering the fact that autophagy was not measurable, the indicators for autophagy activation were judged by expression of ATGs. In our study, GEPIA indicated that the expression of some key autophagy genes in GC were significantly higher than that in normal tissue (**Figure 3**). These results were consistent with previous observations in established solid tumors (32, 65). However, previous findings on the regulatory effect of autophagy inhibition on CXCL10 expression are not consistent. For instance, two studies showed that ATG5 knockdown significantly suppressed influenza-virus induced CXCL10 expression in macrophages (66, 67). Two other studies reported that deletion of some other key autophagy genes, FIP200 or BECN1, led to increased CXCL10 production in mammary tumor cells (68) or melanoma cells (36). Nevertheless, the regulation of CXCL10 expression in GC cells has not yet been reported.

Data from our study showed that autophagy inhibition induced CXCL10 expression in AGS cells. Autophagy inhibition was achieved by two approaches: genetic approach (ATG5 knockdown or ATG7 knockdown) and chemical treatment (CQ). Of note, ATGs is critical for the formation of autophagosome. Autophagy deficiency has been confirmed in cells lacking ATG3 (69), ATG5 (70), BECN1 (71), ATG7 (52), ATG9A (72), ATG16L1 (73), FIP200 (74), and AMBRA1 (75). In addition, CQ, a widely used autophagy inhibitor, is known to inhibit autolysosome formation and lysosomal protein degradation (76). In our study, both genetic approach (ATG5 knockdown or ATG7 knockdown) and chemical treatment (CQ) significantly induced CXCL10 expression in AGS cells, but the mechanism for induction of CXC10 expression was still unclear. Furthermore, our data showed that ATG5 knockdown facilitated T lymphocyte migration by increasing CXCL10 expression.

We next investigated the mechanism underlying the induction of CXCL10 expression by autophagy inhibition. We found that JNK activator decreased and JNK inhibitor increased CXCL10 expression in AGS cells. In addition, autophagy inhibition significantly decreased the activity of JNK signaling pathway. Thus, these data suggest that autophagy inhibition induces CXCL10 expression by suppressing the inhibitory effect of JNK signaling in AGS cells. In contrast, Mgrditchian et al. reported that BECN1 deletion induced CCL5 expression by activating the JNK signaling pathway, which in turn recruited more NK cells into melanoma tumors (36). This difference in the effect of autophagy inhibition on JNK signaling may be associated with tumor types.

Next, we investigated whether autophagy inhibition also induced CXCL10 expression under hypoxia mimetic conditions. Because of the inadequate oxygen supply and increased energy consumption within the tumor microenvironment, hypoxia is one of the most important characteristics of solid tumors, especially in the advanced stages (77). In the hypoxic microenvironment, autophagy flux is enhanced along with increased tumor growth (78). Advanced tumors have been shown to use autophagy to promote tumor survival (79, 80). Our current observations that ATG5 knockdown induced CXCL10 expression in $CoCl_2$-treated AGS cells support a scientific basis of autophagy inhibition as a potential combinational therapy strategy for immunotherapy.

Apart from recruiting T lymphocytes into solid tumors and enhancing the sensitivity to anti-tumor therapy, autophagy deficiency was also reported to cause some cancer related pathology (81, 82). For instance, the mutation of ATGs was reported in tumor cells (83). Because of the function of autophagy in counteracting cellular stress, some ATGs were considered as tumor suppressors in rodent tumor models (45, 84–86). In addition, Yang et al. indicated that fluorouracil inhibited the growth of GC cells via ATG6 activation (87). In this case, autophagy also sometimes seems as a protective mechanism in tumor initiation period. Overall, autophagy might regulate tumorigenesis in a tumor stage-specific manner.

In summary, to the best of our knowledge, this is the first report on the regulatory effects of *in vitro* autophagy inhibition on CXCL10 expression in GC cells and its potential mechanism in recruiting T lymphocytes into the tumor. These findings provide novel insights into understanding the functions of autophagy in immunotherapy. Furthermore, our results highlight the potential of autophagy inhibition to be used in combination with immunotherapy approaches such as immune checkpoint blockade. Our findings also suggest CXCL10 as a potential novel candidate for targeted therapy against GC.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. Kaplan Meier-plotter can be found here: http://kmplot.com/analysis/. The GEPIA (Gene Expression Profiling Interactive Analysis) can be found here: http://gepia.cancer-pku.cn/index.html.

AUTHOR CONTRIBUTIONS

QM and LH designed the study. QM and YZ performed the experiments and statistical analysis. QM drafted the manuscript. QM, YZ, and LH revised the manuscript. 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/fonc.2020.00886/full#supplementary-material

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REVIEW
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Immune Signatures and Survival of Patients With Metastatic Melanoma, Renal Cancer, and Breast Cancer

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Despite remarkable recent progress in treating solid cancers, especially the success of immunomodulatory antibody therapies for numerous different cancer types, it remains the case that many patients fail to respond to treatment. It is therefore of immense importance to identify biomarkers predicting clinical responses to treatment and patient survival, which would not only assist in targeting treatments to patients most likely to benefit, but might also provide mechanistic insights into the reasons for success or failure of the therapy. Several peripheral blood or tumor tissue diagnostic and predictive biomarkers known to be informative for cancer patient survival may be applicable for this purpose. The use of peripheral blood ("liquid biopsy") offers numerous advantages not only for predicting treatment responses at baseline but also for monitoring patients on-therapy. Assessment of the tumor microenvironment and infiltrating immune cells also delivers important information on cancer-host interactions but the requirement for tumor tissues makes this more challenging, especially for monitoring sequential changes in the individual patient. In this contribution, we will review our findings on immune signatures potentially informative for clinical outcome in melanoma, breast cancer and renal cell carcinoma, particularly the outcome of checkpoint blockade, by applying multiparametric flow cytometry and mass cytometry, routine clinical monitoring and functional testing for predicting and following individual patient responses to therapy.

Keywords: immune signatures, biomarker, melanoma, renal cancer, breast cancer

INTRODUCTION

The long-standing controversy as to whether the immune system performs immunosurveillance against cancer, as originally proposed by Burnet (1), and the accompanying skepticism as to whether immune-based treatments would ever be effective (2) was finally laid to rest with the development of clinically effective immunomodulatory antibody treatments [immune checkpoint inhibition, ICI (3)], culminating in the Nobel Prize for Physiology or Medicine in 2018. Nonetheless, there are countless reasons why some cancer patients may not respond at all, or later become refractory to ICI, almost matched by the large number of published papers discussing

this issue (4). For routine application and selection of the best therapy with the least cost and fewest side-effects, a major unmet need is to define robust biomarkers predicting meaningful response. These would ideally be as simple as possible and predict the likelihood of response not only prior to but also during therapy. For the purpose of monitoring response to therapy, and for ease of application in routine clinical settings, biomarkers established from a small sample of peripheral blood would offer many advantages over tissue biopsy. Parameters measurable in peripheral blood mononuclear cells (PBMC) include antigen presentation capacity, T cell antigen-specificity, activation and differentiation/activation states, cytokine and chemokine production, quantity and quality of regulatory T cells (Tregs) and of so-called myeloid-derived suppressor cells (MDSCs), as well as circulating cancer cells themselves, cell-free DNA and exosomes from the tumor. What would be more difficult but theoretically not impossible to determine using blood would be the presence of tumor-associated antigens and MHC expression on the cancer cells, their mutational burden and neoantigen landscape, the expression of cell membrane ligands directly involved in the regulation of T cell function, as well as more mundane parameters such as tumor burden. Although tumor tissue is certainly highly informative when searching for such immune biomarkers, one evident limitation is that these are rarely available for all patients and at different times during therapy. Hence, peripheral blood, which can be repeatedly obtained during therapy in a minimally invasive manner, is an attractive alternative, despite not representing the place "where the action is." Here we summarize predominantly our own work on constellations of peripheral biomarkers informative for responses to ICI (mostly anti-CTLA-4 or anti-PD-1 in melanoma). We contrast these with tumor-infiltrating immune cells (TIICs) in breast and kidney cancers where comparisons between peripheral and tissue data are more readily possible. The overall aim of the work reviewed here was to generate minimal clusters of the simplest possible biomarkers with maximal predictive ability for routine application in the clinic (**Figure 1**).

PERIPHERAL BIOMARKERS FOR MELANOMA ASSESSED AS *IN VITRO* T CELL RESPONSES TO TUMOR-ASSOCIATED ANTIGENS

With the above in mind, our interest in establishing immunological biomarkers informative for survival of patients with metastatic melanoma predated the introduction of ICI and stemmed from early studies on melanoma patients surviving for an unusually prolonged time on conventional therapy or other non-classical therapies. At that time, we undertook a small RNA vaccination study that sought to immunize individual melanoma patients with personalized mixtures of shared cancer testis and lineage antigens identified as expressed by the resected tumor (5). These included NY-ESO-1, Melan-A, MAGE-A3 and survivin as well as several others. We incubated pre-vaccination PBMCs from each patient with mixtures of overlapping peptides representing each entire molecule to which the patient would be vaccinated, and then restimulated with the same peptides thereafter. The assay readout was CD4+ and/or CD8+ T cell activation as assessed by simultaneous intracytoplasmic staining for 6 pro- and anti-inflammatory cytokines (IL 2, IFN-γ, TNF, IL 4, IL 5 or IL 10, and IL 17). Thus, this demanding assay system assesses the capacity of the immune cells in the individual patient's blood to pick up, process and present antigen by antigen-presenting cells (APC) in a manner triggering memory T cell activation and proliferation, and indicates whether the response is mediated by CD4+ or CD8+ T cells, and whether predominantly pro- or anti-inflammatory cytokines are produced, as well as revealing which potential tumor-associated antigens (TAA) can be recognized by the patient's T cells. This approach had first been successfully applied to document increasing frequencies of TAA-reactive CD8+ T cells in a patient responding to intra-lesional injection of IL 2 (6). Using this same assay, we next accessed our biobank of cryopreserved PBMCs from late-stage melanoma patients on conventional therapy and retrospectively associated responses to TAA by patients surviving for longer than usual (>2 years at that time), less than usual (<6 months) or in between. We found that although all patients' PBMCs responded to the positive control peptides (matrix protein and nucleoprotein peptides from influenza), the frequency of patients responding to NY-ESO-1 and/or Melan-A in the "long-survivor" group was significantly greater than in the "short-survivor" group. Patients responding to more than one TAA did better than those responding to none or only one. Interestingly, responses to NY-ESO-1 mediated by either CD4+ or CD8+ T cells were associated with longer survival, whereas CD8+ but not CD4+ T cell responses to Melan-A, were beneficial (7). Responses to two other TAA tested were not informative because almost all patients responded to MAGE-A3 and almost none to survivin (8). Prospective studies confirmed this association and went further to show that not only the identity of the antigen and responding T cell subset but also the nature of the T cell response against that antigen was informative for survival in these patients (7). In more recent independent studies, we have again observed predictive capacities of NY-ESO-1- and Melan-A-reactivities also for the outcome of melanoma patients under ICI with anti-PD-1 ± CTLA-4 antibodies (Zelba et al., personal communication) raising the question of potential advantages of T cells recognizing shared tumor antigens as one of several modules in future treatment strategies. Ongoing trials targeting in particular NY-ESO-1 might help to answer this question (for example NCT01967823, NCT03029273, NCT02775292).

PERIPHERAL BIOMARKERS FOR MELANOMA ASSESSED BY SURFACE MARKER PHENOTYPING OF IMMUNE CELLS

A more conventional approach, easier to standardize and apply in routine clinical practice than the functional assays described above, monitors the presence of different immune

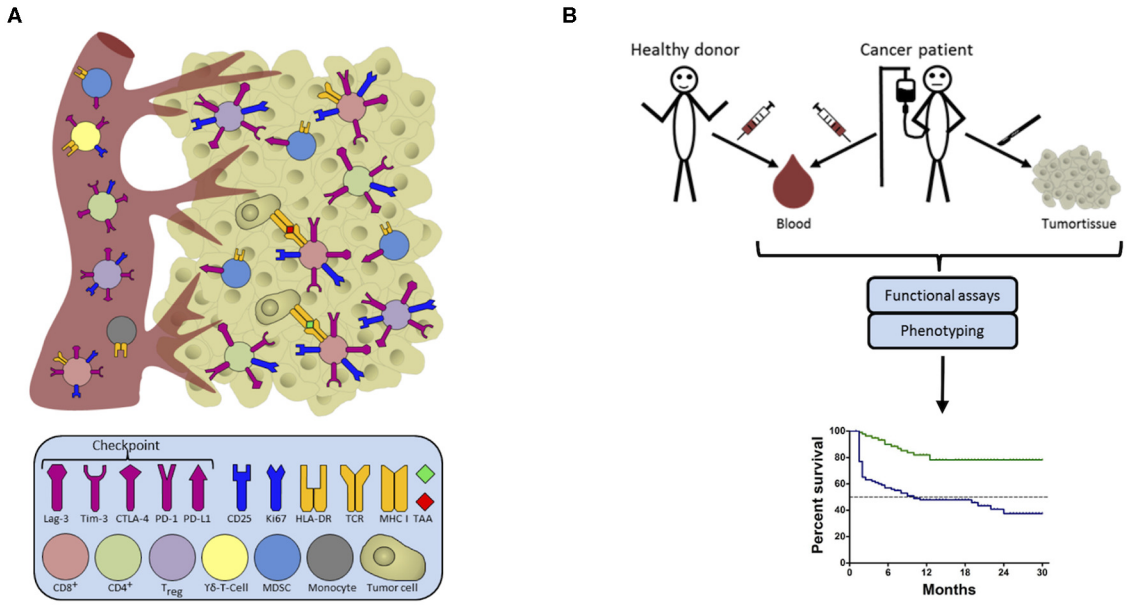

FIGURE 1 | Candidate biomarkers in the host-cancer/cancer-host interaction. **(A)** Intra-tumoral leucocytes commonly consist of a highly diverse pool of cells which may allow prognostic or even predictive associations with the course of disease/treatment outcome. Some of these cells involved in cancer immunosurveillance migrate between tissues and can thus also be detected in peripheral blood. The figure shows cells in the blood on the left, and in the tumor on the right, color-coded to represent the different cells involved, along with their surface receptors. **(B)** Blood is an ideal source of material for the determination of clinically relevant biomarkers as it is easy to access repeatedly, and allows comparison with healthy donors. Functional assays combined with phenotyping provide constellations of immune parameters constituting an immune signature with a closer correlation with survival than any single factor. From a practical point of view, we should aim to replace functional assays by rapid *ex vivo* phenotyping approaches to pave the way for defining novel biomarkers for use in a routine clinical setting.

cells in the peripheral blood by flow cytometry. To maximize data density from small blood samples, single cell, multi-parameter analysis has made great strides recently. In an early study, using a 38-channel time-of-flight mass cytometry (CyTOF) approach in 2013 we investigated the peripheral immune landscape (using PBMCs) in what was at the time the largest cohort of stage IV melanoma patients and age-matched healthy individuals subjected to this new technique (9). We compiled a detailed immune signature of T cells, NK cells, B cells and myeloid cells and their subsets and found that superior survival was characterized by relatively high proportions of differentiated NK-cells and a balanced distribution of monocytic MDSC (mMDSC)-like and APC-like phenotypes (HR: 0.2) (10). The predictive capacity of a comparable myeloid APC-like phenotype was reported by Krieg et al., in a similar high-dimensional CyTOF immunomonitoring study in melanoma under PD-1 blockade (11). Not only classical T cells, but also T cells carrying the alternative γδ T cell receptor can exert strong anti-tumor, but also under certain circumstances pro-tumor functions, as reviewed by others elsewhere (12). We suggest that these cells must also be considered when generating informative immune signatures because we found in a discovery study that low frequencies of Vδ1+ γδ T cells correlated with prolonged overall survival (OS) (13).

PERIPHERAL BIOMARKERS FOR MELANOMA WITH IPILIMUMAB TREATMENT

For the purpose of clinical exploitation not relying on complex biological assays or specialist multi-parameter flow cytometry, simpler assays would be most useful and most likely to find widespread employment. As ipilimumab came into routine use as the first ICI agent licensed in 2011, we asked whether the cell surface immune signatures and intracellular FoxP3 staining would remain informative for patients receiving this agent, relative to conventional markers like LDH serum levels (14). We accessed our PBMC biobank from a large multi-center study to assess immune cell frequencies and clinical metadata before therapy start, in order to investigate potential correlations at the single and multiple factor level. We identified a model comprising a compound signature of low serum LDH-levels, absolute monocyte counts, mMDSC frequencies, high absolute eosinophil counts, Treg frequencies and relative lymphocyte counts associated significantly with a favorable outcome following ipilimumab treatment. For patients with a risk score of 0 in this model, the 2-year survival rate was 40.8%, whereas for those with a risk score $\leq$ 130 it was only 17.3%, and, strikingly, no patient with a risk score $>$ 130 survived $>$15 months (15). Our data confirmed previous work reporting on the

poor prognosis of patients with high LDH (16, 17), MDSC levels (17–19) or eosinophils (20) under ipilimumab.

In a follow up analysis of partially overlapping cohorts, we investigated changes of 22 factors (15 immune cell populations and seven routine blood counts) at two time points under therapy (2–8 and 8–14 weeks after start of ICI). We identified amongst others, significant increases in the expression of the proliferation marker Ki67 on regulatory T cells (Tregs), CD4+ and CD8+ T cells and in Treg frequencies and absolute eosinophil counts in most of the observed patients, while frequencies of nonclassical (CD16+) monocytes were significantly decreased at a later follow-up time point. However, neither dynamic alterations in Tregs nor mMDSCs correlated with patients' OS (but retained their prognostic capacity under therapy when the cohort was dichotomized according to their median frequencies at the respective time point). Interestingly, early increases of absolute lymphocyte counts and delayed increases of peripheral CD4+ and CD8+ T cell frequencies within the pool of lymphocytes were significantly associated with a better outcome of ICI {1 year survival rate: 93.3%, response rate [best overall response (BOR) following immune-related response criteria (irRC)]: 71.4%} (21). Next, we investigated, also in partially overlapping cohorts, patients' peripheral blood CD4+ and CD8+ T cell differentiation signatures and PD-1 expression because that population was previously found in melanoma to harbor a pool of clonally expanded, tumor-reactive cells (22, 23). We found that an immune-activated CD8+ T cell compartment, characterized by higher frequencies of CD8+ effector memory type 1 (EM1) cells (CD45RA– CCR7– CD27+ CD28+) and lower frequencies of CD8+ T_{EMRA} cells (CD45RA+ CCR7– CD27– CD28–) before starting CTLA-4 blockade correlated significantly with a more favorable outcome in univariate analyses (1 year survival rates: 46.4 vs. 35.4% for high vs. low CD8+ EM1 cells; 46.7 vs. 35% for low vs. high CD8+ T_{EMRA} cells). Interestingly, the frequency of PD-1 expression on peripheral CD8+ EM1 cells was not informative for therapy outcome at baseline, but a decrease of this population during therapy correlated with an improved clinical response (BOR following irRC) (24). However, due to limited sample material, we did not have the opportunity to investigate whether PD1+ EM1 CD8+ T cells that recognized tumor antigens increased during therapy in responding metastases. We also do not know whether this population harbored (clonally expanded) tumor-reactive cells nor whether such cells, if present, might have been dysfunctional. Reading et al., provide a detailed discussion of the role of CD8+ memory T cells in tumor immunity in this context (25).

Investigations of γδ T cells revealed that these cells also possessed value as biomarker candidates for the outcome of ipilimumab therapy. We found higher peripheral frequencies of Vδ1+ and lower frequencies of Vδ2+ cells in stage IV patients before start of therapy than in an age- and sex-matched control cohort of healthy subjects; this effect was even more pronounced in short-term survivors (< 9 months OS). In line with these findings, low Vδ1+ and high Vδ2+ T cell frequencies prior to therapy start correlated significantly in a univariate analysis with prolonged OS under therapy (1 year survival rates: 53.3 vs. 37.9% for low/high Vδ1+ and 54.2 vs. 39% for high/low Vδ2+) (13). Further investigation of the predictive capacity but also the functionality of γδ T cells under single-agent PD-1 treatment or in combination with CTLA-4 inhibitory therapies is currently ongoing under the aegis of the German Research Unit 2799 (Receiving and Translating Signals via the γδ T cell receptor; https://for2799.de/). In that context, it is important to be aware of potential pitfalls in the characterization of circulating and tissue-resident γδ T cells because the application of commercially available reagents to classify these unconventional T cells is not always trouble-free. Based on the published literature and our own experience, we have recently provided an overview of how such pitfalls might be circumvented and suggested basic requirements for harmonization and standardization of γδ T cell immunomonitoring approaches (26).

PERIPHERAL BIOMARKERS FOR MELANOMA WITH PEMBROLIZUMAB TREATMENT

We have recently extended some of the above analyses to melanoma patients treated with single agent anti-PD-1 antibodies and investigated routine baseline blood parameters and clinical meta-data in a multi-center study before starting anti-PD-1 blockade. High relative eosinophil counts, relative lymphocyte counts, low serum LDH-levels and the absence of metastasis in other than soft-tissue/lung were independent baseline characteristics that associated with favorable OS. The more of these favorable baseline factors were evident in a given patient, the better was his/her survival probability (1 year survival rates: 83.9% for best factor combination; 14.7% for the poor factor combination) (27).

In a recent study from Bochem et al. (28), we investigated peripheral blood T-cell phenotypes, searching for biomarker candidates predicting treatment outcome in melanoma patients under PD-1 inhibition. Patients with lower than median frequency of peripheral PD-1+ CD56+ T-cells had a significantly longer OS (1 year survival rate 78.4 vs. 52.8% for low vs. high frequencies), progression free survival (1 year progression-free survival rate 35.1 vs. 27.8% for low vs. high frequencies) and superior clinical benefit (59.5 vs. 27.8% for low vs. high frequencies; BOR following RECIST 1.1 criteria) compared to the reciprocal group. Interestingly, neither frequencies of "classical" CD56– CD4+ nor CD56– CD8+ T-cells, nor of the PD-1+ population within the CD4 or CD8 subsets was associated with clinical outcome (28). Only little is known about PD-1+ CD56+ T-cells in human cancers. Thus, future investigations are required for a better characterization of this heterogeneous cell population that presumably comprises large fractions of "non-classical" T cells, like NKT-like cells or γδ T cells.

To overcome limitations in the PD-1 detection in sample material obtained from patients under PD-1 therapy, we found it important to employ an experimental protocol to deal with steric hindrance between still-bound therapeutic antibodies and competition with the diagnostic antibody. This might be the

reason why accurate PD-1 quantification in such samples has been problematic. Saturation of the patient's T cells with the therapeutic PD-1 antibody followed by secondary detection of the latter was necessary to allow accurate quantification of PD-1 on the cell surface (29).

PERIPHERAL-VS.-TISSUE BIOMARKERS FOR BREAST CANCER

To investigate whether other solid cancers behave similarly to melanoma in terms of the prognostic and predictive value of peripheral immune biomarkers, we elected to study breast cancer. We had already shown many years ago that Her2/neu peptides 776–788 and 884–899 were naturally-processed and presented TAA (30, 31). Due to our interest in the impact of age and immunosenescence on cancer immunity, we elected to study newly-diagnosed older women and found that the ability of patient's PBMCs to respond to TAA *in vitro*, in this case to her2/neu peptides, was also informative for breast cancer (32). Results paralleled findings in melanoma, demonstrating that prognostic impact depended on the pro- anti-inflammatory cytokine balance in the responding T cells (33). Moreover, the main markers in peripheral blood, namely, levels of mMDSCs, were also important indicators of survival in breast cancer as well as melanoma, and a combination of mMDSC levels and her2-reactivity even more so (32), as was the level of circulating plasmacytoid dendritic cells (34). It may be clinically important to note that cell surface marker immune phenotyping in older breast cancer patients identified correlations between baseline immune profile and geriatric assessment (35). Thus, frailer patients had higher levels of granulocytic cells but lower levels of cells with suppressor phenotypes including mMDSCs and Tregs, with none of these immune populations correlating with chronological age, but rather with frailty itself. The implications of these findings remain to be clarified, but clearly suggest that immune signatures correlating with clinical outcome depend on the physical state of the patient and can (in the case of elderly patients) be partly identified by geriatric frailty assessments (36). Whether the same is true for tumor-infiltrating immune cells in breast cancer is not yet established, but differential densities of CD8+ and CD163+ cells in the tumor core and margins were found to have significant prognostic value for survival (allowing better patient stratification than TNM staging, tumor size, lymph node invasion or histological grade). Patients having favorable immune signatures had favorable clinical outcomes despite poor clinicopathological parameters (37). These findings parallel many others in different cancers (38, 39). Of note in the light of our studies discussed above, low levels of intra-tumoral T cells and more granulocytic cells were present in clinically frail patients with shorter disease-specific survival (36). Together, these results are consistent with the notion that peripheral biomarkers are informative for clinically-relevant outcomes also in breast cancer, and may at least partially reflect what is seen in the tumor itself.

PERIPHERAL-VS.-TISSUE BIOMARKERS FOR RENAL CANCER

In renal cell carcinoma (RCC), expression of both PD-1 and PD-L1 within the primary tumor is associated with bad prognosis (40–42). In a recent study, we assessed the expression of five inhibitory receptors on T cells from RCC patients by flow cytometry (43). We found that PD-1, LAG-3 and Tim-3 were the three most upregulated checkpoint receptors on non-Treg CD4+ and CD8+ TILs as compared to autologous peripheral T cells, whereas PD-1, CTLA-4 and LAG-3 were dominant on tumor-associated Tregs. At the single cell level, PD-1 and LAG-3 were also the most often co-expressed receptors on CD4+ and CD8+ TILs. Still, there was a noticeable variability in the expression of the receptors between individuals, especially for LAG-3. Two main groups of tumors were identified. The first group (approximately half of the tumors, generally at more advanced T stages) was characterized by a high fraction of LAG-3+ T lymphocytes as well as other tumor-associated immune cells. A second group was constituted by tumors with rare expression of LAG-3 on all immune cell types. Our data are well in line with the results obtained by Giraldo et al., who showed that high densities of PD-1+ cells, and also of LAG-3+ cells, were associated with poorer prognosis in primary and metastatic RCC (40). PD-1 was slightly upregulated in peripheral T cells from RCC patients as compared to PBMCs from healthy donors, but for most other checkpoints, expression was only significantly increased in TILs, indicating that tumor-associated T cells, but not blood T cells, are more appropriate for checkpoint expression assessments.

In short-term functional experiments using RCC TILs activated with CD3 antibody in the presence of checkpoint-specific monoclonal antibodies, we found that simultaneous blocking of PD-1 and LAG-3 was more efficient in facilitating IFN-γ production than blocking of PD-1 alone or in combination with Tim-3. Here again, variability was observed between tumors. The frequency of IFN-γ producing CD8+ cells was increased ∼2-fold for some patients, whereas it was nearly unchanged for others. This suggests that further parameters, possibly patient-specific, may be responsible for T cell unresponsiveness. Obviously, assessment of TIL functionality is technically challenging, and the development of simpler *in vitro* models could significantly improve testing. If successful, a following essential step would be to establish whether *in vitro* testing can readily predict clinical response to checkpoint blockade (43).

Whether checkpoint receptors (and their ligands) are expressed as similar levels in various tumors needs to be systematically addressed in middle to large scale patient cohorts. As an example, Li et al., recently showed that PD-1 is upregulated at comparable levels in TILs vs. PBMCs of eight different tumor types, including RCC (44). In contrast, Tim-3 expression was clearly lower in TILs from breast carcinoma, as compared to e.g., RCC or cervical cancer. Co-expression analysis of five inhibitory receptors also showed that some dominant combinations were

observed on CD8+ T cells in most tumor types, whereas secondary patterns appear more tumor specific.

Note that the tumor digestion procedure in particular when enzymatic digestion is performed (45) but also the antibody clones and fluorochromes used [our unpublished observations and (26, 29, 46–48)] as well as the staining procedure (extra- or intracellular staining of CTLA-4) and the settings used for *in vitro* functional testing might all influence the analyses. Regarding *in vitro* functional analyses, different groups, including ourselves, have observed that the functional impact of the addition of blocking antibodies against checkpoint molecules is rather modest. Hence, here again, the field would certainly benefit from at least partial standardization of reagents and protocols, especially for flow or mass spectrometry multiparametric single cell studies, so that results obtained across various studies are more easily comparable.

CONCLUSIONS AND PERSPECTIVES

Although much effort is rightly being poured into analyzing the tumor microenvironment in order to understand the biology of cancer cell-host cell interactions, the routine application of such analyses for practical purposes is limited. While resected or biopsied tissue may also be useful for establishing baseline predictive biomarkers of response to therapy, monitoring of patient status at follow-up is challenging unless liquid biopsies can be employed. Using a minimally-invasive approach that can be repeated at will offers great advantages for immunomonitoring that may enable early detection of treatment response (or side effects) and enable therapies to be modified to replace ineffective treatments with others that might be more successful or tolerable. Combining immune biomarkers with routine clinical laboratory measures, as we have accomplished thus far and reviewed here, is merely an unsophisticated start to this effort, but possesses the advantage of feasibility for many groups in the field. Future work will be able to focus more closely on both tumor-derived and host-derived factors as determined in liquid biopsies. The former include circulating tumor cells (49), cell-free tumor DNA (50), exosomes containing tumor antigens (51), and soluble factors produced by the tumor; the latter include tumor antigen-specific T and B cells, innate immune cells and regulatory elements. Compound constellations of such markers will allow us to refine the clusters of parameters that we are beginning to find informative for monitoring cancer patients on immunotherapy (15, 21). Ideally, a blood-based "doctor's office" test would facilitate more rapid, safer and cheaper immune monitoring for therapy selection and modification.

AUTHOR CONTRIBUTIONS

KW-H, CG, and GP contributed jointly to conception and design of the study. GP wrote the first draft of the manuscript. KW-H and BW contributed the sections about the checkpoint blockade era in melanoma. GP wrote the sections discussing data from the pre-checkpoint era in melanoma and the section about breast cancer. CG contributed the discussion of renal cancer data. All authors contributed to manuscript revision, read, and approved the submitted version.

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REVIEW
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From Cancer to Immune-Mediated Diseases and Tolerance Induction: Lessons Learned From Immune Oncology and Classical Anti-cancer Treatment

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Success in cancer treatment over the last four decades has ranged from improvements in classical drug therapy to immune oncology. Anti-cancer drugs have also often proven beneficial for the treatment of inflammatory and autoimmune diseases. In this review, we report on challenging examples that bridge between treatment of cancer and immune-mediated diseases, addressing mechanisms and experimental models as well as clinical investigations. Patient-derived tumor xenograft (PDX) (humanized) mouse models represent useful tools for preclinical evaluation of new therapies and biomarker identification. However, new developments using human *ex vivo* approaches modeling cancer, for example in microfluidic human organs-on-chips, promise to identify key molecular, cellular and immunological features of human cancer progression in a fully human setting. Classical drugs which bridge the gap, for instance, include cytotoxic drugs, proteasome inhibitors, PI3K/mTOR inhibitors and metabolic inhibitors. Biologicals developed for cancer therapy have also shown efficacy in the treatment of autoimmune diseases. In immune oncology, redirected chimeric antigen receptor (CAR) T cells have achieved spectacular remissions in refractory B cell leukemia and lymphoma and are currently under development for tolerance induction using cell-based therapies such as CAR Tregs or NK cells. Finally, a brief outline will be given of the lessons learned from bridging cancer and autoimmune diseases as well as tolerance induction.

Keywords: immunotherapy, immune tolerance, checkpoint inhibitors, chimeric antigen receptors (CARs), autoimmune disease

INTRODUCTION

In view of the high complexity of the immune system, it is hardly surprising that therapeutic intervention for a disease involving immune dysfunction may result in changes in immune responses that prove beneficial for other immune-mediated diseases. Over the last four decades, this has been the case with therapeutic agents developed for use in cancer—both the early non-selective

agents and the recent highly specific biologicals—which are increasingly being found to exert benefit in autoimmune and auto-inflammatory diseases. The resulting commonalities have led to development of new models and approaches to biological therapies covering the whole spectrum of immune responses.

CLASSICAL ANTI-CANCER DRUGS

From Anticancer to Autoimmune Disease Therapy

Cytotoxic immunosuppressive drugs go back to the 1950s when cyclophosphamide, an alkylating agent reacting with purine bases to form double-strand adducts which cross-link DNA to trigger apoptosis, was introduced for the therapy of solid and hematological malignancies. Because of its immunosuppressive activities, cyclophosphamide has subsequently been used for the treatment of systemic lupus erythematosus, vasculitis and other autoimmune diseases, but its non-specific cytotoxicity severely restricts its clinical use, a common limitation for the broader use of many anti-cancer agents (1).

Around the same time, several antimetabolites were developed for use in cancers, the agent subsequently used to the greatest extent being methotrexate (**Table 1**). This drug is a folate analog which inhibits the enzymes dihydrofolate reductase and thymidylate synthase, thereby depleting tumor cells of the purine and pyrimidine precursors required for DNA and RNA synthesis (23). The subsequent history of methotrexate use, well illustrates the courses of a number of drugs introduced for cancer therapy which have found applications in other diseases. It was initially found to be of use in psoriatic arthritis and in this autoimmune disorder continued to exert therapeutic efficacy at doses considerably lower than those required in cancer. Michael Weinblatt overcame the widespread reservation about using an anti-cancer drug for autoimmunity and performed randomized controlled trials with methotrexate in rheumatoid arthritis (RA) (24). The drug has long since become the standard of treatment for rheumatoid arthritis, but at the relatively low doses used, its mechanism of action is thought to be due to enhanced conversion of AMP to extracellular adenosine, an endogenous anti-inflammatory substance which reduces macrophage cytokine release. Recently, though, it has been shown to inhibit JAK1/2 kinases, which are involved in inflammatory cell signaling (25).

Rituximab

Rituximab (**Table 1**) was one of the first therapeutic monoclonal antibodies to be introduced to the clinic in the 1990s. Directed toward CD20 on the surface of B cells, its selective efficacy at four weekly doses of 375 mg/m^2 in non-Hodgkin lymphoma (NHL) of B cell origin is based on the fact that CD20 is expressed on both healthy and NHL B-cells, but not on immature or developing B cells (26). With a long half-life, rituximab can be found in plasma and bound to circulating B cells for up to 6 months, making it useful for treatment of chronic diseases (27). Thus, from the outset of its development, despite the fact that significant decreases in circulating immunoglobulins were not observed in

TABLE 1 | Overview of drugs used for oncological and immunological indications.

Target	Drugs	Oncological use		Immunological use	
		Indications	**Potential mechanism**	**Indications**	**Potential mechanism**
Dihydrofolate reductase/ thymidylate synthase	Methotrexate	Breast cancer, leukemia, lung cancer, lymphoma, osteosarcoma (2)	Antimetabolite, depletes tumors of precursors for RNA/DNA synthesis (2)	Psoriasis, rheumatoid arthritis (3)	Conversion of AMP to extracellular adenosine; JAK1/2 kinase inhibition (3)
CD20	Rituximab, Ocrelizumab	B-cell non-Hodgkin's lymphoma, B-cell chronic lymphocytic leukemia (Rituximab) (4)	B cell depletion by induction of apoptosis, antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) (5)	Multiple sclerosis (Ocrelizumab) (6), severe refractory systemic lupus (Rituximab) (7), ANCA-associated vasculitis (Rituximab) (7), RA (Rituximab) (7)	B cell depletion by induction of apoptosis, antibody dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC) (8)
Proteasome	Bortezomib	Multiple myeloma (9)	Induction of apoptosis and inhibition of tumor cells, reduction of cytokine and VEGF production (10)	Potential use for myasthenia gravis, severe SLE (11, 12)	Induction of apoptosis of plasma cells, reduction of cytokine production (13)
PI3K/mTOR	Everolimus, Sirolimus, Temsirolimus	Advanced renal cell carcinoma (14), gastroeneropancreatic neuroendocrine tumor (15), subependymal giant cell astrocytoma (16), breast cancer (17)	Reduction of cell growth and proliferation by inhibition of mTOR pathway (18)	Renal transplantation to prevent organ rejection (19)	Suppression of T cell proliferation by inhibition of mTOR pathway (20)
IDH	Enasidenib	Acute myeloid leukemia (21)	Inhibition of 2HG synthesis (22)	Not identified yet	

lymphoma studies, there was considerable interest in studying rituximab for B cell depletion in autoimmune diseases in which generation of autoantibodies is a major pathological issue. Initial studies were carried out in IgM-associated polyneuropathies associated with a lymphoblastic B cell clone in the bone marrow which has a low proliferation rate and is not susceptible to conventional immunosuppressive but expresses CD20 (28). Intriguingly, in multi-morbid patients with lymphoproliferative diseases, beneficial effects of rituximab were also observed on autoantibody-related autoimmune manifestations. Following the discovery by Edwards and Cambridge in 1998 that auto-reactive B cell clones are promoted by macrophage activation and inflammation, clinical trials were initiated in RA (29). Rituximab in combination with methotrexate was licensed for use in RA in 2006 at 2 × 1,000 mg separated by 2 weeks. It has subsequently been licensed for ANCA-associated vasculitis and severe refractory systemic lupus (SLE). B cells and the generation of autoantibodies are also major players in the development of multiple sclerosis (30, 31). Consequently, rituximab also showed efficacy in the treatment of multiple sclerosis, leading to the development of ocrelizumab (**Table 1**) a humanized antibody directed toward CD20 that was approved for the treatment of multiple sclerosis patients (6, 32).

The realization that rituximab has clear efficacy in various inflammatory and autoimmune diseases, sparked off a search for other drugs which could selectively modulate B cell function. Notable among these is belimumab, which binds to B cell activating factor (BAFF) or B-lymphocyte stimulator (BLyS). This mediator is required for the normal development and survival of B cells. In SLE and also multiple sclerosis patients, however, BAFF is overexpressed, contributing to autoimmune B cell proliferation (33). Binding of belimumab to BAFF prevents it from binding to autoimmune B cells, resulting in B cell apoptosis (34). Belimumab was introduced for the therapy of SLE in 2011, the first new drug specifically approved for this indication in 56 years. A variety of follow-up drugs are under development (35).

Bortezomib and Proteasome Inhibitors

Bortezomib (**Table 1**), a dipeptide boronate, is a selective inhibitor of the 20S proteasome, a subunit of the 26S proteasome, which degrades intracellular proteins labeled by linear ubiquitination for subsequent hydrolysis of the peptides generated (36). Its development arose out of research led by Alfred L. Goldberg into the role of protein breakdown in the muscle wasting or cachexia seen in many systemic diseases such as cancer, sepsis and renal failure. The discovery of the role of the proteasome in the activation of the key transcription factor, NFκB, diverted the research toward development of anti-inflammatory, anti-neoplastic compounds (36). Inhibition of NFκB prevents apoptosis in tumor cells with a high protein turnover, causes ER stress and as a result of proteasome inhibition, misfolded proteins accumulate intracellularly (37). Based on these effects, bortezomib was approved for the treatment of multiple myeloma in 2003. Several other proteasome inhibitors are also under development for oncological indications (38).

Inhibition of intracellular protein degradation also modifies antigen presentation and the generation of antibodies, including autoantibodies through inhibition of the immunoproteasome, a specialized form of proteasome, mainly expressed in lymphocytes and monocytes. Consequently, antibody-producing plasma cells, which also have high protein turnover, are sensitive to inhibition by bortezomib and experimental studies suggest its potential use in the treatment of the autoimmune diseases, myasthenia gravis (MG) and severe SLE (11). A number of cases have been reported in which bortezomib was tested clinically. Currently a prospective, non-randomized clinical trial is in progress in which bortezomib is being tested in MG, SLE and RA patients refractory to current therapeutic regimes (12). Unfortunately, cells adapt to survival in the presence of proteasome inhibitors and other approaches are being taken to inhibit different types of proteasome complexes found within cells (38). One such approach involves inhibitors of the E3 ligases involved in ubiquitin activation and one, pevonedistat (MLN4924) has already entered clinical trials for acute myeloid leukemia (39). Many research groups are developing PROTACs (Proteolysis Targeting Chimeric Molecules), bispecific molecules which both act as ligands for E3 ligase and bind to the target protein to be tagged with linear ubiquitin for degradation by the proteasome (40). This would be of benefit both for tumor-targeted therapy and potentially for the inhibition of autoantibody production.

PI3K/mTOR Inhibitors

Mammalian target of rapamycin (mTOR), the downstream effector of phosphatidylinositol-3-phosphate kinase (PI3K), is a component of the epidermal growth factor receptor (EGFR) signaling pathway induced by natural ligands such as EGF, leading to cell growth and proliferation. The mTOR-AKT-PI3K pathway is dysregulated in many cancers (41). Everolimus, sirolimus (rapamycin) and temsirolimus (**Table 1**) inhibit mTOR and thereby cell proliferation. In this context, everolimus and temsirolimus showed efficacy in the treatment of advanced renal cell carcinoma (RCC) (14). Everolimus is also approved for the treatment of gastroenteropancreatic neuroendocrine tumor (15), subependymal giant cell astrocytoma (16) and breast cancer (17). Everolimus and sirolimus are further approved for prevention of organ rejection after renal transplantation, since inhibition of the mTOR pathway suppresses T cell proliferation. However, mTOR inhibition also increases the capacity of phagocytic cells to release cytokines such as IL-12 leading to the priming of pro-inflammatory TH1 and TH17 responses (20). Thus, the inflammatory side effects that can occur in transplant recipients treated with rapamycin are possibly due to this interaction with cytokine release by phagocytic cells. Another severe adverse outcome of transplantation is malignancy, a major cause of post-transplant mortality. Since mTOR inhibitors exert various anti-proliferative effects, transplant patients suffering from such malignancies can benefit from both the immunosuppressive and the anti-carcinogenic potential of mTOR inhibitors. In keeping with this, a lower rate of *de novo* malignancy under mTOR inhibition after solid organ transplantation has been observed (42, 43). Everolimus is also effective in therapy-resistant autoimmune hepatitis (44) and given in combination with

methotrexate, it provides clinical benefit in RA (45), but is not approved for these indications.

Metabolic Inhibitors

The incentive to develop effective, more potent and less toxic drugs stimulated the search to identify pathways that are critical for the survival of, or even exclusive use by cancer cells. In this respect, isocitrate dehydrogenase (IDH) enzymes were identified since they normally metabolize isocitrate to α-ketoglutarate. In a mutated state—as found in AML patients and in low-grade gliomas—IDH also converts α-ketoglutarate into the oncometabolite 2-hydroxyglutarate (2HG) that causes cell differentiation defects by impairing histone demethylation (22). Enasidenib (**Table 1**), a first-in-class inhibitor of mutated IDH2, was approved for the treatment of acute myeloid leukemia (AML) (21). In addition, immunometabolism-modulating drugs that can improve immune cell survival or modify the interactions between cancer cells and immune cells have become a focus of investigation. Epacadostat, an indoleamine 2, 3-dioxygenase 1 (IDO1) inhibitor, controls tryptophan metabolism to foster immune cell activity. However, epacadostat in combination with pembrolizumab failed to provide superior outcome in melanoma when compared to pembrolizumab alone (46). In contrast to the other drugs discussed in this review, the use of these metabolism-modifying anti-tumor agents for autoimmune diseases is in its infancy. It is questionable whether IDH inhibitors are suitable for the treatment of autoimmune diseases since metabolic inhibition could lead to a decrease in immune cell activity, although metabolic interactions can significantly modify the inflammatory status of immune cells. Pro-inflammatory immune cells such as macrophages, for instance, are characterized by upregulated glycolysis, impairment of oxidative phosphorylation, and disruption of the Krebs cycle at two steps, after citrate and succinate formation (47). Citrate is used in fatty acid biosynthesis, which permits the increased synthesis of inflammatory prostaglandins. Succinate activates the transcription factor HIF-1α, a regulator of a wide range of genes, including IL-1β, CCL2, and CXCL8 (48–50). The inhibition of IDH could lead to an increase in citrate, potentially accompanied by an increase in inflammatory prostaglandins and to a decrease in succinate. This is potentially linked to a reduced synthesis of pro-inflammatory cytokines and to inhibition of glycolysis, possibly accompanied by a shift in immune cells toward a more anti-inflammatory status. However, further studies are needed to investigate whether metabolic inhibitors are suitable for the treatment of autoimmune diseases.

Lessons Learned

The development of cytostatic anti-tumor agents for use in autoimmune diseases such as psoriasis and RA emphasizes the importance of careful dissection of the (broader) mechanisms of action of drugs which modulate immune responses, particularly those mechanisms that are not immediately relevant to the targeted oncological indication. These include intracellular signaling processes, but also cell growth, metabolic and cell surface binding interactions. This is not only crucial for an understanding of the breadth of pharmacological activity of these agents, but for their potential repurposing for other important immune disorders and also for potential immunotoxicity. Thus, to translate cytotoxic, biological and cellular agents from oncology to autoimmune applications, clarification of their mechanisms can lead to dosing improvements, novel targets and unexpected uses (**Figure 1**). In the following, some examples are provided.

Rituximab is a prime example of increased understanding of both the mechanism of action on B-cells and their role in different autoimmune diseases opening up totally new markets for the drug and for a whole new class of B cell inhibiting drugs, including belimumab. This class is likely to be extended with proteasome inhibitors, such as bortezomib, which are effective in myeloplasias and appear to bear promise for treatment of diseases in which autoantibody generation is high. Undoubtedly, with the widespread efforts to identify novel immune-oncological drugs and new targets for modulation of immune-mediated diseases, there will be an increased dove-tailing of research programs to identify targets, such as the well-characterized PI3K/mTOR inhibitors, which find parallel therapeutic applications for both cancer and inflammatory and autoimmune disorders.

The broad ramifications for immune-mediated disease therapy of drugs developed as immunotherapies for cancer are well illustrated by immune checkpoint inhibitors, such as those acting at PD-1. These have been extensively discussed in a recent review (51). Shown to be active in a variety of cancers, including melanoma, metastatic lung cancer, kidney cancer and Hodgkin's lymphoma, agents targeting PD-1 or PD-L1 have also been found effective for lupus, psoriasis and inflammatory bowel disease, as well as being investigated for potential use in chronic infection and sepsis (51, 52). PD-1-related immune checkpoint inhibitors also illustrate the complications that arise with the pharmacological modification of immune homeostasis, such as skin, renal and hepatic toxicities.

The development of anti-cancer drugs for immune-mediated diseases thus, highlights the relevance of altering the dosing regimen to reduce potential anti-tumor-related toxicity, but retaining therapeutic effects in inflammatory or autoimmune conditions. In translating immunotherapeutic agents from cancer therapy to treatment of chronic inflammation and autoimmunity, toxicities are inevitably less acceptable. Understanding mechanism of action (MoA) of methotrexate at lower doses led to substantial reduction in toxicity while applying this drug.

mTOR is a good example of a target which has experienced "indication-hopping," having been developed for immunosuppression and immunomodulation and then as an anti-cancer and inhibitor of cellular senescence. A recent report indicates that doses of everolimus can also be readjusted, depending on the indication (cancer or transplant rejection), to reduce unnecessary toxicity (53). The further demonstration that everolimus, like rapamycin, can slow immunosenescence in the elderly suggests that a downward readjustment of the dose may result in a well-tolerated dosing regimen in chronic immune-mediated disorders in the elderly (54).

Another illustration of an agent developed at a high dose for cancer treatment which was subsequently pursued at a low

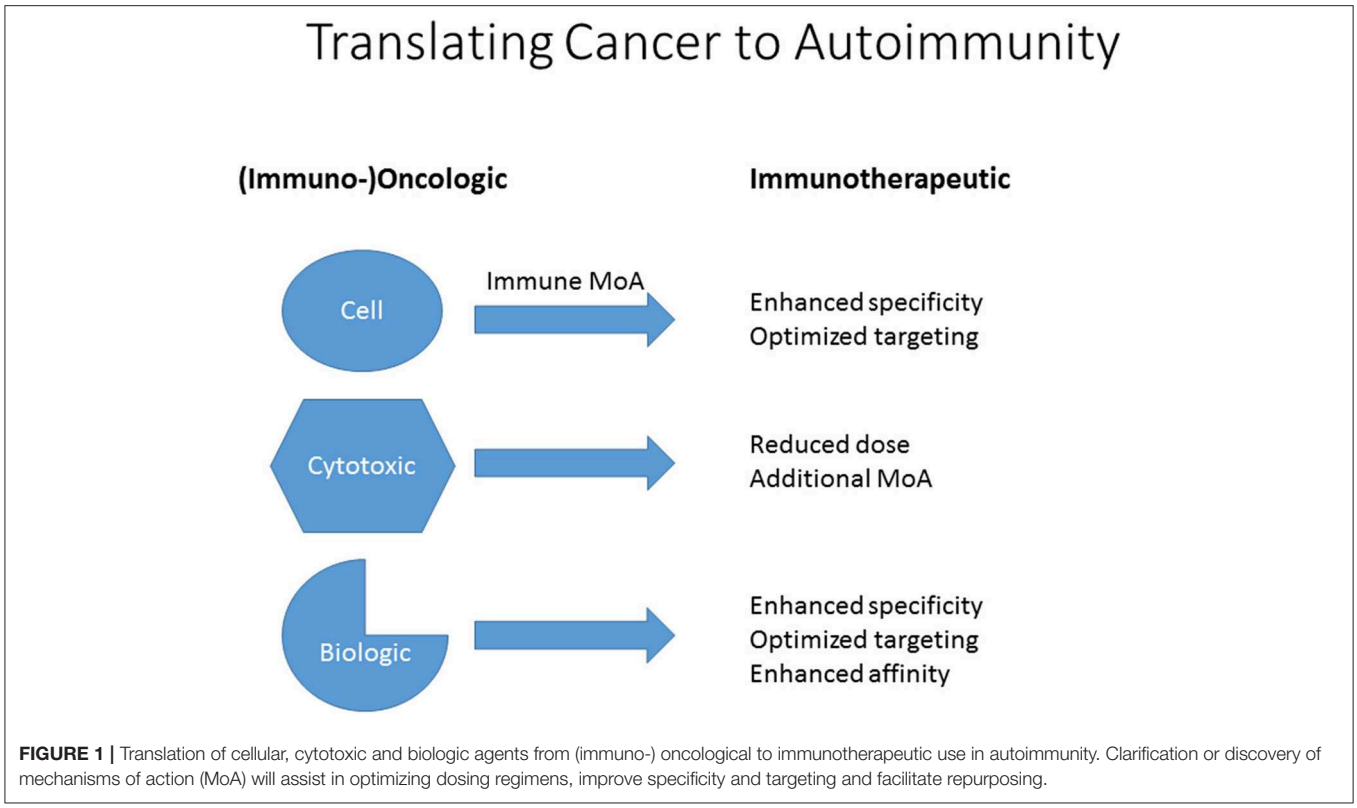

FIGURE 1 | Translation of cellular, cytotoxic and biologic agents from (immuno-) oncological to immunotherapeutic use in autoimmunity. Clarification or discovery of mechanisms of action (MoA) will assist in optimizing dosing regimens, improve specificity and targeting and facilitate repurposing.

dose for immune-mediated diseases is the cytokine interleukin-2 (IL-2). Recombinant IL-2 was first developed as a stimulant of T cell immunity by administration at a high dose with autologous lymphokine activated killer cells for the treatment of metastatic melanoma and kidney cancer. Subsequently, it was found that Treg cells express the IL-2 receptor CD25 constitutively, and that IL-2 is more critical for the development and survival of Tregs than for effector T cell function (55). This discovery has given a pronounced incentive to the development of drugs acting at CD25 on Tregs for the treatment of immune-mediated diseases. In the future, we should expect to see drug companies seeking parallel development of immunotherapies for various indications instead of the classical development for a primary followed by a secondary indication. "There is clearly a strong rationale for further expanding the opportunities for cross-fertilization of ideas and approaches between cancer immunology and autoimmunity, so that further synergies between the two fields can accelerate the development of effective immunotherapies"(55).

CELL-BASED THERAPIES

From CAR T Cells in Immune Oncology to CAR Tregs for Tolerance Induction in Immune Mediated Disease

CAR T Cells

Chimeric antigen receptor (CAR) modified T cells are a novel class of anti-cancer therapy for target-specific recognition and destruction of cancer cells. An extracellular single-chain variable fragment (scFv) antibody is used to bind to the respective cancer target combined with an intracellular CD3zeta chain to activate T cells (56). Linking to a second co-stimulatory domain results in lasting T cell response and prolonged cell survival. The first five generations of CARs have been reviewed (57, 58).

Adaptive immunotherapies using these CAR T cells have achieved spectacular remissions in refractory B cell leukemia and lymphoma. So far, frequent, durable and objective regression in pediatric B cell acute lymphoblastic leukemia (B-ALL), chronic lymphocytic leukemia (CLL), and B cell lymphoma have been reported using anti-CD19 CARs (59–62). In 2017 and 2018, two CD19 CAR T cell products (Tisagenlecleucel (Kymriah®), Axicabtagene ciloleucel (Yescarta®) received marketing approval in USA and Europe, respectively. Treatment cost is >275.000€/product and patient. Clinical trials with CAR T cells in several malignant diseases now constitute a fast-growing field with >1,000 clinical trials registered with clinicaltrials.gov, most of them undertaken in the United States and China and around 10% in Europe. While most of the clinical trials still address hematological malignancies, the number of trials in oncology is increasing continuously (63–65). Management of the severe side effects, such as cytokine release syndrome or neurotoxicity, which appear in 2/3–3/4 of the patients, has been established and reviewed (66).

For the increasing numbers of patients, the reproducible manufacture of high-quality clinical-grade CAR T cell products is becoming a growing challenge, moving from manual to a more automated process (67–70). In Europe, CAR T cell

manufacturing is regulated by the Tissue and Cell Directives published in 2004 (2004/23/EC) and 2006 (2006/17/EC; 2006/86/EC), respectively. Beside autologous CAR T cells for individualized medicine, initial studies have been performed using allogeneic "off the shelf" CAR T cells from healthy donors (71).

CAR Tregs

Tolerance induction is a major goal in cell-based immunotherapy. Gene-modification of CAR Tregs has provided significant advantages with clinical applications in organ transplantation and cell therapies. Early clinical studies recently demonstrated the tolerability, safety, broad spectrum of effects and feasibility of Treg-based cell therapies for excessive immune reactions, such as GvHD, or autoimmune diseases, tissue protection and to prevent progression of inflammatory disorders (72). In particular, new technologies for the production of CAR Tregs with selective potential against aggressive effector cells, reflected by an excessive T cell response and autoimmune reaction, can be attenuated by specific CAR Treg cell activity (72–74). Initially, CARs were used in 2 subgroups of CD28-CD3ζ CAR-modified Tregs, which were redirected against the carcinoembryonic antigen (CEA). This surface target is often overexpressed on human lungs as well as in the intestine, in colon cancer and colonic inflammation (ulcerative colitis) (75–77). Other studies revealed that human CD19-engineered CAR Tregs were able to suppress the cytotoxicity and proliferation of CD19 CAR T effector cells *in vitro*. Mouse tumor (CD19+) experiments demonstrated clearly that tumor-infiltrated CD19-modified CAR Tregs inhibited CD19 CAR T cell-dependent tumor elimination at a ratio of 1 (CAR-Tregs) to 16 (anti-CD19 CAR T effectors) (78). Recently, systematic testing of humanized HLA-A2 CARs revealed their ability to interact with HLA-A*02:01 and to trigger human Treg-mediated suppression *in vitro*. Moreover, transplantation of human HLA-A2-CAR Tregs inhibited HLA-A2-positive effector cell-associated xenogeneic GvHD and decreased rejection of human HLA-A2-positive skin allografts (72, 79). These results suggest the use of humanized alloantigen-specific CARs to engineer retargeting and specificity of clinically applicable Tregs.

Role of NK Cells in Cancer and Autoimmune Disease

Human natural killer (NK) cells (∼10% of PB lymphocytes) are an important subpopulation of innate lymphoid cells (ILCs), which play an essential role in innate defense against virally infected and cancer cells (80–82). Their activation is controlled by a highly sensitive balance between natural cytotoxicity receptors (NCRs) and killer cell immunoglobulin-like receptors (KIRs) responsible for recognition of "non-self" transformed cells without major histocompatibility complex (MHC) or specific antibodies (80, 83, 84). Broad cytotoxic mechanisms and rapid stimulation of immune reactions make this lymphoid cell type suitable as a candidate for use in cancer immunotherapy. In the last decade, a strong focus has been laid on the establishment and validation of chimeric antigen receptor (CAR)-modified effector cells to treat refractory cancer patients but mainly using autologous T cells as a source of potent effector cells. Unlike T cells, NK cells lack the potential to generate graft-vs.-host disease (GvHD) and the absence of this adverse response makes NK cells an ideal alternative to CAR-modified T cells (81). This potentially improved safety of engineered CAR NK cells for cancer immunotherapies, in comparison to CAR T cell therapies, could stimulate broad research and development in the field of cancer immunity (81, 85). CAR-modified NK cells thus represent a potential source of combined gene- and cell therapies, offering potential allogeneic "off-the-shelf" cellular therapy mediating severe anti-leukemic and anti-tumor effects without triggering potentially lethal alloreactivity such as GVHD.

In addition to their ability to fight cancer cells in a targeted and effective manner, NK cells also seem to have immunomodulating, protective properties. Accordingly, allogeneic NK were advantageous in patients with mismatched hematopoietic transplants by dint of their strong graft-vs.-leukemia (GvL) effects and amelioration of leukemia relapses, but also by protection of these patients against GvHD and graft rejection (80, 86, 87). NK cell-dependent immunotherapies largely prevented transplant rejection by sustaining the hematopoietic transplant and exerting a GvL response (80, 88).

The important function of NK cells in autoimmune disease remains to be fully clarified (83, 89). Past studies have provided multiple indications that certain subgroups of NK cells probably exercise a protective mechanism to counteract autoimmune diseases. In this context, distinct NK cell subsets were repeatedly reported to result in a clear attenuation of the overall Th1 response in autoimmune diseases by releasing Th2 cytokines (89). Moreover, NK cells are able to down-regulate the CD4 and CD8 T cell response during chronic viral infections by binding, in particular, of TNF-related apoptosis-inducing ligand (TRAIL) or by secretion of high perforin levels to induce T cell apoptosis (90–92).

A protective effect of NK cells could also be demonstrated in patients with multiple sclerosis (MS) (93, 94), high surface expression levels of CD95 (Fas) being detected on NK cells derived from patients in disease remission which were classified as "NK2" cells. These NK cells secreted high amounts of interleukin-5 (IL-5) and IL-13 (94, 95). Interestingly, NK cells isolated from patients with MS exhibited lower proliferation capacity and restricted effector cell functions (96). One hypothesis suggested that activated NK cell subsets are mainly responsible for decreased production rates of interferon-gamma (IFNg) in resident effector/memory T cells. Accordingly, *ex-vivo* experiments with NK cell-depleted PBMNC showed enhanced IFNg levels after stimulation of T cells which underlines the regulatory role of NK cells in MS (94).

In experimental murine autoimmune encephalomyelitis (EAE), CNS inflammation was abolished and spinal cord and brain damage attenuated by transferring acetylcholine-producing NK cells into the cerebral ventricles which suppressed infiltrating/resistant macrophages and monocytes (97, 98). In contrast, increased inflammation levels were detected after depletion *in vivo* of these NK cells. Experiments *in vitro* showed increased CD4 T cell frequencies followed by enhanced Th1 cytokine secretion as a result of NK cell depletion (98).

Recent studies have shown that the adoptive transfer of CXCR5-negative NK cell subsets improves autoimmune myasthenia gravis (EAMG) symptoms by down-regulation of splenic follicular helper T (Tfh) cells and germinal center B cells, inducing apoptosis of T cells but not of B cells. CXCR5-negative NK cells were found mainly outside the B cell zone, whereas CXCR5-positive NK were localized within the B cell zone and secreted higher IL-17 levels. These data suggest that a distinct (CXCR5-negative) NK cell subset is responsible for inhibition of the autoimmune response in EAMG models (99).

Despite these encouraging results from scientific studies, no data are available from controlled prospective studies. There is still no clear explanation of the role of NK cells in autoimmunity, and further studies are necessary to characterize distinct NK subsets, how they exacerbate inflammatory reactions and which key NK players protect against the progression of excessive inflammation.

Mesenchymal Stem Cells in the Treatment of Autoimmune Diseases

Mesenchymal stem cells (MSC) are a heterogeneous group of multipotent, non-hematopoietic, self-renewable progenitor cells of different cell types which can differentiate into adipocytes, chondrocytes, osteoblasts and myocytes (100–102). Because this type of stem cell has potent immunosuppressive effects on both the innate and acquired immune system, MSCs have been used therapeutically in the last two decades for their immunomodulatory effects and their seemingly low toxicity and side effects in various autoimmune diseases. During this period, thousands of patients were treated with autologous and even allogeneic MSCs for the targeted treatment of various diseases and a large number of clinical studies (see clinicaltrials.gov) have tested the effectiveness and feasibility of MSC-based therapies under clinical conditions, including GvHD, Crohn's disease, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), MS, Type 1 diabetes mellitus (T1DM), organ (kidney) transplantation, cardiovascular diseases, neurological diseases, hematological malignancies and autoimmune diseases (101, 103–105).

Despite advances in the research and development of novel treatments and biological agents, successful treatment of autoimmune diseases remains unattainable. Recently, both the therapeutic benefit of MSCs and their capacity to counteract autoimmune disease progression was reported (106, 107). The immune-modulating effects of MSCs on other lymphoid and myeloid cell types is mediated by the multiple release of mediators, including transforming growth factor beta (TGF-β), prostaglandin E_2 (PGE_2), nitric oxide (NO), soluble HLA-G or indoleamine 2, 3-dioxygenase (IDO). Such effects also occur in the presence of increased plasma levels of tumor necrosis factor alpha (TNFa), toll-like receptor 3 (TLR3) agonists and IFNg (107, 108). As a result CD4+CD25+CD127– and CD4+CD25+Foxp3+ regulatory T cell (Tregs) subsets are stimulated, resulting in enhanced immunosuppression of cytotoxic CD8+ T cells and $CD56^{dim}$CD16+ NK cells (107–110).

A well-studied example of efficacy in the treatment of autoimmune diseases in patients is in systemic lupus erythematosus (SLE), a chronic autoimmune disease with clinical manifestations in all organs of the body, associated with increased morbidity and mortality (111, 112). A clinical study with allogeneic umbilical cord MSCs demonstrated the safety and effectiveness of MSC therapy in refractory SLE patients (113–115). Previous studies in refractory and severe SLE patients revealed a tendency toward clinical remission and an amelioration of serological markers for organ dysfunction (100, 112, 116, 117). Interestingly, only allogeneic MSCs from healthy donors, but not from autologous SLE patients, showed immunosuppressive properties in SLE patients while improving symptoms of SLE disease. Moreover, more precise characterization of patient-derived MSCs indicated phenotypical senescence and a number of dysfunctions in immune regulation and proliferation (113). These data were confirmed in another clinical study in which, after allogeneic MSC transplantation from healthy donors, only the proportion of refractory SLE patients showed clinical remission or extenuated disease symptoms. Other SLE patients did not benefit from an autologous MSC transplantation approach (118), which suggests that MSCs derived from SLE patients have several immunosuppressive dysfunctions. At the present time, nine clinical study protocols can be found for MSC-based treatments of SLE patients (www.clinicaltrials.gov).

MSC-containing transplants have also been successfully performed in the treatment of Crohn's disease, a chronic inflammatory reaction of the gastrointestinal tract. Accordingly, improvement in the disease course was achieved in three of eight MSC-transplanted patients with refractory Crohn's disease following autologous bone marrow-derived MSC transplant. However, five of these eight patients showed an ameliorated Crohn's disease activity index score (100, 119). Complete occlusion of the fistula tract with a simultaneous reduction in the activity index for Crohn's disease and healing of the rectal mucous membranes was observed in the majority of the patients within 1 year (100, 120). This could be confirmed in further long-term observations of the same patients (100, 121). To date, almost twenty clinical studies are available on MSC transplantations in Crohn's disease at different stages listed in the online database (www.clinicaltrials.gov).

Due to the wide clinical application of MSC-based immunotherapies in autoimmune diseases, this innovative research field has also been expanded to investigate the primary immunomodulatory effects of MSCs in more detail. Recent studies demonstrated that release of extracellular vesicles, especially of so-called exosomes, represents an important mechanism of action of MSCs which weakens the symptoms of autoimmune diseases (107, 122). These hypoimmunogenic, blood-brain-barrier-crossing vesicular carriers for intercellular communications contain high amounts of immunoregulatory molecules to trigger self-tolerance. Thus, the MSC-derived extracellular vesicles (MSC-EVs) contain mRNAs, microRNAs (miRNAs), cytokines, chemokines and immunomodulatory factors that seemed to down-regulate chronic inflammation or infections (122). Recently, it was demonstrated that

MSC-EV-mediated efficacy was largely equivalent to the immunosuppressive effects seen after the transplantation of MSCs into patients with autoimmune disease. Moreover, MSC-EV-mediated effects were detected in some autoimmune and inflammation mouse models, as in the protection of hepatocytes in acute liver injury and fibrosis, in the treatment of lung inflammatory diseases, in attenuation of neuroinflammatory and inflammatory eye diseases, in the protection of renal tubular epithelial cells and injured cardiomyocytes (122). In summary, MSC-EVs exert immunosuppression and represent a potentially novel therapeutic remedy.

PRECLINICAL IMMUNE COMPETENT MODELS FOR DRUG DEVELOPMENT OF IMMUNOMODULATORY DRUGS

Definition of the Problem

Healthcare is evolving from reactive disease care to care that is predictive, preventive, personalized and participatory. Selecting and developing the optimal drug for each patient requires both profound understanding of cancer molecular biology, as well as well-established immune competent pre-clinical tests. Being able to transfer results from the lab to clinical studies and beyond is crucial Mimicking the immune system of a human being that has usually evolved over decades in its interaction with a unique environment, dealing with multiple provocations like infections, pollutions etc., is extremely challenging. In order to mimic a realistic human immune response and subsequently allow for the development of immunomodulatory strategies for treatment of cancer and autoimmune diseases, several strategies have been proposed. These involve humanized mouse models and immune competent, human-based *ex-vivo* models (123). In this section, we provide a broad overview of patient-derived, immunocompetent preclinical models, their applicability in drug development and personalized medicine, as well as their advantages and disadvantages.

Humanized Mouse Models for Cancer

Patient-derived, tumor xenograft (PDX) (humanized) mouse models represent the classical tools for systemic preclinical evaluation of new therapies and biomarker identification. Within the past decade, cancer chemotherapy has evolved from non-specific drugs that damage both tumor and normal cells, to more specific agents and immunotherapeutic approaches, which have shown greater effectiveness with less toxicity (124). The understanding of the molecular pathogenesis of cancer, particularly understanding of the critical importance of complex interaction of tumor cells with tissue resident cells, has increased remarkably. This has led to a dramatic increase in the number of experimental agents and clinical trials for human cancers. Unfortunately, our preclinical models perform poorly as predictive platforms for the ultimate success of clinical candidates, reflecting the complexity of cancer (125). The new class of immune modulating drugs, like immune checkpoint inhibitors or cellular therapies such as CAR T cells, require the development of predictive, immune competent preclinical models (125, 126).

CAR-engineered T cells have been largely successful in treating hematological malignancies in the clinic. Unfortunately, CAR T cell therapy can cause dangerous side effects, including off-tumor toxicity, cytokine release syndrome, and neurotoxicity. Animal models of CAR T cell therapy often fail to predict such adverse events and frequently overestimate the efficacy of the treatment. Nearly all preclinical CAR T cell studies have been performed in mice, including syngeneic, xenograft, transgenic, and humanized mouse models. Syngeneic or immunocompetent allograft mouse models use CAR T cells, tumors, and target antigens that are all murine derived (127, 128). Many CAR T cell studies are done in human xenograft models, where it is hard to delineate between xenogeneic rejection, allogeneic response of human CAR T cells to the tumor, and the actual CAR T cell therapeutic efficacy in causing tumor regression. Furthermore, the lack of host immune system does not allow testing of the TME, the tumor's metastatic potential, or the host response to CAR T cells. Only a few studies have used xenograft mice to study the effects of Tregs on CAR T cell efficacy, but studies including other immunosuppressive cells are lacking. The syngeneic or immunocompetent allograft mouse models use CAR T cells, tumors, and target antigens that are all murine derived. However, the syngeneic model has its drawbacks, as mouse biology does not always accurately recapitulate human biology. For example, murine immune systems differ from that in humans, and syngeneic models have been largely unable to mimic CRS (128). However, several very successful drug developments have been based on murine cancer models. Humanized mouse models reflecting parts of human immune responses can be used. Patient-derived xenograft (PDX) mouse models (NOD, Prkdc^{scid}, and Il2rγ^{-}) were developed (129) and used for checkpoint inhibitor studies. For example, BALB/c-Rag2nullIl2rγnullSIRPαNOD (BRGS) pups are humanized through transplantation of cord blood (CB)-derived CD34+ cells in order to test anti-PD-1 immunotherapy (130). Recently, the limitations of these models became clearer. The genetically and/or immunological modified laboratory mouse, transplanted with a cultured tumor cell line or primary isolated tumor cells, has been the predominant preclinical model used to assess potential therapeutic efficacies. However, these mouse models often do not adequately reflect tumor progression and the cellular, immunological and genetic heterogeneity found within human cancers. Furthermore, laboratory mice also present with a vastly restricted immune profile compared to humans (131).

To address the failure rate of clinical trials in oncology, preclinical models that accurately predict clinical outcomes are urgently needed. Therefore, the so-called "Avatar" concept for co-clinical trials has emerged. PDX Avatar *in-vivo* models are generated from the tumors of patients enrolled in a clinical trial, and these models are treated simultaneously with the same agents administered to the patients in the clinical trial. Coupled with tumor genomic profiling data, Avatar co-clinical trials are designed to aid in the design of personalized therapeutic regimens in real time (132).

Human *ex vivo* Models for Cancer and Autoimmune Disease Models

The Avatar concept is also applicable to *in vitro* and *ex vivo* models (133). This article focusses on *in vitro* and *ex vivo* patient-derived models with increasing tumor heterogeneity and complexity and describes the application of the models in drug research and development.

In vitro cancer models extend from commercially available cancer cell lines to patient-derived primary disseminated cancer cells, which can be used to generate patient-derived cancer cell lines (PDCL). The most widely used preclinical models are conventional cell lines, such as the NCI-60 standard panel developed in the late 1980s (134). However, the accumulation of genetic aberrations in cancer cell lines with increasing passage number (135) and the lack of tumor heterogeneity highlight the limitations of cell line-based models and pave the way to patient-derived cell models. Patient-derived tumors are dissociated enzymatically and/or mechanically or circulating tumor cells are isolated from blood as a biological correlate of metastasis (136–139). These slow proliferating, dissociated tumor cells exhibit the heterogeneity of the original tumor and are known to be of prognostic relevance (123). Unfortunately, establishment of cell lines from these primary tumor cells is inefficient and often requires cycles of re-implantations as xenografts in mouse models. Still some cell lines from breast cancer, melanoma and small cell lung cancer have been developed and used successfully. Since the tumor is disintegrated during the procedure, the microanatomy of the tumor microenvironment is lost. Spheroid or organoid generation from these primary tumor cells are of significant interest for the evaluation of patient-specific targets and for screening of drugs in early drug development. The growth of patient-derived cells in 3D cultures as spheroids features physiological relevant cell-cell interactions. In particular, the development of 3D tumor co-cultures from cancer cells in combination with fibroblasts, endothelial cells, immune cells, bone cells or adipocytes enables cross-talk between tumor cells and the stromal cells (140). Tumor organoids have been created from different entities, including colorectal, stomach, liver and pancreas cancers. The use of cryopreserved tumor material, organoids and well-defined patient-derived xenografts from biobank materials is advantageous for drug screening (141).

The complexity and spatial aspects of intra-tumor heterogeneity is reflected best in organotypic tumor tissue slices. In comparison to organoids, organotypic tissue slices retain their natural microenvironment, reflecting the situation of a single patient, and could be regarded as an individual Avatar for this patient tumor response. The tissue is not dissociated and hence tumor cells and tumor microenvironment are maintained in an non-manipulated and autologous condition. Various slicing methods have been described, namely manual choppers, the Krumdieck tissue slicer, and vibratomes. The IMI-funded consortium project PREDECT (http://www.predect.eu) studied slice-explants from a variety of sources. Using slices derived from breast, prostate and lung cancer models, sustained viability of cultured slices was seen for up to 72 h (142). The possibility to compare tissue (tumor) slices from different species is an advantage in translating data from mouse to humans and may help to transfer and validate targets established in mouse models. However, organotypic tissue slices are prepared and cultured heterogeneously using different methods. In principle, a prevalidation study of lung tissue slices showed applicability of suitable standardization (143). Validation for *in vivo* data in co-clinical studies may help to use this tool for efficient P4-medicine (predictive, preventive, personalized and participative). Systemic effects of treatments or metastatic processes in a human setting have been difficult to monitor *in vitro*. However, new developments using human approaches *ex vivo*, to model cancer in microfluidic human organs-on-chip, for instance, promise to identify key molecular, cellular and immunological features of human cancer progression in a fully human setting.

Patient-Derived Models in Drug Testing and Personalized Medicine

A fast and effective way to evaluate a compound in drug testing or predict responses of a patient to specific anti-cancer drugs is to use high throughput approaches. These procedures are based mainly on simple test models which provide robust data on efficacy and targeted binding of the compound. However, most cell lines lack specific targets and are thus, no longer relevant. Patient-derived cell lines or more complex spheroid containing immune cells help to select candidates at an early stage for preclinical assessment and to provide data for stratified medicine approaches (144). A key step was the development of droplet-based chip platforms encapsulating primary cancer cells in nanoscale spots of hydrogels, allowing for comparison of the *in vitro* data obtained from the chip with clinical data, as well as with gene expression data. In a proof of concept study, the testing of 24 anti-cancer drugs in patient-derived brain cancer cells were well correlated with their oncogene overexpression (c-Met, FGFR1) and *in vivo* xenografts. Further developments use spheroids. Thus, tumor spheroid systems of the PANC-1 cell line in co-culture with pancreatic stellate cells are currently used in minipillar histochips as a tool to analyze stroma-targeting drugs (145).

Extensive preclinical studies are requested by public authorities to demonstrate the efficacy and safety of the test item. The paradigm changes in anti-cancer drug development from "one-size-fits-all" to patient-specific therapies have changed dramatically the requirements for translation to the clinic. Tumor entity driven targets within the cancer cells or in the tumor milieu have made drug testing on simple cancer cell line assays outmoded and demand the development of complex human based immune competent models. Molecular characterization of individual tumors is also paving the way to predictive therapies for individualized medicine. However, these biomarkers, obtained from transcriptomic and proteomic signatures, require evaluation of their predictively in clinical settings. For example, Her2 amplification is a strong predictive marker for trastuzumab treatment of breast cancer, but lacks predictivity in gastric cancer (146). PDX models using patient-derived cells are still the most relevant models to validate biomarkers and tumor relevant targets as they maintain the histopathological features, gene expression profiles, copy number variations and metastatic outgrowth of the original

tumors (147). In humanized PDX models, even the testing of drugs targeting immune cells is possible. Safety evaluation for off-target effects, however, needs to be well thought through. New targets regularly arise, which cannot be replicated in animal models nor adequately represented by immune competent models. The implementation of human (tumor) tissue slices may help to gain robust confirmation of the clinical potential of such drugs. Human tumor tissue *ex vivo* reflects the tumor heterogeneity and contains tissue-resident immune cells. It is thus, highly recommended as a validation tool (148). Evidence-based therapy suggestions to clinicians for metastatic cancer is a major challenge since epigenetic changes in cancer cells, altered tumor microenvironment and differences in the cellular composition of the metastatic tissue make it nearly impossible to draw conclusions from therapy predictions made on primary tumors. New technologies enable the detection of circulating tumor cells in easy accessible blood preparations and raise the possibility of characterizing these cells with a more metastatic phenotype and to gain insight into tumor progression (149–151). Inadequate scientific data on early metastatic progression weakens predictivity of therapy options in a metastatic setting. Finally, every model comes with its limitations and test strategies have to be matched to the mode of action of the test item and the individual patient. Integrative test strategies to evaluate efficacy of anti-cancer drugs need the cost-efficient combination of models. Furthermore, the test strategy considers various levels of test complexity as they may be used in a tiered approach.

Lessons Learned

Test models for immunomodulatory drugs in cancer and autoimmune disease models need to reflect the complexity of the disease. In contrast to immunomodulatory drugs in cancer, treatments for autoimmune diseases are focused on restoring immune tolerance. CAR T cell-derived immunotherapies, chimeric autoantibody receptor T (CAR T) cells, and CAR regulatory T (CAR Treg) cells bring new hope of treatment choices for autoimmune diseases. However, learning from T cell therapy in cancer, attention should be paid to the inflammatory microenvironment in autoimmune disease. Foxp3 expression in the CAR Tregs may be downregulated in this microenvironment and the phenotype may lose its immune suppressive function. It is obvious that there is no single model that reflects all relevant features. However, the use of the Avatar concept could bring significant progress and enable clinical style studies *ex vivo* as well as *in vivo* in humanized mice. While *in-vitro* and *ex-vivo* models usually lack the systemic response and adaptive immune response, murine models are never fully human and always lack a fully human response. Therefore, critical disease mechanisms or therapeutic targets should be validated by a combination of different models which generate reliable and predictive information. First steps have been taken to identify gaps in immune safety assessment within the EU consortium, imSAVAR. Specific modes of actions of immune modulatory drugs are being addressed, for which models or methods to predict adverse immune effects are not available. For this, existing models will be refined and new models and biomarkers developed. A part of the project will be to establish a platform providing biological samples from different resources that can be integrated into the new model systems.

CONCLUSION

Our knowledge and understanding of the innate and adaptive immune system currently provides a picture of a multi-component system that is essential for immediate defense against pathogens, as well as for the stimulation of the adaptive immune system. In addition, the constant maintenance of self-tolerance is crucial. However, it is clear that a wide variety of infectious and acquired diseases are closely linked to failure to establish healthy innate immunity. Diseases such as auto-inflammatory diseases are often caused by congenital dysfunction in immune responses. This increased understanding has permitted the development of novel targeted, cell-based therapies and drugs that are now used in normal clinical practice. As our knowledge of the different inhibitory and stimulatory immune mechanisms associated with autoimmune diseases progresses, we shall see significant improvement in early detection and diagnosis, as well as in the use of adequate treatment options.

AUTHOR CONTRIBUTIONS

SK, SS, and UK were mainly responsible for the performance of this review. MP acted as a native English specialist in editing the manuscript and also contributed to the content of this manuscript. All authors contributed to the article and approved the submitted version.

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Cancer Stem Cells—Origins and Biomarkers: Perspectives for Targeted Personalized Therapies

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The use of biomarkers in diagnosis, therapy and prognosis has gained increasing interest over the last decades. In particular, the analysis of biomarkers in cancer patients within the pre- and post-therapeutic period is required to identify several types of cells, which carry a risk for a disease progression and subsequent post-therapeutic relapse. Cancer stem cells (CSCs) are a subpopulation of tumor cells that can drive tumor initiation and can cause relapses. At the time point of tumor initiation, CSCs originate from either differentiated cells or adult tissue resident stem cells. Due to their importance, several biomarkers that characterize CSCs have been identified and correlated to diagnosis, therapy and prognosis. However, CSCs have been shown to display a high plasticity, which changes their phenotypic and functional appearance. Such changes are induced by chemo- and radiotherapeutics as well as senescent tumor cells, which cause alterations in the tumor microenvironment. Induction of senescence causes tumor shrinkage by modulating an anti-tumorigenic environment in which tumor cells undergo growth arrest and immune cells are attracted. Besides these positive effects after therapy, senescence can also have negative effects displayed post-therapeutically. These unfavorable effects can directly promote cancer stemness by increasing CSC plasticity phenotypes, by activating stemness pathways in non-CSCs, as well as by promoting senescence escape and subsequent activation of stemness pathways. At the end, all these effects can lead to tumor relapse and metastasis. This review provides an overview of the most frequently used CSC markers and their implementation as biomarkers by focussing on deadliest solid (lung, stomach, liver, breast and colorectal cancers) and hematological (acute myeloid leukemia, chronic myeloid leukemia) cancers. Furthermore, it gives examples on how the CSC markers might be influenced by therapeutics, such as chemo- and radiotherapy, and the tumor microenvironment. It points out, that it is crucial to identify and monitor residual CSCs, senescent tumor cells, and the pro-tumorigenic senescence-associated secretory phenotype in a therapy follow-up using specific biomarkers. As a future perspective, a targeted immune-mediated strategy using chimeric antigen receptor based approaches for the removal of remaining chemotherapy-resistant cells as well as CSCs in a personalized therapeutic approach are discussed.

Keywords: cancer stem cells, senescence, targeted therapy, CAR cells, biomarkers, precision therapy

INTRODUCTION

In 2018, according to the GLOBOCAN study, the malignant neoplasms with the highest mortality were lung (1.76 million deaths), stomach (783,000 deaths), liver (782,000 deaths), breast (627,000 deaths), and colorectal cancers (551,000 deaths) as well as blood cancers including leukemia (309,000 deaths) (1). All of these cancers are heterogeneous tumors containing cells with various stem cell properties, as described below. Already in 1877, Virchow's student Cohnheim noticed this cell population and pointed out that it possesses an embryonic character (2). Today, those cells are called cancer stem cells (CSCs) or tumor-initiating cells (TICs) and are seen as drivers of tumor establishment and growth (2–5), often correlated to aggressive, heterogeneous and therapy-resistant tumors (6, 7). Upon application of therapeutic regimens such as chemo- or radiotherapy the composition of tumor cell subpopulations changes (6, 8). At first, tumor cells with a high proliferative capacity are targeted and depleted causing a decrease in tumor size while CSCs survive (9). Additionally, some tumor cells will become senescent [therapy-induced senescence (TIS)], and subsequently can cause a change in the tumor microenvironment (TME) with tumor promoting effects due to the senescence-associated secretory phenotype (SASP) (6, 10–12).

It is well-known that CSCs are resistant to treatment and can cause tumor relapses (13). However, under the therapeutic pressure and changed microenvironment CSCs can be newly generated. In this case, these cells do originate from non-CSCs or from therapy-induced senescent tumor cells (14–18). It is therefore of importance to characterize these cells in detail and to understand their origin at the time of tumor initiation and tumor relapse.

This review underlines the role for a thorough investigation of tumors especially in the post-therapeutic period. Such post-therapeutic or therapy follow-up diagnostics are not conducted in the clinic on a regular basis, yet. The importance of specific biomarkers that analyze several parameters, such as CSCs phenotypes, senescence and TME composition, will allow the detection of therapy-resistant CSCs that cause tumor recurrence. A precise elimination of those cells of risk in a timely fashion using targeted cellular therapeutic approaches as the second line therapy is discussed in this study.

CSCs AND THEIR ORIGIN AT TUMOR INITIATION

Tumor initiation can either be driven by transformed differentiated cells or transformed tissue resident stem cells (19) (compare **Figure 1**). The transformation can take place during tissue regeneration and can additionally, be initiated and/or accelerated as a response to infections, toxins, radiation or metabolic influences causing mutations (20, 21). During the transformation process, oncogenes are overexpressed and tumor suppressors are inactivated promoting uncontrolled growth of the cells (19). As a consequence, cells de-differentiate and acquire stem cell characteristics (19). The transformation of tissue resident stem cells or their progeny is believed to presuppose a different set of genomic changes allowing uncontrolled, niche-independent proliferation (5, 22). As stem cells already possess unlimited growth potential, it is believed that the transformation of stem cells and their progeny requires only few genomic changes (5, 22, 23). For example, the low mutagenic changes, identified in more than 10% of gastric cancers suggest that these tumors arise from tissue resident stem cell populations (24). Two stem cell populations have been identified in gastric cancers: slow cycling cells expressing the transcription factor Mist1 in the gastric corpus and Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-expressing cells in the gastric antrum (25–27). Both populations have been linked to cancer generation in mouse models (24, 26, 27). In colon cancers, recent studies in mice have shown that even differentiated intestinal epithelial cells can be potential CSCs (28). The fact that adult differentiated cells, tissue resident stem cells or their progeny can promote tumor generation has also been shown in the liver. Cell tracking, *in vitro* and *in vivo* studies showed that liver cancer can originate from adult hepatocytes (29–32) as well as from hepatoblasts and hepatic progenitors (31, 32).

Tumor type, prognosis and aggressiveness are also influenced by the origin of the tumor, as analyzed for instance in breast cancers (33–35). Breast tumors originating from luminal progenitors are associated with a good prognosis, except those overexpressing Her2 (34, 36). Tumors originating from basal-like progenitors show a very aggressive phenotype (34).

In squamous cell carcinomas the differentiation phenotype seems to be influenced by the cell of origin and the kind of driver mutation, both responsible for the invasiveness and aggressiveness of the tumor (37, 38). Loss of the phosphatase and tensin homolog (Pten) as well as the liver kinase B1 (Lkb1) in lung epithelia causes tumor formation of highly penetrant tumors. These tumors are rarely metastatic and are characterized by a differentiated phenotype (37). Basal cells located within the trachea and main bronchi have been shown to self-renew and to form heterogeneous spheres (39). These basal stem cells can cause basal cell hyperplasia or epithelial hypoplasia, finally resulting in squamous cell metaplasia or dysplasia, which are discussed as precursors of squamous cell lung carcinomas (SCC) (39, 40). Studies by Fukui et al. suggest that high basal cell signatures correlate to a clinically aggressive phenotype in lung adenocarcinoma (40). Adenocarcinomas are considered to originate from sub-segmental airways of the bronchioalveolar stem cells or Type I and Type II pneumocytes (39). These bronchioalveolar stem cells are quiescent in healthy lungs but can enter proliferation cycles and could be targets of mutations causing transformation (39, 41). In mouse models, data indicate that small cell lung cancers (SCLC) can also originate from other cell types, i.e., neuroendocrine cells (42).

While in solid tumors the origin is heavily discussed, in hematological tumors the situation seems to be clearer. In acute myeloid leukemia (AML), the cell of origin is thought to be a hematopoietic stem or progenitor cell (43). However, a subgroup of human AML has been shown to share expression profiles with lymphoid T-cell progenitors. The authors showed that

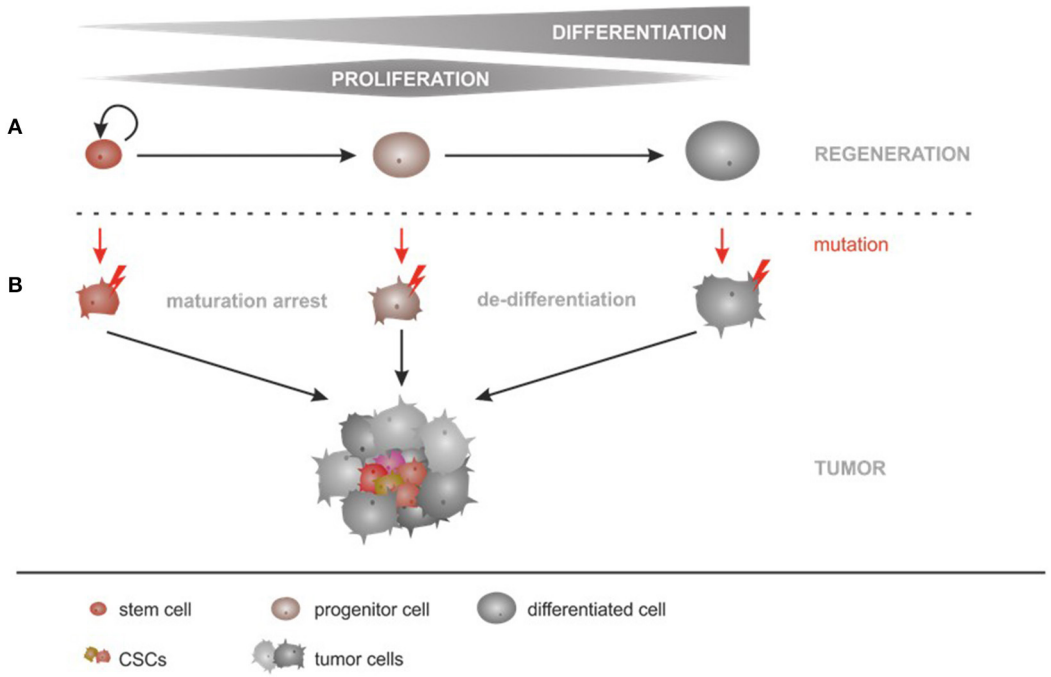

FIGURE 1 | The origin of CSCs at tumor initiation: The two hypotheses of CSC generation. **(A)** The proliferation and differentiation of adult tissue resident stem cells is part of the physiological regeneration program that maintains tissue homeostasis. Adult tissue resident stem cells divide asymmetrically and generate transient amplifying cells, which possess a high proliferative capacity. These cells terminally differentiate; a process during which they will lose their proliferative capacity to finally support organ homeostasis. **(B)** Tumors can be generated by step-wise accumulation of several mutations (red lightening) that transform differentiated cells and cause a de-differentiation. Tissue resident stem cells as well as their progeny can accumulate mutations that lead to uncontrolled and niche independent growth. Heterogeneous tumors are generated. CSCs share phenotypic characteristics and several markers have been described in solid as well as in liquid cancers.

under oncogenic conditions, DN2 (double negative 2) T-cell progenitors process into lymphoid, biphenotypic, and myeloid leukemia cells (43–45). In chronic myeloid leukemia (CML), the cell of origin is characterized by the expression of the Bcr-Abl oncogene, generated from a chromosomal translocation between chromosome 9 and 22 (46, 47). This molecular aberration defines the chronic phase in CML, which progresses into blast crisis upon additional mutations that promote self-renewal (46, 47). While leukemic stem cells (LSCs) are well-defined and characterized in AML and CML, the concept of CSCs in acute lymphoblastic leukemia (ALL) and also in non-hodgkin lymphoma (NHL) is less established (48–50).

Tumors generated on the basis of CSCs are believed to follow a unidirectional hierarchy, in which only the CSC population is able to initiate tumor growth (51). At the time point of tumor initiation, it is suggested that cancer stem cells divide asymmetrically to maintain the CSC pool (52). These asymmetric divisions generate transient amplifying cells, which are undergoing symmetric divisions; therefore having a high proliferative capacity (51, 52). Based on recent data from hematological cancers (AML), the hierarchical model proposed by Bonnet and Dick (43) is most likely a simplified description. It is now believed that the organization of CSCs (in solid as well as in hematological cancers) is more complex (52–56). In contrast to the CSC model in which only a small subpopulation of cells is able to promote tumor initiation and growth, the clonal evolution model states that genetically unstable cells accumulate genomic and genetic alterations over time causing an increase in tumor aggressiveness, resistance and heterogeneity (5, 57). Both models are not mutually exclusive, which can be explained by the cellular plasticity (plasticity model) that suggests, that different cellular states can interconvert (as explained later) (5, 57).

Because CSCs have been shown to cause tumor initiation and tumor relapses, the search for biomarkers that characterize these cells and allow therapeutic as well as prognostic prediction or follow-up is ongoing. The most prominent markers of solid and hematological tumors are described in the following section.

Biomarkers for CSC Populations in Solid Cancers

In solid cancers, the clinical use of CSC specific biomarkers is very limited, besides the use of the carcinoembryonic antigen (CAE), fragments of the cytokeratin 19 (YFRA 21-1) (58) and the alpha-fetoprotein (AFP) that is expressed by cancer stem cells (58, 59). Importantly, most markers expressed in CSCs can also be found in adult tissue resident stem cell populations, human embryonic stem cells (hESC) or adult tissues (60). Additionally, most markers label heterogeneous stem cell populations pointing to the fact that their characterization and isolation has to be based on marker combinations using several surface markers or combinations of extracellular as well as intracellular markers; to

TABLE 1 | Examples of lung cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD44 (and its variants)	(61–66) (39, 67–69)* (70)**	(71)	(71–80)	(61, 64, 70, 81) (39, 69)*
CD87	(82)			
CD90	(83) (39, 67)*			
CD133	(84–99) (39, 67–69)* (70, 100)**		(74, 101–104) (69)*	(91, 105–112) (39, 67, 68)* (70)**
CD166	(62, 66, 113) (39, 68)*			(113)
Surface markers, not CD				
EpCAM	(62, 66, 86, 114, 115)	(116–120)	(121)	(117, 122–124)
Intracellular markers				
ALDH	(65, 84, 114, 125–129) (39, 68, 69, 130)*	(131)	(132–134)	(62, 128, 135) (39, 69, 130)* (70, 126)**
Nanog	(70)			(70, 126) (69)*
Oct-3/4	(96) (67, 69)*		(67)*	(136) (69)*

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size), diagnostic, or prognostic (survival, resistance etc.) approach. Starsindicate reviews (*) and contradictory results (**).

TABLE 2 | Examples of breast cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD24	(137)			
CD29 (ß1 integrin)	(137, 138)			
CD44 (and its variants)	(139–149)	(150–154)	(76, 150, 152, 154–166)	(166–171) (172, 173)**
CD49f	(174–176) (177)*		(178)	(175, 178, 179)
CD61	(137, 180)			
CD70				(181)
CD90	(182)			
CD133	(183) (184)*	(185–187)	(188–190) (184)*	(191–193) (184)*
Surface markers, not CD				
CXCR4	(194)			
EpCAM		(186)	(186)	
LGR5	(195)			(195)
ProC-R	(196)			
Intracellular markers				
ALDH	(147, 148, 197, 198) (199, 200)*		(198, 201, 202) (199)*	(171, 192, 197, 203–208) (200)* (209, 210)**
BMI-1			(143, 211–218) (219)*	
Nanog			(142)	(220, 221)
Notch	(222–224)	(222, 225)	(187, 212, 222, 224, 226–230)	(222, 226, 231–234) (235)*
Oct-3/4		(142)		(220, 221)
Sox2		(142)		
Signaling pathways				
Wnt/ß-Catenin	(195, 236, 237)	(236)	(237)	

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size), diagnostic, or prognostic (survival, resistance etc.) approach. Stars indicate reviews (*) and contradictory results (**).

identify and isolate cells that promote tumor initiation, resistance and relapse.

Below, a short summary of the most prominent markers is provided. CSC markers that could have potential usefulness within therapeutic, diagnostic, and prognostic approaches are pointed out (compare **Tables 1**–**7**) and focus on most deadliest tumors of lung, liver, breast, stomach, and colorectal as well as AML and CML. **Tables 1**–**7** provide an extensive list of markers expressed in CSCs. A comparison shows that several markers are expressed in several tumor types.

CD44

CD44 is a biomarker which is not only expressed in solid but also in hematological cancers (see below). Its expression is associated with increased proliferation, self-renewal and metastasis (61, 149, 462, 463). For example, in colorectal cancers, expression of CD44/CD166 characterizes a cell population able to form tumor spheres, suggesting anchorage-independent proliferation of these cells (333). In other studies, $CD44^{high}/CD133^{high}$ cells showed increased tumorigenic capabilities (334). Also in breast cancers, the percentage of $CD44^{+}/CD24^{-}/CK^{+}/CD45^{-}$ cells was shown to be increased in malignant lesions compared to non-malignant lesions (139). A significant decrease in proliferation and migration of breast cancer cells was observed after the knock-down of CD44 (140). In gastric cancers, the knock-down of CD44 reduced sphere formation and caused decreased tumor growth in severe combined immunodeficiency mice (246). In many tumors (e.g., breast and liver), CD44 is expressed as isoform and its expression has been associated with increased cancer stem cell properties (141). In lung cancers, CD44v9 expression correlates significantly with early-stage lung adenocarcinoma and epidermal growth factor receptor (EGFR) mutations (464). Variants of CD44 are also expressed in gastric cancers and promote tumor initiation (248).

The CSC marker CD44 has been indicated as a biomarker for diagnostic, therapeutic, and prognostic approaches (compare **Tables 1**–**5**). In gastric cancer patients, $CD44^{+}$ circulating tumor cells correlated with a poor prognosis (465). In colorectal

TABLE 3 | Examples of gastric cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD24	(238) (239)*	(240)*	(241)	(242–244) (239, 245)*
CD44 (and its variants)	(246–251) (239, 240, 245, 252)*	(247, 251, 253, 254) (240)*	(255–257) (239, 240, 245)*	(247, 251, 254, 258–260) (239, 240, 245, 252)*
CD90	(251) (239, 245)*			
CD133	(247, 249, 250) (239, 240, 252)*	(254, 261) (240)*	(257) (239, 240)*	(254, 262–265) (239, 240, 252)*
Surface markers, no CD				
CXCR4	(266)		(267)*	(268–271)
EpCAM	(248, 249, 272) (239, 240, 252)*		(273)	(265, 272)
LGR5	(274) (252)*	(240)*	(275, 276) (252)*	(275, 277–279)
LINGO2	(280)			(280)
Intracellular markers				
ALDH	(249, 281, 282) (239, 240, 252)*			(260, 281, 282)
Letm1	(283)			(283)
Musashi2	(284)			(284)
Nanog	(285) (239, 286)*	(287) (240)*		(287, 288) (286)*
Oct-3/4	(239, 252)* (289)**			(247, 265, 288) (289)**
Sox2	(247) (239, 240, 252, 290)*	(240)* (291)**	(292)	(247, 288, 293) (265)**

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (*) and contradictory results (**).

TABLE 4 | Examples of liver cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD24	(294–296) (297, 298)*		(298)*	(295)
CD44	(299, 300) (298)*			(300–303) (298)* (304)**
CD90	(295, 300, 305–308) (297, 298)*			(295, 300, 304, 309) (298)*
CD133	(295, 296, 300, 310–313), (297, 298)*	(314)		(295, 300, 304, 311, 314–319), (320)**, (298)*
Surface markers, not CD				
EpCAM	(297, 298)* (294, 300, 304, 311, 321)	(322)	(298)*	(300, 301, 304, 311, 319, 321–327) (298)*
Intracellular markers and pathways				
AFP	(311, 321)		(328)	(311, 321, 329), (330)*
Nanog	(312, 313, 331), (298)*		(298)*	(331) (298)*
Notch	(295, 296, 305)		(295)	
Oct-3/4	(313, 331), (298)*			(309, 331), (298)*
Sox2	(313) (298)*			
Wnt/ ß-catenin	(295, 313)		(295)	(313) (330)*, **

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (*) and contradictory results (**).

cancers, a prognostic quantitative real-time PCR was established to analyze the expression of CD44v2 showing that a high expression correlated with a worse prognosis (339). In gastric cancers, the expression of CD44 and CD90 correlated with distant metastasis and could therefore be used as a diagnostic biomarker (251) and was suggested as a biomarker for treatment response (253). Therapeutic approaches targeting CD44 have been made using e.g. adenoviral delivery of siRNA *in vitro* (337). Furthermore, CD44-targeting drug conjugated aptamers (76) or hyaluronic acid coated onto nanoparticles have been in the focus of research (155). Antibody-based photosensitizer conjugates for combined fluorescent detection and photo-immunotherapy (PIT) of CD44-expressing cells in triple-negative breast cancers (TNBC) (150) or other antibody-based approaches tested in safety studies (466–468).

CD133

The biomarker CD133 (Prominin-1) is expressed on hESCs and rarely found on normal tissue cells (60). The marker has been additionally identified in tumors of breast, liver, stomach, and colon (compare **Tables 1**–**5**) and has also been described as a marker that characterizes cells with high tumorigenicity and a high ability to form spheroids (184, 469). In breast cancers, its expression correlates with N-cadherin expression that was found to be significantly higher in patients with metastasis (191). In lung cancers, the expression of CD133 has been correlated to epithelial to mesenchymal transitions (EMT), in combination with other additional stem cell markers, such as BMI1 (84).

The expression of CD44 and CD133 in colorectal cancers can predict metastasis (470), however, no correlation to patient outcome could be detected (471). In breast cancers, CD133 mRNA was suggested to be suitable for prognosis prediction (193, 472) and CD133 protein has been correlated to a poor prognosis (193). Pre-clinical therapeutic approaches cover antibody-based targeting of colorectal (341, 342) as well as breast cancers (188) (compare **Tables 1**–**5**).

EpCAM

The epithelial cell adhesion molecule (EpCAM, CD326) is expressed on CSCs in various tumor types including colon and hepatocellular cancers (473–476). Furthermore, it is expressed

TABLE 5 | Examples of colorectal cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD24	(332)			
CD44	(333–335) (336)*		(337, 338)	(339)
CD133	(334, 340) (336)*	(340)	(338, 341–343)	(340, 344)
CD166	(333) (336)*			(333)
Surface markers, not CD				
EpCAM	(335) (336)*		(345, 346) (347)*	
LGR5	(335, 348–350) (336)*	(351)	(352)	(353, 354)
Intracellular markers				
ALDH	(335, 355, 356) (336)*			(355) (357)*
Letm1	(358)			(358)
Nanog	(359, 360) (336)*		(361)	(361, 362)
Oct-3/4	(363, 364) (336)*			(363, 365)
Sall4		(366)		(366)
Sox2	(359, 367, 368) (336)*			(367–369)

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Starsindicate reviews (*).

TABLE 6 | Examples of AML cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD33		(370)	(371–392)	(393)
CD123	(370, 394–396)	(395, 397–399)	(373–376, 397, 400–412)	(394, 399, 403, 413)
Surface markers, not CD				
CLL-1	(414–416)	(370)	(414, 417–419)	(415, 420)
TIM3	(421)		(422)	(420, 423)
Intracellular markers				
ALDH	(424)			(424, 425)
Nanog	(426)	(427)		(426)
Oct-3/4	(428)	(429)		(429–431)
Sox2				(431, 432)

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach.

in non-transformed tissues such as epithelial cells (476), and various stem and progenitor cells (477, 478). EpCAM is involved in proliferation and differentiation as well as in cell signaling and formation and maintenance of organ morphology (479). In cancer tissue, EpCAM is homogeneously expressed on the cell surface, while in epithelia it is expressed on the basolateral side (476).

In breast cancers, the expression of EpCAM is correlated to CSC-like phenotypes that promote formation of bone metastases in mice (480). In lung cancers, the expression of EpCAM is often associated with the expression of CD44 and CD166. Triple positive cells show increased clonogenicity, spheroid formation, self-renewal capacity, and show increased resistance to both 5-fluouracil and cisplatin (62).

As one of the first CSC markers, EpCAM has been evaluated as a therapeutic biomarker (compare **Tables 1**–**5**). Targeting EpCAM with different antibody formats has been performed in colorectal as well as breast cancers (347). In colorectal cancers, a therapeutic approach targeting $EpCAM^+$ cells with aptamers has been performed in pre-clinical conditions (345, 346).

Intracellular Biomarkers as Regulators of Stemness in Solid Cancers

Both embryonic and CSCs show unlimited growth, invasive capacity and are characterized by an undifferentiated cellular state (481). This feature depends on transitions between epithelial and mesenchymal states, regulated by a network of intracellular pluripotency transcription factors. As reviewed by Hadjimichael et al. and also described by others pluripotency in ESC is regulated by a core-network of transcription factors, consisting amongst others of Oct-3/4, Sox2, Nanog, Klf4, and c-MYC as well as signaling pathways such as the Jak/Stat, Wnt/ß-catenin, Hedgehog/Notch, TGF-ß as well as FGF signaling pathways (367, 482, 483). The core-pluripotency network consisting of Nanog, Oct-3/4 and Sox2 (described in detail below) activates genes of self-renewal and suppresses genes involved in differentiation (482). Pluripotency factors as well as signaling pathways have been indicated as biomarkers for CSCs as shortly described below (compare **Tables 1**–**5**). Of note, the tables do not include all biomarkers, however describe the most abundant ones reported in the literature.

Sox2

The transcription factor Sox2 belongs to the SRY-related HMG-box (SOX) family, and is involved in the maintenance of an undifferentiated cellular phenotype (367). Its aberrant expression in cancers often leads to increased chemotherapy resistance and asymmetric divisions, as observed in colorectal cancers (368). In those, Sox2 expression correlates with a stem cell state and with a decreased expression of the caudal-related homeobox transcription factor 2 (CDX2), which could serve as a prognostic marker for a poor prognosis (367, 368). In gastric cancers, expression of Sox2 correlates with the tumor stage as well as with a poor prognosis (247, 288). The formation of tumor spheroids *in vitro* also correlates to the overexpression of CD44 and CD133 as well as the transcription factors Sox2, Nanog and Oct-3/4 (247).

TABLE 7 | Examples of CML cancer stem cell markers and their use as diagnostic, predictive, or therapeutic biomarkers.

Marker	Stem cell marker	Biomarker diagnostic	Biomarker therapeutic	Biomarker prognostic
Surface markers, CD				
CD25	(433–437) (438–440)*	(439)*	(441)	
CD26	(433–437, 442–445) (438–440)*	(443, 446) (439)*	(434, 447, 448)	(443)
CD33	(433, 434) (438–440)*			
CD36	(434, 435) (438)*		(435)	
CD117	(433, 434, 437) (439, 440)*			
CD123	(434, 449–451) (439, 440)*		(449, 450)	
Surface markers, not CD				
IL1RAP	(433–437, 452, 453) (438–440)*	(439)*	(452, 453)	(437)
Intracellular markers				
JAK/STAT	(433) (438)*			
Wnt/β-catenin	(454–456) (438, 457)*		(454, 458, 459) (457)*	
FOXO	(460) (438)*		(460)	
Hedgehog/Smo/Gli2	(461) (438)*		(461)	

The table lists examples of cancer stem cell markers and indicates those which have been tested as biomarkers within a therapeutic (metastasis, tumor stage, size, resistance), diagnostic (i.e., resistance), or prognostic (survival, resistance etc.) approach. Stars indicate reviews (*).

However, in another study, Sox2 levels were downregulated in gastric cancers in comparison to normal tissue and high Sox2 expression correlated with decreased metastasis and a better prognosis for the patient due to increased p21 levels (293). Therefore, the oncogenic functions of Sox2 are controversially discussed in gastric cancers, in which Sox2 might also have tumor-suppressor functions. These different functions seem to depend on the cancer origin and cellular context (484).

Oct-3/4

Oct-3/4, also known as POU5F1, belongs to the POU homeobox gene family and is also a regulator of pluripotency in mammalian stem cell population. Oct-3/4 is upregulated in several cancers and may support the neoplastic transformation and resistance (485). In colorectal cancer cells, Oct-3/4 causes increased migration and liver metastasis (363, 486) correlating with poor survival (365). As reviewed by Prabavathy et al. Oct-3/4 expression is correlated to increased self-renewal and metastasis in lung cancer cells (67). A meta-analysis showed that Oct-3/4 expression in lung cancer was associated with poor outcomes concerning the differentiation degree, the TNM Classification of Malignant Tumors (TNM) and lymphatic metastasis (136). In hepatocellular carcinoma (HCC) Oct-3/4 expression was correlating with tumor size and recurrence (309).

Nanog

Nanog is a homeobox domain transcription factor widely expressed in human cancers (487). In colorectal tumors its expression was significantly increased in $CD133^+$ cells, and on the basis of a univariate analysis, Nanog expression correlated linearly to liver and lymph node metastasis and the TNM stage. It might therefore be useful as a prognostic biomarker in post-operative liver metastasis (362). In breast cancer, expression of Nanog and Oct-3/4 has been correlated to a poor prognosis of the patient as well as EMT (220, 221). In HCC cell lines Nanog expression drives selfrenewal and invasion, metastasis, and drug resistance (298).

Biomarkers for CSC Populations in Hematological Cancer

CSC biomarkers of AML and CML have been listed in **Tables 6**, **7**. They indicate commonly used markers and point out possible functions of these markers as biomarkers in prognosis, therapy, and diagnosis. Below a short introduction of the most relevant markers is given.

CD44

As mentioned above, CD44 is a common marker shared by many cancers (60). In hematological cancers, CD44 expression is functionally associated with chemotherapy resistance (488, 489). The expression of CD44 in AML is significantly correlated with a poor overall survival (OS) (490). Furthermore, CD44 was shown to be significantly higher expressed in non-remission AML patients (490). A highly relevant function of CD44 for LSCs is the adhesion to the bone marrow niche (491).

CD123 and CD33

In hematological malignancies, such as AML, CD123 as well as CD33 have been described as the "classical" CSC markers (492, 493). CD123 is a marker expressed on LSCs (395, 397, 494), but not exclusively (395, 398). In AML patients, CD123 expression correlates to the therapy response rate (413, 495), the relapse risk (403), and a shorter disease-free period and OS (399, 413). CD123 has been associated with increased proliferation and differentiation (494, 496).

CD33 is historically, the most commonly used marker for AML stem cells, with clinical implementation of CD33 targeting, dating back to the Food and Drug Administration (FDA) approval of gemtuzumab ozogamicin (GO) in 2000 (497). CD33 is highly expressed on blasts in around 85–90% of AML patients (433, 438, 497) and also expressed at higher densities in CML (433, 438) but less on healthy hematopoietic stem cells (HSCs). These cells are additionally characterized by expression of CD25, CD26, and Interleukin-1 receptor accessory protein (IL-1RAP) and also other markers (440).

CLL-1

The C-type lectin-like molecule-1 (CLL-1) is a promising alternative to the "classical" LSC targets (414). The majority of

AML patients shows CLL-1$^+$ LSCs, a marker not being expressed on HSCs (370, 414–416). Compared to CD33, CLL-1 was not only more frequently and stronger expressed on LSCs, but also not or more weakly expressed on normal tissues leading to reduced off-target effects after treatment with a respective antibody-drug conjugate. Therefore, CLL-1 might be a more suitable and specific LSC target than CD33 (414). A high expression of CLL-1 is associated with poor prognosis (420) and a faster relapse (415) in AML. Interestingly, controversial observations have been made using CLL-1 as a biomarker after chemotherapy. The diagnostic value of CLL-1 is discussed controversially: while Zhang et al. showed that CLL-1 was increased after chemotherapy (371), others showed that there is no difference between CLL-1 expression at diagnosis and at relapse (415) or even detected a decreased CLL-1 expression at relapse (370). The relevance of CLL-1 as a prognostic biomarker for chemotherapy failure or relapse is therefore still unclear. Its expression is not detectablewithin the chronic phase of CML (440).

TIM-3

Another "non-classical" LSC biomarker is T-cell immunoglobulin and mucin 3 (TIM-3), that is highly expressed on LSCs but not expressed on healthy HSCs (498). It is correlated to a poor prognosis (420) and treatment failure (423). Stem cell properties of TIM-3$^+$ cells were confirmed by engraftment in a xenograft mouse model (421).

Intracellular Biomarkers as Regulators of Stemness in Hematological Cancers

The core-network of pluripotency associated transcription factors as well as signaling pathways have also been analyzed in hematological cancers. Fifty AML patients have been analyzed for the expression of Sox proteins, which are overexpressed in 10–22% of the patients. The analysis showed that high levels of Sox proteins may have a prognostic value (432). The analysis of Oct-3/4 expression correlated with an unfavorable prognosis and is associated with FMS-like tyrosine kinase 3-internal tandem duplications (FLT3-ITD) (430).

Activation of stemness-associated pathways especially in CML has been shown to promote extensive proliferation and has been linked to the onset of blast crisis, which is associated with a loss of differentiation of the leukemia initiating cells. An important impact on this effect has the Wnt/ß-catenin pathway (46) that promotes HSC proliferation, independent of the bone marrow niche (5, 22, 499). Especially, resistance to the tyrosine kinase inhibitor imatinib has been shown to correlate to an increased nuclear localization of ß-catenin (454, 458, 500). Inhibitors targeting the Wnt pathway have been shown to be of advantage for example in long-term cell cultures (500). Additionally, the hedgehog pathway has been suggested to be involved in chemotherapeutic resistance in CML, which is also characteristic for chronic phase CML cells (47). Mouse studies also indicate the involvement of the hedgehog pathway (46, 47), which has been implicated as a therapeutic biomarker for CML (456, 461).

To summarize, CSCs at tumor initiation originate from either differentiated cells or adult tissue resident stem cells (5, 19, 22). Several data indicate that the origin strongly correlates to the aggressiveness of the tumor. Therefore, extra- and intracellular biomarkers that characterize CSCs have been identified and implemented to be of diagnostic and prognostic advantage. However, stem cells are subject to a high degree of plasticity modulated by the TME (19), that is significantly changed by chemo- and radiotherapies and composed of several different cell types. In the following section, focus will be laying on senescent tumor cells as part of the TME as they have long-term influence on TME and CSC development and progression.

THE ESCAPE OF CANCER STEM CELLS FROM THERAPY

At the moment first-line therapeutic treatments in progressed tumors include in the most cases surgery, chemo- as well as radiotherapies (501) (compare **Figure 2**). Those have been shown to induce DNA damage and to trigger senescence in cancer cells, a process known as therapy-induced senescence (TIS) (10, 502, 503). TIS will cause a decreased tumor size and attracts immune cells such as neutrophils, monocytes as well as T-cells toward senescent tumor site (503). However, over a long-term period the anti-tumorigenic effects of TIS are lost and the cancer might gain stemness causing tumor relapses (**Figure 2**).

Therapy-Induced Senescence: Its Hallmarks, Biomarkers, and Its Role in CSC Generation

Agents that induce DNA damage such as chemo- and radiation therapies have been identified to trigger senescence in differentiated cancer cells (10). TIS has been in the research focus, because it significantly contributes to the long-term outcome of patients (12). The DNA damage response ultimately activates one or several tumor suppressors pathways [p53, p16 (Ink4a), p21 (Waf1), and retinoblastoma (RB)], that trigger and maintain the senescence growth arrest (504). However, it is important to mention that the senescence phenotype can also be induced in cancer cells which lack functional p53 and RB protein (504). TIS and senescence in general, are recognized as a double-edged sword, that on the one hand leads to the attraction of immune cells, inflammation, and elimination of senescent tumor cells and correlates with a positive post-treatment prognosis and treatment outcome (505–507). On the other hand, senescence can play a strong pro-tumorigenic role that supports CSC generation, as described below.

Senescent cells are characterized by biochemical and morphological changes such as flattening and/or nuclear enlargement (508). There are several classical biomarkers of cellular senescence and they comprise: senescence-associated beta-galactosidase (SA-ß-gal) activity, expression of p53 protein, the amount of p53 in the nucleus, increase in expression of p14 (Arf), p16 (Ink4a) and p21 (Waf1), SASP, and often senescence-associated heterochromatic foci (SAHF) (12, 505, 507, 509–515). Furthermore, senescent cells display low Ki67 levels and show levels of heterochromatin protein 1 (HP1) gamma (516), as well

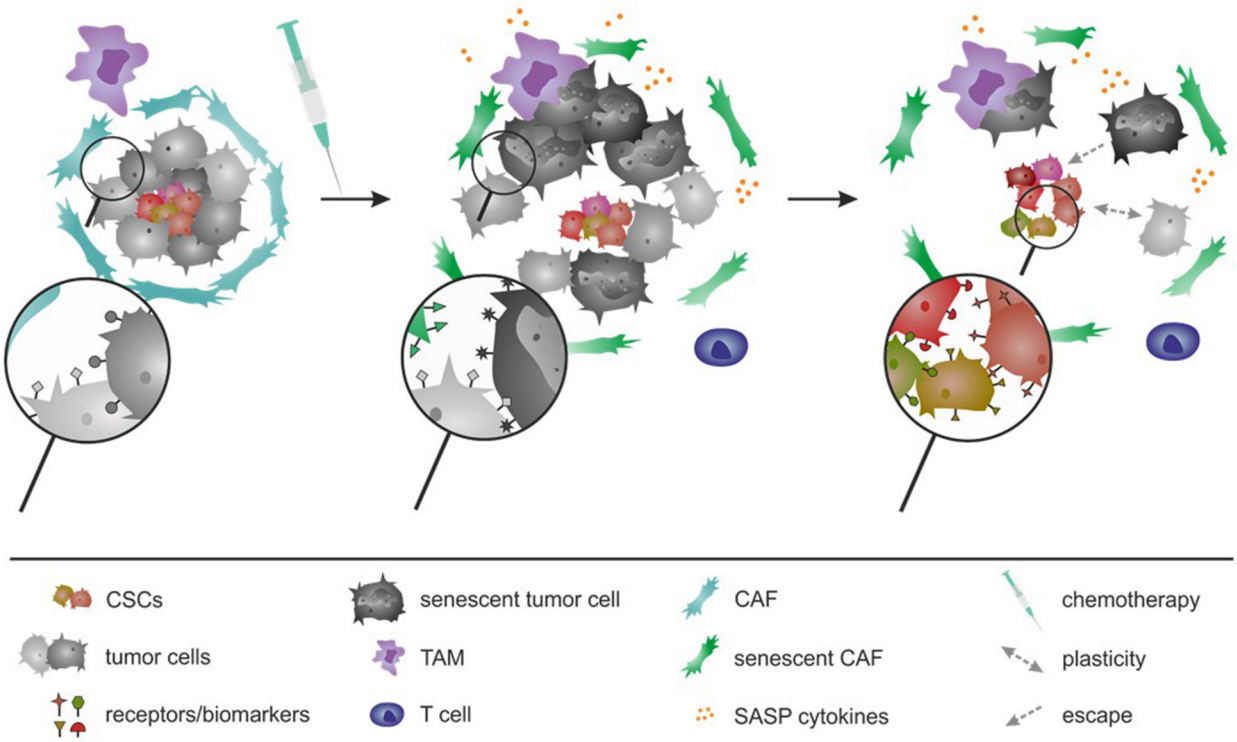

FIGURE 2 | Kinetic of tumor development in pre-, early-, and late-therapeutic period upon application of chemo- and/or radiation therapy: current situation in the clinic. **(A)** In the pre-therapeutic situation, heterogeneous tumors are composed of several cell types, including CSC, tumor cells, TAMs, and CAFs; all characterized by biomarkers. **(B)** In the early post-therapeutic period, upon application of the first-line treatment (that currently uses mostly chemo- or radiotherapeutic regimens) several important changes occur in the tumor, in particular: tumor cells or CAFs die due to the therapy or become senescent, whereas CSCs mostly survive the treatment. Senescent cells (tumor cells and CAFs) attract immune cells toward the senescent site via SASP. The SASP therefore plays a positive role and attracts immune cells in this early post-therapeutic situation. Attracted immune cells promote the clearance of dead, of necrotic, and senescent tumor cells and CAFs. **(C)** In the late post-therapeutic situation uncleared senescent tumor cells and senescent CAFs and SASP thereof play a negative (pro-tumorigenic) role and support tumor development. SASP molecules provide stimulating factors for CSCs for further uncontrolled proliferation as well as their maintenance. Also, remaining senescent tumor cells acquire additional mutations that promote activation of a stemness phenotype and finally a tumor relapse.

as di- or tri-methylated lysine 9 of histone H3 (H3K9Me2/3) and histone H2A variant (macroH2A) (505, 517, 518). The usefulness of telomere length as a biomarker of senescence has been questioned (505).

Biomarkers that underline the effect of a therapeutic approach based on the induction of senescence have to be evaluated carefully and quite often simultaneously. The investigation of senescence markers after post-operative chemotherapy in muscle-invasive bladder cancer (MIBC) revealed that the simultaneous expression of several markers involved in the p53 pathway has to be checked to correctly assess the pathological outcome of MIBC (509). The analysis revealed that the expression of p14 (Arf) was associated with an impaired response to chemotherapy and poor prognosis, whereas p21 (Waf1) expression was related to reduced tumor cell proliferation (509).

TIS can play an anti-cancerous role (503). As demonstrated in our studies in premalignant and malignant liver disease, the induction of senescence leads to a so-called "senescence surveillance" mechanism, which relies on innate and adaptive immune cells. These cells clear senescent premalignant cells, thereby protecting premalignant liver from cancer development (535, 536). Interestingly, in further studies, we could show that the chemokine (monocyte chemoattractant protein 1, MCP-1) axis is of importance for the induction and maintenance of senescence and for the sufficient immune surveillance in the liver (525). Several biomarkers of senescence were found to correlate with a disease-free survival or with an improved OS in several solid cancers (516, 524). One such indicator, a lysosomal-beta-galactosidase (GLB1) that hydrolyzes beta-galactose from glycoconjugates and represents the origin of SA-ß-gal, was reported as a reliable senescence biomarker in prostate cancer (516). Inhibition of the cyclin-dependent kinase 4/6 (CDK)-RB pathway by a novel drug, SHR6390, resulted in reducing the levels of Ser780-phosphorylated RB protein and correlated with the G1 arrest as well as with cellular senescence in a wide range of human RB^+ tumor cells *in vitro* (520). Xiang et al. identified seven senescence-associated genes (SAGs, **Table 8**) significantly decreased in senescent cells and increased in HCC

TABLE 8 | Biomarkers of therapy-induced senescence (TIS).

Biomarker	References
Senescence-associated beta-galactosidase (SA-β-Gal)	(12, 14, 504, 505, 510, 516–520)
P53	(14, 504, 520, 521) (507)*
Retinoblastoma (RB) Protein (CDKN2A; Ser780phosphorylated RB protein; cyclin-dependent kinase (CDK) 4/6-retinoblastoma)	(12, 14, 504, 519–521) (507)*
P14 (human) P19 (mouse)	(12, 509, 510, 514, 515, 519) (505, 507, 513)*
P16 (INK4A; CDKN2)	(12, 14, 509, 512, 515, 519, 522) (505, 507, 513, 514, 516)
P21 (WAF1)	(14, 509, 522) (505, 507, 513)*
Senescence-associated heterochromatic foci (SAHF)	(12, 509, 510, 515, 519) (505, 507, 513)
Heterochromatin protein 1 (HP1) gamma	(509, 516, 518)
Telomere length	(505)*
Di- or tri-methylated lysine 9 of histone H3 (H3K9Me2/3)	(505, 517, 518)
Histone H2A variant (macroH2A)	(505, 517, 518)
Lysosomal-beta-galactosidase (GLB1)	(516)
Inhibition of growth (ING) family of proteins (ING−1,−2,−3,−4,−5)	(523)
Senescence-associated genes (SAGs) family: [18B (KIF18B), Citron kinase (CIT), Centrosomal protein 55 (CEP55), minichromosome maintenance complex component 5/7 (MCM), Cell division cycle 45 (CDC45), enhancer of zeste homolog 2 (EZH2)]	(524)
Senescence-associated secretory phenotype (SASP)	(12, 14, 510, 519) (505, 507, 509, 522)
Soluble TNF-receptor-II	(11, 523)
Chemokine (C-C motif) receptor/ligand 2, (CCR2/CCL2); Monocyte chemoattractant protein 1 (MCP-1) axis	(525)
IL-1	(526)
IL-6	(527–531)
IL-8	(528, 531, 532) (526, 527)
Regulated on activation, normal T cell expressed and secreted (RANTES)	(533, 534)

Examples of the most important biomarkers of TIS are listed. Stars indicate reviews (*).

tissues (524). Interestingly, those SAGs were strongly associated with OS, especially in Asian populations, and had a better predictive accuracy in comparison to serum AFP in predicting OS of HCC patients (524). Recently, Smolle et al. reviewed and underlined the role of members of the inhibition of growth (ING) family. These act as tumor suppressors, regulating cell cycle, apoptosis, and cellular senescence. The authors proposed them as clinically useful biomarkers in the detection and prognosis of lung cancer (523).

In line with the positive role of senescence, evidence exists regarding the role of TIS in turning "cold" tumors toward a "hot" phenotype that results in activating immune responses against tumor antigens (503). As reported in $Her2^+$ breast cancer patients treated with Trastuzumab and chemotherapy, the treatment-induced epitope spreading was characterized by increased antibody responses not only to the tumor antigen Her2, but also to endogenous CEA, insulin-like growth factor-binding protein 2 (IGFBP2), and p53 (521).

TIS is a very important protective mechanism that is induced immediately after chemo- or radiation therapy. TIS mediates the recognition and clearance of senescent tumor cells by immune cells (503, 535). Induction of TIS after the therapy is associated with a better prognosis and OS (524). However, if senescent tumor cells are not cleared in a timely fashion, their role at a later time points shifts from positive to negative, as discussed in the section below.

Negative Role of TIS: Cancer Progression

Several studies report a pro-tumorigenic effect of TIS leading to cancer recurrence and support of tumor development (503). Uncleared senescent cells acquire additional mutations, thereby escaping the cell cycle arrest and transform into malignant cells (536). Moreover, factors secreted by senescent cells are also reported to play a strong tumor-promoting role (526).

Was et al. suggested that senescent human colon cancer cells (HCT116) that appear during a doxorubicin-based therapy enter a "dormant" cellular state, survive the treatment, and cause tumor re-growth (537). Importantly, the recent findings by Scuric et al. suggest a long-term effect of chemotherapy and/or radiation exposure upon TIS (11). In this study, markers of cellular senescence, including higher DNA damage and lower telomerase activity, were observed many years later in breast cancer survivors (11). Elevated levels of a soluble tumor necrosis factor (TNF)-receptor-II, a pro-inflammatory biomarker and one of the main SASP molecules, were also detected (11). A negative effect of SASP was correlated to a p53 single-nucleotide polymorphism (SNP) at codon 72 which is correlated to increased risk of breast cancers (538). Using a humanized mouse model, Gunaratna et al. showed that SASP caused an increased invasion of pro-inflammatory macrophages (522). However, the inflammation proceeded into a chronic inflammation with pro-tumorigenic action and tumor-associated macrophages (TAMs) contributed to angiogenesis and increased tumor growth rates (522). Also, senescent cancer-associated fibroblasts (CAFs) and, in particular, expression of Caveolin-1 (CAV1) promote tumor invasion in pancreatic cancer (539). Moreover, in clinicopathological characteristics of patients, a high CAV1 expression directly correlates with higher levels of serum tumor antigens, with the rate of advanced tumor stage, and with significantly worse outcomes in both overall and disease-free survival (539).

It has been suggested that cancer therapies, especially chemo- and radiotherapies, possess long- and late-term pro-tumorigenic side effects and could therefore contribute to the relapse of the malignant disease they were intended to treat (540). Such long-term effects could be caused by the decreased removal of senescent cells, as described below.

Cancer Stemness: Senescence Escape

As mentioned above, cells undergoing senescence can still escape the senescence program and become malignant while acquiring additional mutations (519, 535, 536) (**Figure 2**). In our studies, we observed a spontaneous mutation [a deficiency in p19 (Arf)] in Ras-expressing hepatocytes, which resulted in a full-blown HCC development using a Ras-induced precancerous liver disease model (535, 536). The reversibility of TIS can be caused through the inactivation of tumor suppressors p53, p16 (Ink4A), p19 (Ink4d), and/or RB (504, 507, 519). Additionally, the over-expression of CDC2/CDK1 and survivin can promote cancer stem cell survival and can also promote the development of polyploidy (507). In general, mutations in CDKN2A, coding for p16 (Ink4a, CDKN2A), p21 (Waf1, CDKN1A), and p27 (Kip1, CDKN1B) as well as E2F3 and EZH2, and a high c-MYC expression might result in low percentages of senescent cells (504, 519). Moreover, particular mutations completely protect melanoma cells from cell cycle arrest upon chemotherapy: DMBC29 melanoma cells that carried a $EZH2^{S412C}$ mutation, expressed c-MYC at a low level and a wild type of CDKN2A did not undergo senescence, in contrast to many melanoma cells treated with vemurafenib and trametinib (519).

An escape of cells from senescence was also identified by Milanovic et al. in B-cell lymphoma studies (14). In those studies, the researchers showed that senescent cells substantially upregulated an adult tissue stem cell signature and activated Wnt signaling (14). This senescence-associated stemness was an unexpected cell-autonomous phenotype that caused the generation of cells with a higher tumorigenic potential *in vitro* (14).

However, escape from senescence is not the only pathway that promotes an increase in the cancer stemness phenotype. Stemness within the tumor tissue is also regulated indirectly by signaling molecules which support the maintenance of stemness in CSCs as well as non-CSCs, as described in the following sections.

Cancer Stemness: SASP and CSC Maintenance

The stemness phenotype within a tumor can also be mediated via SASP (526). Several studies address the strong pro-tumorigenic phenotype (526) whose cytokines can mediate the maintenance of CSCs. The most prominent interleukins (IL) of SASP are IL-1,−6, and−8 (526). These cytokines can influence the CSC phenotype and functionality and therefore influence the plasticity phenotype of CSCs.

Using breast cancer cell lines, Di et al. showed that an induction of senescence in mesenchymal stem cells by hydrogen peroxide treatment causes an increased secretion of the inflammatory cytokine IL-6, which led to a higher migratory capacity of breast cancer cells *in vitro* as well as in xenotransplants (541). An increase in the aggressive metastatic chemoresistant phenotype upon inflammatory cytokine stimulation such as IL-1ß, IL-6, and RANTES (regulated on activation, normal T cell expressed, and secreted) was also observed by others (533, 534). Our own work indicated that IL-8 blocks differentiation of hepatocellular premalignant cells, a pathway mediated via mammalian target of rapamycin complex 1 (mTORC1) kinase, that causes an increase in chemotherapy resistance (532). An increase in tumorigenicity and EMT of breast cancer cells has been shown to correlate to an increased expression of CD44 or CSC-like properties and be caused by the senescence-associated IL-8 and IL-6 (527–529). Pathways that might be involved in such cellular reprogramming processes are the JAK2/STAT3 signaling pathway (542), the IL-6/STAT3 and NOTCH cross-talk signaling (187, 530) as well NFκB-IL-6 signaling axis, responsible for the generation of CSCs (531). Interestingly, interference with those pathways by aspirin increased chemosensitivity and decreased self-renewal in breast cancer cells (531). In colorectal cancer cells the inflammatory cytokine IL-6 mediates deacetylation, which subsequently activates NANOG transcription and accumulation of stemness phenotypes, correlating with malignant progression and poor prognosis (543).

To summarize, TIS on the one hand has positive effects that eliminates differentiated tumor cells and also causes invasion of immune cells with anti-tumorigenic functions. On the other hand, senescence causes negative effects that are reflected by pro-tumorigenic functions causing CSC development and a gain of cancer stemness (**Figure 2**).

An additional level of complexity is added by the plasticity of CSCs as well as non-CSCs, which also causes increased cancer stemness, resistance, and relapse. Examples are given in the next paragraph.

Cancer Stemness: Plasticity of CSCs and Non-CSCs

Cancer stemness is not only triggered by senescence escape and acquisition of stemness phenotypes or supported by maintenance of stemness (544) but also by the plasticity of CSCs and non-CSCs, altogether causing tumor relapses after treatment, as described below.

Plasticity is regulated by the TME that is very heterogeneous and consists of CAFs, TAMs, and neutrophils as well as of cancer-associated adipocytes, tumor-infiltrating lymphocytes, and cancer cells with or without stem cell characteristics (545). Therefore, a clear separation between SASP effects and plasticity cannot be made as several direct and also indirect regulatory networks are involved (**Figure 2**).

Mechanistically, plasticity of cells is a characteristic that ensures robust tissue regeneration and homeostasis (546, 547) and describes the phenotypic and molecular changes of tumor cells increasing stemness and reflecting the tumor's ability to self-renew (18, 548). This phenotype is ultimately closely linked to EMT (15, 548). As described, the transition from the epithelial to mesenchymal state is associated with defined regulatory networks, chromatin remodeling and gene expression programs that are specific to the epithelial, mesenchymal or hybrid cellular state (15–18). Plasticity increases the complexity by suggesting that CSCs can switch between different cellular states, characterized by the expression of surface markers as well as transcription factors (18, 56). Examples for this come from the analysis of different tumor cells: Chaffer et al. demonstrated that $CD44^{low}$ cells (non-CSCs) can switch to a $CD44^{high}$ phenotype (CSCs) resulting in mammosphere formation, a phenotype that could be induced by upregulation of the zinc finger E-box binding homeobox 1 (ZEB1) protein

expression induced by TGF-ß (548), which is a major cytokine of the TME (545). In NSCLC cell lines, two distinct CSC subpopulations have been described by expression of CD133 and the aldehyde dehydrogenase (ALDH) (549). ALDHs compose an enzyme superfamily with metabolic functions. The analysis of its activity is often used to identify CSCs (550, 551). Analyzing CD133 and ALDH activity, Akunuru et al. separated cancer stem/progenitor cells ($CD133^+$, $ALDH^{high}$) from non-CSCs ($CD133^-$ or $ALDH^{low}$) and showed that non-CSCs can interconvert into CSCs. The latter process is activated by TGF-ß signaling or signaling by the zinc finger protein SNAI (Snail) transcription factor family. The described process underlines the dynamic plasticity of CSC/non-CSCs cells (549). After TGF-ß treatment, the authors observed an increase in IL-1ß and IL-6 as well as an increase in $CD133^+$ and $ALDH^{high}$ subpopulations (549).

Interferon-ß (IFN-ß) as well as Oncostatin M (OSM), also cytokines within the TME, have been shown to regulate CSC phenotypes (552). Activation of IFN-ß signaling pathways in non-CSCs blocks the expression of CD44 and Snail, which causes a decrease tumor sphere formation and additionally inhibits invasion (552). In contrast, OSM induces a stemness phenotype in non-CSCs (552). One of the major regulators of colorectal tumor plasticity (either CSCs or cancer cells) are the Wnt-ß-catenin and the KRAS/BRAF/ERK pathways, which have been implicated to regulate tumorsphere formation, self-renewal as well as resistance, as reviewed by Pereira et al. (553) and Zhan et al. (554). Activation of Wnt-signaling increased sphere and clone formation as well as drug resistance (555, 556). Acquisition of stemness was also described by Perekatt et al. using transgenic mice to analyze the function of Wnt-signaling in tumorigenesis and de-differentiation in the gut (28). The authors show that the inactivation of Smad 4, a factor that regulates the differentiation program, promoted the development of adenomas with characteristics of activated Wnt signaling over long-term periods (28). Such Wnt activation can correlate with increased treatment resistance as reviewed by Mohammed et al. (557). Also in gastric cancer, activation of the Wnt pathway causes an increase in CD44 as well as Oct-3/4 expression and correlates with an increased proliferation (558).

As described above, a gain of stemness due to SASP and CSC maintenance or by plasticity of CSCs and non-CSCs, can cause increased resistance (**Figure 2**). CSCs (pre-existing or post-therapeutically generated *de novo*) can escape the treatment by the expression of drug exporters and detoxification proteins, entrance into dormancy as well as resistance to DNA damage induced cell death (4, 15, 185, 559, 560). Their survival causes tumor relapses (**Figure 2**). To interfere with the relapse, several strategies have been under investigation to block CSC resistance and growth (9, 13), as described below (**Figures 3**, **4**).

ERADICATION OF CSCs: NEW TARGETED APPROACHES

Targeting CSCs has been in the focus of research for many years (13). As reviewed by Shibata and Hoque, the combination of CSC-targeted therapies and conventional non-targeted therapies can result in a decreased chemoresistance (9). Approaches of CSC-targeted therapies include kinase inhibitors as well as targeting stem cell associated pathways such as Wnt and β-catenin, some of which have already entered the clinical phase (9, 13). Immunological approaches that target CSCs via MHC-restricted killing include adoptive cell transfer, targeting checkpoint inhibitors as well as antibody-based approaches and vaccination. MHC-unrestricted killing based on NK-, γδT-, and chimeric antigen receptor (CAR) T-cell approaches have been established (561, 562). Currently, these approaches are performed after failures of the first-line therapies.

Based on the promising results of CAR T-cellular therapy in treating hematological diseases, CAR T-cell-based approaches have also moved forward into the therapy of solid cancers (563, 564). Although, CAR T-cell-based approaches face difficulties in treating solid cancers, their therapeutic use could be a promising alternative (563, 564).

CAR THERAPIES TARGETING CSCs

Targeting $CD133^+$ CSCs

Targeting $CD133^+$ CSCs in solid cancers has shown quite promising preclinical results either using monotherapeutic approaches (565, 566) or using combinational approaches together with cytostatic agents (567). Recently, a clinical trial testing CD133-directed CAR T-cells in patients with ALL, AML, breast, brain, liver, pancreatic and ovarian cancers as well as colorectal cancers has been completed (NCT02541370, **Table 9**). Initial results showed feasibility, safety, and efficacy of CD133-directed CAR T-cells in patients. Especially, HCC patients who were not responsive to sorafenib showed a median progression-free survival of 7 months (568). In all patients the duration of response ranged from 9 to 63 weeks; three patients showed a continued response at the time of publication. Stable disease was observed in 14 out of 23 patients for 9 weeks to 15.7 months and 21 patients did not show detectable signs of metastasis (568).

Additional studies (**Table 9**) are ongoing for the treatment of relapsed or refractory AML (NCT03473457), relapsed or late staged sarcoma (NCT03356782), as well as glioma (NCT03423992). A case study of a patient receiving CD133-directed CAR T-cells after previous chemo- and radiotherapy as well as EGFR-directed CAR T-cell therapy reported a partial response for a period of 4.5 months (569). However, severe toxicities affecting the skin, the oral mucosa, and the gastrointestinal tract were reported (569).

Targeting $CD44^+$ CSCs

Although CD44 is a very prominent CSC antigen, only few CAR-based approaches targeting CD44 have been developed. Early approaches that entered clinical trials included monoclonal antibodies and antibody-conjugates. First studies involving ^{186}Re-conjugated antibody against the splice variant CD44v6 showed advantageous effects at first, however a long-term stable disease was only observed in one patient (570, 571). Likewise, the CD44-directed monoclonal antibody RG7356 showed only modest success in clinical trials with AML patients (572) and solid tumors (468). Tijink et al. coupled the CD44v6-directed antibody bivatuzumab to the cytotoxic antimicrotubule agent

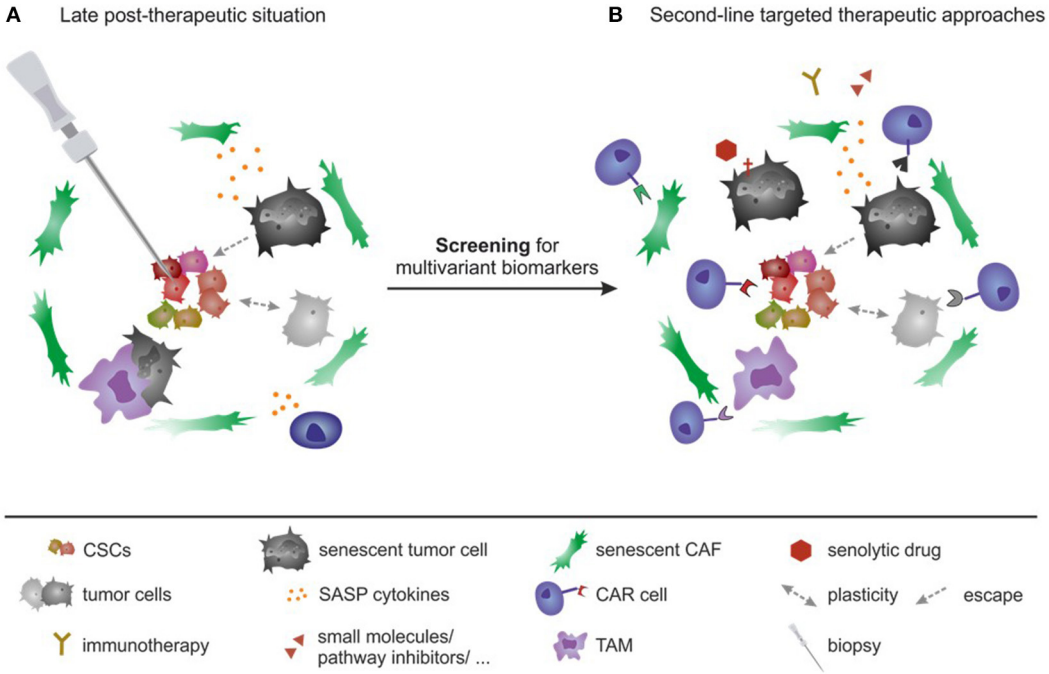

FIGURE 3 | Targeted personalized second-line therapy as a future perspective. **(A)** Analysis of post-therapeutic biopsy samples: follow-up studies need to be included into regular clinical post-therapeutic relapse analysis. After therapy, local biopsies of remaining tumor tissue and/or satellite tissue should be taken periodically (even after several years post-therapy) and a multivariant analysis for biomarkers has to be performed, including the analysis of CSC biomarkers, pro-inflammatory cytokines, senescent markers as well as markers for CAFs. **(B)** Targeted second-line therapy needs to be performed based on the analysis described in **(A)** and will include a specific targeted eradication of remaining cells that could promote tumor relapse and metastasis. Targeted therapies comprise CAR-based approaches targeting CSCs as well as senescent cells or CAFs and TAMs. They also include senolytic drugs to deplete senescent cells independent of CAR approaches.

mertansine to produce an antibody-prodrug conjugate (573). Bivatuzumab mertansine was administered to seven patients and two of them showed stable disease during the therapy phase. However, one patient with squamous cell carcinoma of the esophagus died after treatment due to toxic epidermal necrolysis, which caused the premature cancelation of this trial (573). Because of this fatality, two clinical trials that were conducted in parallel for patients with metastatic breast cancer (574) and head and neck squamous cell carcinoma (575) had to be terminated.

Still, there are some promising approaches involving CD44v6-directed CAR therapies. For instance, cytokine-induced killer (CIK) cells carrying a CAR against CD44v6 showed anti-cancer effects against sarcoma *in vitro* and *in vivo* (576). Furthermore, a phase I/IIa clinical trial using CD44v6-directed CAR T-cells for AML and multiple myeloma patients is currently recruiting (NCT04097301) (**Table 9**).

Targeting EpCAM$^+$ CSCs

Pre-clinical as well as clinical studies targeting EpCAM$^+$ cancer cells using monoclonal antibodies or CAR constructs have been performed to date using co-culture and xenograft approaches (577–579) (**Table 9**). Combination therapy of EpCAM-directed CAR NK-92-cells and regorafenib, a potent multikinase inhibitor, resulted in a synergistic antitumor effect using for example colorectal cancer cells or xenograft models (580). CAR T-cells targeting EpCAM have been shown to significantly block tumor growth in xenografts and to secrete cytotoxic cytokines, including interferon-γ (IFN-γ) and tumor necrosis factor alpha (TNF-α) *in vitro* (579). Additionally, an injection of EpCAM-directed CAR T-cells led to delayed disease progression in immunodeficient mice with peritoneal ovarian and colorectal xenografts (581). Currently, there are several clinical trials with EpCAM-directed CAR T-cells listed for patients with various malignancies: three trials are ongoing (NCT02915445, NCT03563326, and NCT03013712), one trial is not yet recruiting (NCT04151186), and four trials are listed with unknown status (NCT02725125, NCT02728882, NCT02735291, and NCT02729493) (**Table 9**).

LSC-Directed CAR Therapies

In the field of CAR therapeutics, CD123 and CD33 are frequent targets for AML-specific CAR cells (**Table 9**). CAR T- and CAR NK-92-cells redirected against CD33 have entered clinical trials (**Table 9**). Case reports show a good tolerability of CD33-directed CAR NK-92-cells (372), but disease progression after treatment with CD33-directed CAR T-cells was still present (387). Currently, numerous clinical trials using CAR T-cells targeting CD123 are ongoing. NCT03672851 with two participants had to be terminated due to adverse effects (582). Furthermore, first studies implement CLL-1 as a target of CAR T-cells [**Table 9**; (419), NCT04010877 and NCT03222674].

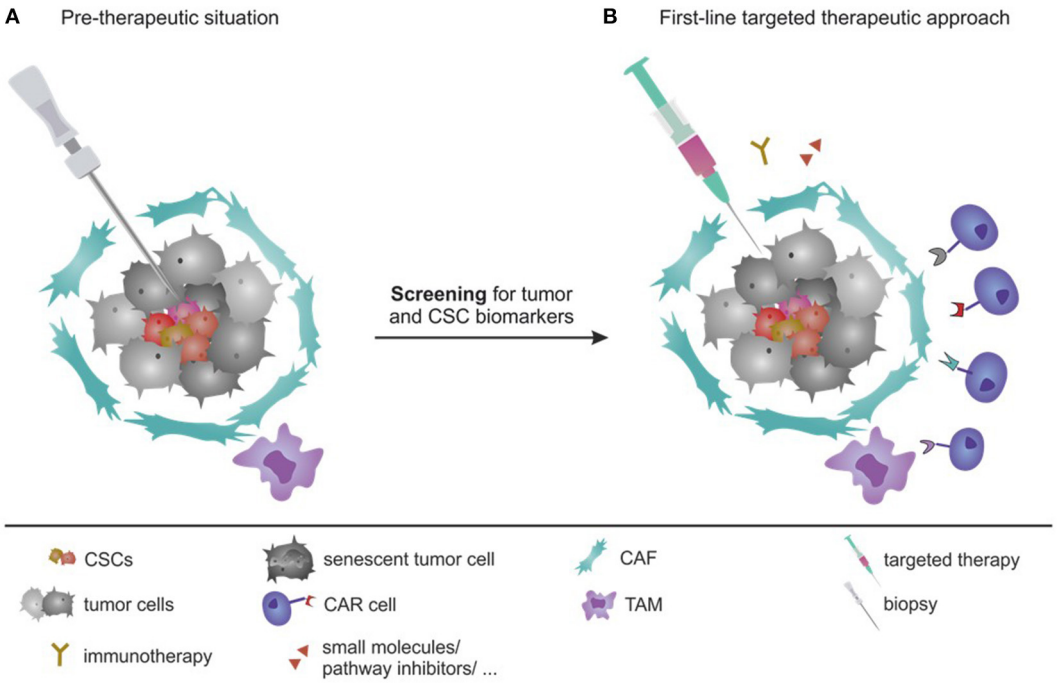

FIGURE 4 | Targeted personalized first-line therapy as a future perspective. **(A)** Pre-therapeutic period: local biopsies before the therapy would allow to determine the heterogenic composition of the tumor, consisting of several biomarkers to be analyzed (CSC, CAFs, and TAMs biomarkers, tumor cell antigens, as well as e.g., T-cell compositions). **(B)** First-line targeted personalized therapeutic approach—therapeutic regimens could combine several approaches: the chemotherapy and small molecules (both selected based on tumor genotype), combined with immunotherapies (antibodies and checkpoint inhibitors based on tumor and analysis of T-cell phenotype), as well as CAR cell-based therapies targeting CSCs, CAFs, and TAMs. Combination therapy will allow a precise and efficient targeting of the heterogenic tumor composition from the beginning on.

NEXT GENERATION CARs AND TARGETING OF CSCs IN COMBINATIONAL THERAPIES

For the more efficient CSC elimination, different approaches that have been developed can be used, i.e., tandem CAR T-cells (TanCAR) (583) as well as single universal (U) tricistronic transgene CAR T-cells (UCAR T-cells) (584). Multi-targeting of Her2, IL-13 receptor subunit alpha-2 (IL13Rα2), and ephrin-A2 (EphA2) was shown to overcome antigenic heterogeneity in 15 primary GBM samples and to increase the therapeutic success using xenograft models (584). Targeting two or more antigens may increase the risk for on-target/off-tumor toxicity, since most of the antigens are not only expressed on malignant cells, but also on healthy cells (60, 585). Improved safety, specificity, and flexibility can be obtained using universal CARs (UniCAR) or split, universal and programmable (SUPRA) CARs (585–589). Both consist of an inert and universal CAR construct without a single chain variable fragment (scFv) adaptor molecule domain in combination with a multiple tumor-targeting scFv adaptor molecule (585, 588, 589). In both cases, the activity of CAR T-cells can be regulated by the dosage of the scFv adaptor molecules or by introducing competitive molecules, such as leucine zippers as a regulator for the SUPRA CARs (588, 589). Additional safety of CAR T-cells can be achieved by the induction of suicide genes, e.g., iCasp9 (590, 591) or by inhibitory CAR (iCAR) constructs, in which signaling domains consist of an immuno-inhibitory receptor [e.g., CTLA-4 or PD-1; (592)]. An antigen only expressed on the surface of healthy cells is a target of iCAR and therefore the the attack of non-tumorigenic cells is greatly reduced (592). Specificity can be improved by using synthetic Notch (synNotch) receptors. The binding of synNotch specific to the antigen induces the cleavage of an intracellular domain and activates in turn the transcription of a second CAR, specific to another tumor antigen (593).

To enhance the targeting of solid tumors using CAR-based approaches, the combination treatment with conventional chemotherapeutic drugs could be a novel strategy to enhance antitumor response. To test this approach, NK-92 cells were modified with an EGFR-directed CAR construct against renal cell carcinoma (RCC) cell lines (594). In combination with the multikinase inhibitor cabozantinib, EGFR-directed CAR NK-92 cells showed synergistic effects *in vitro* and *in vivo* (594). Cabozantinib also caused a decrease of the anti-inflammatory PD-L1 surface expression in renal cell carcinoma cell lines (594). Furthermore, cabozantinib is known to reduce tumor infiltration of immuno-modulatory subpopulations like regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) (594, 595).

TABLE 9 | Overview of clinical trials using current CAR-cell-based approaches in solid and hematological cancers targeting CSC.

Phase	ID number	Approach	Target	Cell-based therapy	Condition
I	NCT03423992	CAR T	CD133, EGFRvIII, IL13RvIII2, Her-2,EphA2, GD2,	Autologous CAR T-cells	Recurrent malignant glioma
I	NCT03563326	CAR T	EpCAM	WCH-GC-CAR T	Neoplasm, stomach metastases
I	NCT02915445	CAR T	EpCAM	CAR T-cells	Malignant neoplasm of nasopharynx TNM stagingdistant metastasis (M), Breast cancer recurrent
I	NCT03766126	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03672851	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03190278	UCAR T	CD123	Allogeneic CAR T-cells	Relapsed/refractory AML
I	NCT04106076	UCAR T	CD123	Allogeneic CAR T-cells	Newly diagnosed AML
I	NCT02159495	CAR T	CD123	Autologous/allogeneic CAR T-cells	AML (various) or blastic plasmacytoid dendritic cell neoplasms
I	NCT03585517	CAR T	CD123	CAR T-cells	Relapsed/refractory AML
I	NCT04014881	CAR T	CD123	CAR T-cells	Relapsed/refractory AML
I	NCT03114670	CAR T	CD123	Donor-derived CAR T-cells	Recurred AML after allogeneic hematopoetic stem cell transplantation
I	NCT03796390	CAR T	CD123	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03126864	CAR T	CD33	Autologous CAR T-cells	Relapsed/refractory AML
I	NCT03795779	cCAR T	CLL1-CD33	CAR T-cells	Relapsed and/or refractory, high risk hematologic malignancies
I	NCT02799680	CAR T	CD33	Allogeneic CAR T-cells	Relapsed/refractory AML
I/II	NCT04097301	CAR T	CD44v6	Autologous CAR T- cells	AML, multiple myeloma
I/II	NCT02541370	CAR T	CD133	Autologous or donor-derived T-cells	Liver cancer, pancreatic cancer, brain tumor, breast cancer, ovarian tumor, colorectal cancer, acute myeloid, and lymphoid leukemias
I/II	NCT03356782	CAR T	CD133	Autologous CAR T cells	Sarcoma, osteoid sarcoma, ewing sarcoma
I/II	NCT03013712	CAR T	EpCAM	Autologous CAR T-cells	Colon cancer; esophageal carcinoma; pancreatic, prostate cancer; gastric cancer, hepatic carcinoma
I/II	NCT03556982	CAR T	CD123	Autologous/allogeneic CAR T-cells	Relapsed/refractory AML
I/II	NCT03222674	Multi-CAR T	CD33, CD38, CD123, CD56, MucI, CLL-1	Autologous CAR T-cells	Relapsed/refractory AML
I/II	NCT04010877	Multiple CAR T	CLL-1, CD33, and/or CD123	Autologous/allogeneic CAR T-cells	AML
I/II	NCT04109482	CAR T	CD123	Autologous CAR T-cells	Relapsed or refractory blastic plasmacytoid dendritic cell neoplasm, acute myeloid leukemia, and high risk myelodysplastic syndrome
I/II	NCT02944162	CAR NK	CD33	NK-92-cells	Relapsed/refractory AML
I/II	NCT01864902	CAR T	CD33	Autologous or donor-derived T-cells	Relapsed/refractory AML
I/II	NCT03971799	CAR T	CD33	CAR T-cells	Children and adolescents/young adults (AYAs) with relapsed/refractory acute myeloid leukemia (AML)
II/III	NCT03631576	CAR T	CD123/CLL-1	CAR T-cells	Relapsed/refractory AML
-	NCT03473457	Single or double CAR T	CD33,CD38, CD56, CD123, CD117, CD133,CD34, or MucI	CAR T-cells	Relapsed/refractory AML
II	NCT02729493	CAR T	EpCAM	Autologous CAR T-cells	Relapsed or refractory liver cancer
II	NCT02725125	CAR T	EpCAM	Autologous CAR T-cells	Relapsed or refractory stomach cancer
N.A.	NCT04151186	CAR T	EpCAM,TM4SF1	CAR T-cells	Solid tumor

Source: http://clinicaltrials.gov/.

The combination of the multikinase inhibitor sunitinib and CAR T -cells targeting carbonic anhydrase IX (CAIX) has been shown to be of advantage as sunitinib reduces immunosuppressive components of the TME (596). Improvements could also be made using Her2-directed CAR NK-92-cells (92/5.137.z) in combination with apatinib (597). Treatment with CAR NK-92 alone resulted in an efficient elimination of small $Her2^+$ tumor xenografts *in vivo*, but not in an elimination of larger solid tumors in gastric cancers (597). A combinatorial treatment with apatinib increased CAR NK-92 cell infiltration into these larger tumor xenografts and resulted in an enhanced antitumor efficacy of the cells (597).

In AML, early approaches focused on the targeting of single markers; combinatorial therapies, targeting more than one marker, have been tested here as well (598). Haubner et al. analyzed optimal combinations of different LSC markers and concluded that CD33/TIM-3 or CLL-1/TIM-3 combinatorial targeting is most suitable since these markers maximally cover AML cells and are minimally co-expressed on HSCs (370). Interestingly, the combination of CD33 and CD123 was found unsuitable (370). Approaches that already implement combinatorial targeting of AML LSCs include tri-specific killer engagers against CD33 and CD123 (373), compound CAR T-cells against CD33 and CD123 (374) or CLL-1 and CD33 (i.e., NCT03795779), universal CAR T-cells against CD33 and CD123 (375), and CAR CIK-cells against CD33 and CD123 (376).

FUTURE PERSPECTIVES

Studies obtained in the last 5–10 years confirmed the importance and the urgent need of diagnostic screening of the TME not only before the treatment, but also at several stages in the post-therapeutic period. This is within the context of personalized therapies that are based on the idea to identify the best therapeutic approach for the patient. This approach should be based on the tumors molecular signature, involving the TME. The best and the most appropriate therapeutic options, which match each individual patient's requirements will increase the therapeutic efficacy and will cause fewer side effects.

The particular value of post-therapeutic local biopsies is that they enable the evaluation of tumor relapse risk on the basis of multivariate biomarkers and also provide information on therapeutically addressable targets within the remaining tumor tissue. In-time detection of tumor-promoting cells, which re-emerge in the post-therapeutic period (**Figure 3**), will allow an application of the individualized and precise second-line therapy in a timely fashion. Detection of tumor cells with stemness phenotypes will allow for their directed and specific targeting using the second-line treatments, depending on a different mode of action (4, 560). This secondary specific therapy can include, targeted therapies such as e.g., immunotherapies, CAR NK-, and CAR T-cells that mediate a precise eradication of several types of cells: CSCs, CAFs, and/or remaining senescent cells. To increase the specificity and therapeutic outcome and to decrease severe side effects, CAR-based therapeutics are constantly being optimized, as discussed in the section above. Special needs are: improvement of target specificity in combination with decreased off-target effects. In addition, secondary therapies could also include senolytic drugs that selectively kill senescent cells as it was discussed in a recent comprehensive review by Short et al. (599). These therapies cause very low or minor side-effects after their administration (599). In the post-therapeutic period, however, it is important to focus on the biomarkers of CSCs as well as the biomarkers of senescent tumor cells, tumor-promoting SASP molecules, CAFs and TAMs. These cells and molecules strongly influence tumor relapse and their monitoring and their in-time elimination is crucial (**Figure 3**). As currently available blood test systems are not sensitive enough to detect local changes in the TME, other methods for instance local biopsies and subsequent multivariant analysis of obtained tissues should be used whenever possible and even after many years upon the first-line therapy (**Figure 3**).

The analysis of multivariant biomarker, however is not only of importance within the post-therapeutic situation. A detailed understanding of the tumor composition before the treatment could allow straight forward first line therapies (**Figure 4**). Target analysis includes CSCs, CAFs, tumors cells and TAMs, and other tumor-promoting cells. Therapeutic options such as chemotherapy and radiotherapy in combination with small molecules and immunotherapies (CAR cells) could tremendously improve the outcome of the first-line approaches and predict relapses (**Figure 4**). Combinations already in the first-line therapy are especially required in advanced stages of malignant disease.

In conclusion, our review gives an overview of the most important biomarkers of CSCs in the TME. Furthermore, we underline the value of local biopsies and precise diagnostics and screening of biomarkers in both pre- and post-therapeutic situations (**Figures 3**, **4**). We suggest the implementation of those strategies in the first and second-line personalized therapy required to eradicate the remaining tumor-promoting senescent tumor cells, CAFs, TAMs, and finally CSCs to protect from tumor recurrence.

The high costs are one point of contention regarding the biopsies and their analysis as well as the implementation of immunotherapies into the first and secondary line targeted therapies. However, considering the costs for therapies, comprising resection, and medication strategies, as well as the patient's sufferings due to a re-emerged full-blown cancer, the targeted therapy will help to save the patients and clinics from high personnel, emotional, and medicinal costs.

AUTHOR CONTRIBUTIONS

SF, UK-B, and TY performed a conceptualization for the review and defined the future perspectives. LW, A-KK, HS, RK, SD, AS, A-RB, TY, SF, and UK-B analyzed the publications and created

the figures and tables. All authors contributed to the article and approved the submitted version.

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Flow Cytometric Analyses of Lymphocyte Markers in Immune Oncology: A Comprehensive Guidance for Validation Practice According to Laws and Standards

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Many anticancer therapies such as antibody-based therapies, cellular therapeutics (e.g., genetically modified cells, regulators of cytokine signaling, and signal transduction), and other biologically tailored interventions strongly influence the immune system and require tools for research, diagnosis, and monitoring. In flow cytometry, *in vitro* diagnostic (IVD) test kits that have been compiled and validated by the manufacturer are not available for all requirements. Laboratories are therefore usually dependent on modifying commercially available assays or, most often, developing them to meet clinical needs. However, both variants must then undergo full validation to fulfill the IVD regulatory requirements. Flow cytometric immunophenotyping is a multiparametric analysis of parameters, some of which have to be repeatedly adjusted; that must be considered when developing specific antibody panels. Careful adjustments of general rules are required to meet legal and regulatory requirements in the analysis of these assays. Here, we describe the relevant regulatory framework for flow cytometry-based assays and describe methods for the introduction of new antibody combinations into routine work including development of performance specifications, validation, and statistical methodology for design and analysis of the experiments. The aim is to increase reliability, efficiency, and auditability after the introduction of in-house-developed flow cytometry assays.

Keywords: flow cytometry, procedures, accreditation, quality control, laboratory diagnostics, validation

INTRODUCTION

Medical routine and study laboratories are subject to a large number of regulations. Recommendations on standard practices for flow cytometry (FCM) validation procedures must comply with legal obligations, the European Regulation 2017/746 on *in vitro* diagnostic medical devices (EU-IVD-R), which also contains mandatory requirements for *in vitro* diagnostic medical devices (IVD) developed and manufactured in healthcare facilities within the European Union (1).

FCM is applied in different analytical fields that comprise assays for research use only (RUO), preclinical applications (PCA) as well as routine methods provided as medical laboratory services. Quality standards for RUO assays and PCA depend on specific rules set by authorities or research and development (R&D) institution, respectively. A common framework for research reporting is the "Minimum Information about a Flow Cytometry Experiment" (2); preclinical rules depend on the context.

Immune therapies for tumors require manifold flow cytometric support. Firstly, while detection of circulating tumor cells is still experimental (3), diagnosis of leukemias and lymphomas is well-established, and a few IVD test kits already exist. Secondly, monitoring of hematological and solid tumor response to therapy is increasingly important, especially in antibody therapies, e.g., reduction of normal of malignant B cell counts following antibody therapy (4), detection of checkpoint inhibitor receptor expression (5), or quantification of CAR-T cells following CAR-T cell therapy (6). Next, detection of adverse effects of novel therapies on lymphocyte subpopulations and their functions supports best medical practice and provides additional knowledge in novel treatments (7).

Our recommendation aims to provide guidance to fulfill legal and normative obligations of EU-IVD-R and EN ISO 15189 (ISO), respectively. Technical terms given in the following recommendations were taken from International vocabulary of metrology (VIM)—Basic and general concepts and associated terms (8). Technical terms from the EU IVD-R are preferred because of their mandatory character in cases of lack of conformity with VIM.

FCM encompasses a wide range of different methodological approaches. It is not in the scope of this article to provide detailed experimental protocols that consistently cover all FCM-based applications. Rather, our focus is on aspects that (i) address specific problems of FCM for novel diagnostic requests, (ii) are common to most FCM-based assays intended for use as a medical laboratory service, and (iii) are minimal experimental requirements that are mandatory to fulfill the above mentioned legal and normative obligations.

Abbreviations: CAR-T, Chimeric antigen receptor transduced T lymphocytes; CE, Conformité Européenne; CI, Confidence interval; CLSI, Clinical and Laboratory Standards Institute; CME, continuing medical education; CPD, continuing professional development; CV, coefficient of variation; DLR, diagnostic likelihood ratio; EQA, external quality assessment; EQC, external quality control; ESCCA, European Society for Clinical Cell Analysis; EU-IVD-R, European Regulation on *in vitro* diagnostic medical devices; FCM, flow cytometry; FMO, fluorescence minus one; FSC, forward scatter; ICCS, International Clinical Cytometry Society; ICSH, International Committee for Standardization of Hematology; IMDRF, International medical device regulators forum; IQC, internal quality control; ISAC, International Society for Advancement of Cytometry; IVD, *in vitro* diagnostic medical devices; LDT, laboratory developed tests; LIMS, laboratory information management systems; LoB, limit of blank; LoD, limits of detection; LoQ, limit of quantification; MRD, minimal residual disease; QQ-plot, quantile-quantile-plot; RoE, risks of error; SD, standard deviation; SOP, standard operating procedure; SSC, side scatter; TOST, test of one-sided significance; VIM, International vocabulary of metrology.

LEGAL AND REGULATORY OBLIGATIONS

EU-IVD-R defines IVD as "…any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, piece of equipment, software or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body…" in the field of medical healthcare (1). The CE (Conformité Européenne) mark certifies that an IVD is in compliance with the European *In vitro* Medical Device Directive 98/79/EC. According to EU-IVD-R, the use of CE-marked IVDs is mandatory for all laboratories that perform diagnostic tests in patient care. So-called in-house tests can only be employed if no product with CE marking is available on the market that meets the appropriate level of performance, which is the case for many parameters in the field of immune oncology. Laboratories must also comply with EN ISO 15189 or, where applicable, appropriate national regulations. Minimum standards are the general safety and performance requirements according to Annex I of the EU IVD-R. Furthermore, a documented risk management system as well as the definition and evaluation of analytical or clinical performance characteristics must be maintained throughout the entire life cycle of an IVD.

ISO 15189 (9) aims to implement the quality assurance policy into medical laboratory services (10–12). This must consider biological and technical specificities encountered in some technique such as in quantitative cell analysis (cytometry) as recently discussed (13, 14).

There are numerous relationships between the requirements of the EU-IVD-R (1) and ISO 15189 (9), which are further modified by national legislation. ISO 15189 accreditation covers laboratory management and technical issues. The first part addresses general laboratory organization in detail (9). The second part addresses technical issues (**Supplement I**) classified under Ishikawa (Fishbone) diagram (15). Much information is common to any analysis:

- Operator authorization (ISO 15189 chapter 5.1),
- Environment (5.2),
- Instruments and reagents (5.3),
- Sampling and pre-analytics (5.4),
- Validation, metrology, or contamination (5.5), (5.6),
- Post-analytics and reporting (5.7 to 5.9), and
- Laboratory information management system (LIMS; 5.10) (9).

Additional information is highly specific to each analysis: method settings, validation, exclusion of interferences (5.5), and quality control and standardization (5.6).

ISO 15189 allows a flexible scope that is highly recommended to FCM laboratories. Flexible scope allows continuous expansion of the range of flow cytometric parameters. This depends on well-established validation procedures, followed by continuous evaluation and occasional improvements. This must be periodically supervised by audits, reports, and management reviews (14).

Various aspects of laboratory management (Quality management, LIMS, agreements, client feedback, complaints,

etc.) as well as of the analytical process (measurement, "mother nature") follow general rules of laboratory diagnostics and will not be discussed here. In contrast, manpower, material, machine, and method require serious consideration in the field of FCM for which consensual resolution is needed. Various national activities have been published to support laboratories in the validation process, for instance in Brazil (16) or Germany (17, 18).

COMMON PRACTICE IN IMMUNE ONCOLOGICAL FLOW CYTOMETRY

Whereas, the EU-IVD-R determines the necessary properties to be validated, both general and FCM specific guidelines have been developed that provide more detailed information regarding the experimental design and statistical methods for analysis. In particular, the guidelines developed by the Clinical and Laboratory Standards Institute (CLSI "evaluation protocols") are quite helpful (19–21). However, adaptation of the guidelines to flow cytometry is challenging.

Several attempts have been made to develop guidance for method validation experiments for flow cytometry-based assays (22, 23). Although the guidance by Selliah et al. (23) provides a wide range of experiments as well as acceptance criteria, the statistical methodology, including the rules for deriving the necessary sample sizes, do not correspond to the state of the art.

Finally, it must be mentioned that there is still inconsistency in the terms used to describe parameters to validate. For example, in the EU-IVD-R the term "analytical sensitivity" is still used although the definition of limits of detection (LoD) and quantitation (LoQ) offer a more precise description of the underlying concepts. Another example is the use of the term "accuracy." It is differently defined in the pharmaceutical world as describes "the systematical error of a measurement" (24), while in the laboratory medicine community where accuracy encompasses both systematic and random errors. Internationally accepted white papers and protocols have been published on this topic (23, 25). The aim of our paper is to propose a reasonable but also efficient consensus strategy for introducing laboratory-developed panels and performing method validation in clinical FCM laboratories as well as to propose minimal criteria to fulfill.

WHAT MAKES FCM SO UNIQUE?

Guidance for method validation in FCM is hard to establish due to the complex nature of this technology. This includes the requirement for samples, the fact that cell characterization requires multiple parameters which can be evaluated in different combination and the high number of interacting variables in each experiment. This will become even more complicated in future when high-parameter research methods such as clustering become routine (26). There are many different clustering algorithms for evaluation of cytometry results. The Flow Cytometry Critical Assessment of Population Identification Methods (Flow-CAP) challenge has made a comparison of performance for flow cytometry clustering algorithms (27). They found that these programs are not accurate enough and too slow for routine use. While specific programs were found to be accurate, slowness rendered them impractical for routine use in clinical laboratories. New algorithms are being developed that address these problems (28).

Relevant parts of the laboratory process are shown in **Box 1**.

The major error sources in FCM (**Box 2**) are related to (i) sample quality, (ii) protocol and panel design, (iii) methods used for instrument settings, standardization, discrimination of negative or positive populations and absolute counting and (iv) data analysis and interpretation (29). Panels must be well-designed and spectral overlap must be sufficiently recognized and properly compensated (30, 31).

TYPES OF FLOW CYTOMETRIC ASSAYS

Quantitative analyses allow the quantitation of precisely defined cell subsets, even as absolute values. Some EQA and standardization guidelines are available. They can address rare events with a need for high sensitivity (low LoQ).

Quantitation of very rare events has recently been developed for the assessment of residual disease and requires precautions to obtain good repeatability at high sensitivity. A minimum number of parameters and a minimum number of positive events to be recorded are required, which means that the sensitivity up to 0.01 or even 0.001% of leukocytes can only be achieved if at least 3×10^5 to 3×10^6 events are acquired (34). In **Table 1**, cell counts to be analyzed when quantifying rare cells are shown.

Most of FCM analyses are qualitative in nature. They mainly address the identification of cells, such as the diagnosis of leukemia and lymphoma, immune monitoring, or in proliferative or dysplastic disorders. Partial quantitation (%) is then determined and informative but not clinically critical. Standardization and EQC are frequently not available and IQC are rare. Measurement of precision, accuracy, or working range is not relevant.

Functional analyses usually require challenging fresh samples with different stimulants. In this case, quantitation is important but rarely standardized. Calculation of precision is done by

BOX 1 | The laboratory process.

- The pre-analytical phase. Functional assays and some differentiation markers are time- sensitive and require an analysis to be performed within a few hours of blood draw whereas some analyses can still be correctly performed within 72 h. This must be validated for each parameter that is being analyzed.
- In the analytical phase, almost all items to be reported in standard operating procedures (SOP) (including linked documents) are themselves still in need of standardization, including protocol design, international references, operator confirmation, and analytical performances as well as description of the assay principle, validation process, and supervisor authorization.
- The post-analytical phase comprises (i) the technical review of examination results as well as (ii) a plausibility check of the results prior to release. A major issue of post-analytics is to provide valid reference ranges or decision limits.

BOX 2 | Error sources in flow cytometry.

- Daily instrument variation is at risk and must be measured and minimized as much as possible by tracking instrument and reagent stabilities. For clinical labs, CE-labeled cytometers should be used, and manufacturers' advice must be followed.
- Protocol outlines for sample preparation, fluorophore detection and gating strategy are often ill-defined and lack consensus.
- One analysis simultaneously identifies several cell sub-populations and provides as many results. Unlike in most diagnostic tests, one analysis does not mean one result.
- Phenotype definitions are not univocal and are constantly changing. There is no international "gold standard" for determining accuracy in terms of phenotype or absolute quantitative measurements.
- Some analyses such as leukemia typing, or functional investigation require several assays (protocols) and their interpretation require the integration of information from the multiple assays.
- Specificity of antibodies used for the detection of antigens may vary depending on the clone, conjugate, and manufacturer. In contrast, different clones can recognize the same antigen and can be certified through the Human Leukocyte Antigen determination program (32).
- There are many different typical phenotypes that need to be identified in the diagnosis of all possible diseases. Samples are frequently scarce and include bone marrow, punctates, and other biological fluids in addition to various anticoagulated blood. All these samples must be fresh for analysis. It is therefore not possible to have internal quality control (IQC) for each analysis, sample type, or pathological phenotype. However, a few IQC are commercially available, mainly for CD4+/CD8+ T cells or CD34+ stem cells. These IQC can be stored for weeks thanks to stabilizing treatment. Not all cell types could be investigated, and specific needs for immune oncology are not yet met.
- As a result of the continuously evolving landscape of biological understanding, new therapies and technological capabilities, newly optimized antibody combinations must often be incorporated into FCM assays. It is therefore important that protocols must their flexibility.
- Although samples are prepared and analyzed in parallel and several batches can be analyzed in 1 day, each sample is prepared individually with independent risks of error and variability. The analysis of one test within a batch does not depend on the whole batch as it is for microtiter-based serological immunoassays with one common standard curve. The validation of IQC inside the batch does not full guarantee the quality of each analysis. Inversely, a successful analysis on one sample, including eventually one IQC does not necessarily mean the entire batch is valid.
- For the same reasons, external quality assessment (EQA) schemes are rare (http://www.eptis.org). The majority are only available for a small number of analyses, in preserved (meaning altered) conditions. Schemes providing fresh blood samples are rare and expensive (http://www.instandev.de/en.html).
- In absence of international references, absolute counts (in cell concentration or antigen density as well) slightly differ according to the system used as shown in EQA comparisons (33).
- The risk for contamination between samples is not negligible. Samples in a batch can have extreme concentration of at least one cell subset. The sample-to-sample contamination risk depends on the organization of the sample preparation (proximity of the tubes, changes in tips or probe cleaning, and on the efficacy of the probe washing between two consecutive samples.

TABLE 1 | Total number of cells to collect in detection of rare events.

Frequency of Rare Events (1/x)	% of total	Desired coefficient of variation % (rare events required)			
		30 (11)	10 (100)	5 (400)	3 (1,111)
20	5	222	2,000	8,000	22,222
50	2	556	5,000	20,000	55,556
100	1	1,111	10,000	40,000	111,111
1,000	0.1	11,111	100,000	400,000	1,111,111
10,000	0.01	111,111	1,000,000	4,000,000	11,111,111
100,000	0.001	1,111,111	10,000,000	40,000,000	111,111,111
1,000,000	0.0001	11,111,111	100,000,000	400,000,000	1,111,111,111

For very rare cell populations, number of cells to be analyzed increases substantially.

repeating stimulations. The working range can be estimated by testing different concentrations of the stimulant. Sensitivity is estimated by the lower stimulation dose giving a significantly different readout from the negative control. Comparing positive and negative controls offers information of reproducibility of the assays and the frequency of "non-responders" observed for some assays. Measuring accuracy is generally not possible. Inter-laboratory comparison is difficult to organize as samples must be tested within 1 day. Standardization and multi-center clinical evaluations are needed.

VALIDATION OF FLOW CYTOMETRIC ASSAYS

Based on the specific characteristics of FCM mentioned above, procedures must be adapted to render method validation more efficient but realistic in daily practice. First, analytical and clinical validation must be distinguished. Clinical validation (diagnostic accuracy, e.g., sensitivity and specificity) is commonly based on clinical studies. Patient data are usually not accessible for laboratories. This is not the scope of this paper but is briefly shown in **Table 2**.

PARAMETERS FOR VALIDATION

Analytical parameters for a specific assay must be determined independently in each laboratory that performs the assay. This should include, if applicable, analytical sensitivity and specificity, trueness (bias), precision, repeatability, intermediate precision, reproducibility, accuracy (resulting from trueness and precision), limits of detection, limit of quantitation, measuring range, linearity, cut-off, determination of appropriate criteria for specimen collection and handling, control of known relevant endogenous and exogeneous interference (cross-reactions), and robustness. Definitions and specifics for FCM are given in **Table 3**. Analytical performance characteristics given by EU-IVD-R that shall be stated by manufacturers to

TABLE 2 | Clinical performance characteristics given by EU-IVD-R that shall be stated by manufacturers to state "fitness for purpose" need to be maintained during the lifetime of an IVD.

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
CLINICAL PERFORMANCE			
Diagnostic sensitivity	Test positivity in disease, true positive fraction, ability of a test to correctly identify disease at a particular decision threshold (35). In agreement or concordance studies, where the true disease state is not available but the test result of a reference method, the term "percent positive agreement" (PPA) is used instead of sensitivity.	"Diagnostic sensitivity" is used in Europe and "clinical sensitivity" is used in the United States (36). This also applies to "diagnostic specificity". The following question is addressed: To what degree does the test reflect the true disease state? The sensitivity is the fraction of patients correctly identified by the test to have the disease (true test positives) among all patients with the disease (as defined by an independent reference standard). Note that the cut-off should be chosen prospectively according the costs of false positive and false negative results. Data driven approaches like choice of the cut-off according maximum Youden-Index is not recommended because of its high uncertainty. The sensitivity does not depend on the prevalence of the disease, but on the spectrum of patients in the disease or non-disease group, respectively.	Clinical performance assessment requires sufficient analytical evaluation. The initial analytical performance assessment must include "abnormal" samples, which must be distinguishable from normal or negative samples, respectively. Crucial for any diagnostic performance study are well defined clinical conditions that specify positivity. Even though clinical performance assessment is mostly done by clinical studies, laboratories are encouraged to retrospectively evaluate the diagnostic sensitivity of their reported results. In such cases, it is crucial to offer the attending physician structured forms that enable him to provide specific clinical information about the patient and the underlying disease or clinical question. Further information necessary for the evaluation of the results should also be requested. Ideally, the reporting of the diagnostic findings is followed by a follow-up communication with the attending physician, if the latter has information that are relevant to the assessment of diagnostic sensitivity. Since neither clinical studies nor retrospectively assessed diagnostic sensitivity may be suitable to some FCM tests, labs are encouraged to thoroughly perform vertical plausibility checks including all available information in case of follow up investigations.
Diagnostic specificity	Test negativity in healthy, true negative fraction, ability of a test to identify the absence of disease at a particular decision (35). In agreement or concordance studies, where the true disease state is not available but the test result of a reference method, the term "percent negative agreement" (NPA) is used instead of specificity.	The following question is addressed: To what degree does the test reflect the true disease state? The specificity (spec) is the fraction of patients correctly identified by the test to not have the disease (true test negatives), among all patients without the disease (as defined by an independent reference standard). The specificity does not depend on the prevalence of the disease, but on the spectrum of patients in the disease or non-disease group, respectively.	As stated for sensitivity, diagnostic specificity assessment also relies on enough initial analytical performance studies. Clinical studies, a retrospective evaluation and thoroughly plausibility checks are proposed that need to be planned and documented with respect to form sheets provided and assessment strategies. Well-designed panels and protocols provide information for the specificity. Documentation for correlation of cytometry results with other laboratory data for the specific clinical diagnosis is necessary.
Positive predictive value	The percentage of positive test results that are true positives when the test is applied to a population containing both healthy and diseased subjects (35). Note: The positive predictive value varies with the prevalence of the disease in the population tested.	The following question is addressed: How likely is the disease given the test results? The positive predictive value (PPV) describes the perspective of a physician or a patient in view of a positive test result: It is the probability that the patient has the disease (as defined by an independent reference standard) given a positive test result or (post-test probability). The PPV depends on the prevalence of the disease. Its value corresponds to the clinical situation where the test is applied. When a test has a PPV > prevalence, it might have a good diagnostic performance (considering a similar consideration for the NPV in parallel).	Immunophenotyping of certain diseases with special markers, provides information on positive predictive value, such as CD200 for diagnosis of Chronic Lymphocytic Leukemia (CLL). It is specific except nodal MCL – Mantle Cell Lymphoma (37). PPV can be very useful when a combination of monoclonal antibody percentage positivity, fluorescence density, and percentage of cells in a cell population is used. Scoring for Myelodysplastic Syndrome is a good example for this approach (38). Even though sensitivity is low for both "Ogata" and "Red" scores, when combined their high specificity and positive predictive value make these scoring systems a useful tool for clinical diagnosis. Note: The lysis methods can interfere in the results.

(Continued)

TABLE 2 | Continued

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
Negative predictive value	Test negativity in healthy, true negative fraction, ability of a test to identify the absence of disease at a particular decision threshold. Note: The negative predictive value varies with the prevalence of the disease in the population tested.	The following question is addressed: How likely is non-disease given the test results? The negative predictive value (NPV) describes the perspective of a physician or a patient in view of a negative test result: It is the probability that the patient has not got the disease (as defined by an independent reference standard) given a negative test result (post-test probability). The NPV depends on the prevalence of the disease. Its value corresponds to the clinical situation where the test is applied. When a test has a NPV > (100%-prevalence) it might have a good diagnostic performance (taking into account a similar consideration for the PPV in parallel).	The presence or lack of an antigen provide information on Negative Predictive Value (NPV). A good example is 100% NPV (prevalence = 4%, PPV = 5.4%) for neutrophil expression of CD64 for excluding sepsis cited by (39): 100 patients with suspected sepsis were investigated and authors found an excellent negative predictive value for CD64 (100% sensitivity and 100% NPV), although specificity was low in this study (28% specificity). CD34 counts for bone marrow transplantations, depending on the absolute counts, and percentage, also have a PPV and NPV for success of the transplantation. Another example for NPV is the use of specific CD4+ T cell responses to discriminate the latent and active tuberculosis cases. NPV is as high as 92.4% (prevalence = 19.1%, PPV = 80%) for this approach (40).
Likelihood ratio	"Likelihood ratio" means the likelihood of a given result arising in an individual with the target clinical condition or physiological state compared to the likelihood of the same result arising in an individual without that clinical condition or physiological state (1). For a binary test the positive and negative likelihood ration are determined. The positive diagnostic likelihood (DLR+) ratio is the probability of a positive test result given the disease divided by the probability given the non-disease. DLR–: Test negativity in healthy, true negative fraction, ability of a test to identify the absence of disease at a particular decision threshold.	DLR+: The following question is addressed: By how much does the test change knowledge of the disease status? In other words, the positive diagnostic likelihood ratio describes directly the gain in information a test provides (whereas the PPV can only be interpreted when it is set into relationship with the prevalence). Formally, the DLR+ is the ratio of post-test odds and pre-test odds of the disease given a positive test result. Practically, it is calculated as sens/(1-spec) [in case of a binary test]. Meaningful tests should have DLR+ > 1. DLR–: The following question is addressed: By how much does the test change knowledge of disease status? In other words, the negative diagnostic likelihood ratio describes directly the gain in information a test provides (whereas the NPV can only be interpreted when it is set into relationship with (100%-prevalence)). Formally, the DLR– is the ratio of post-test odds and pre-test odds of the non-disease given a negative test result. Practically, it is calculated as (1-sens)/spec [in case of a binary test]. Meaningful tests should have DLR– < 1.	Sometimes presence or absence of one marker effect the likelihood ratio of flow cytometry results as CD49d for CLL prognosis. CD49d is an unfavorable prognostic marker, comparison of likelihood ratio along with other performance measures indicated that omission of CD49d significantly reduces the prognostic power of the prediction models (41). Efforts for development of better analysis and interpretation software in cytometry systems are ongoing. Use of Z-scoring in classification of cells expressing multiple fluorophores, use of spillover in actively scoring events, and the successful classification of multiple fluorophores using a single detector within a flow cytometer is suggested by Lawrence et al. (42) There are too many factors for determination of positive (DLR+) and negative likelihood ratio (DLR–) in cytometry based clinical use. Clinical status of patient, stage of disease, accuracy of the test, environmental and genetic factors, age, gender, accompanying diseases all effect the likelihood ratio. An example for this complicated situation is bronchoalveolar lavage fluid immunophenotyping for CD4+/CD8+ cells in diagnosis and follow up of pulmonary sarcoidosis. A meta-analysis performed for determination of likelihood ratio found PLR as 4.04 while NLR was 0.36 (Likelihood ratios >30 and <0.33 are considered as strong indicators to rule in or rule out a diagnosis, respectively). This suggest that immunophenotyping of CD4+/CD8+ has low ability to discriminate sarcoidosis from non-sarcoidosis (43).

state "fitness for purpose" need to be maintained during the lifetime of an IVD. As commented in this table, although it should be noted that not all performance characteristics can be validated for every flow cytometric setting. And, finally, even if it would be feasible, the full method validation for each modified or novel analysis, each sample type, and each pathological issue would be outrageously expensive and time-consuming. For transparency reasons, we recommend to document which characteristics were not validated and the underlying reasons.

TABLE 3 | Analytical performance characteristics given by EU-IVD-R that shall be stated by manufacturers to state "fitness for purpose" need to be maintained during the lifetime of an IVD.

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
ANALYTICAL PERFORMANCE			
Analytical sensitivity	Quotient of the change in an indication of a measuring system and the corresponding change in a value of a quantity being measured (Slope of an empirical calibration curve (indirect reference measurements).	There are several definitions of "analytical sensitivity" with different meanings. Within this document we use the term "analytical sensitivity" to describe any performance evaluation in terms of LoB, LoD (see below) and/or LoQ (see below), as in the IMDRF framework. Another general term, which is used by CLSI (20), is "detection capability." The term is not used in the CLSI evaluation protocols. It is recommended to refer to LoB, LoD, LoQ (see below).	Sensitivity refers to the precision and accuracy of rare events and dim antigen measurements. It is important for measurable/minimal residual disease analysis for leukemia, lymphoma, and multiple myeloma samples. For this type of samples, to reach to high level of sensitivity, minimal number of cell counts are important. Lower Limit of Detection (LOD) is the lowest number of cells counted. Usually 10–50 events are enough for adequate calculations. At least 50 events are necessary for lower limit of quantitation (LOQ). LOD and LOQ can be obtained by below formula: LOD or LOQ = (MRD Cluster/total cells acquired) × 100% (44). Calibration of flow cytometer is not considered here because this must follow manufacturers advise.
Analytical specificity	Note: analytical specificity resembles the concept named selectivity. Selectivity gives an indication of how strongly the result is affected by other components in the sample (45). The CLSI EP07 (46) uses this term.		Specificity is how well a flow cytometry test determines the specific cell population and/or the antigen evaluated. This includes all stages of cytometry analysis from sample collection to patient report release. Sample type, antibody selections, panel design, analysis, standardized interpretation of results are important for the analytical specificity (23). Heterotypic antibodies and cross-reactivities as well as uncommon target epitopes can cause aberrant results. Specificity of antibodies cannot be verified but should be given by providers, preferentially as CE-labeled IVD.
Trueness (bias)	Closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value (8).	Measurement trueness is inversely related to systematic measurement error. The estimate for the systematic error is the bias. The bias is measured as the difference between an average of quantity values and a reference quantity value used as measure for "true quantity."	Not required/not possible to establish in majority of immune-oncological applications. There is no gold standard. Therefore, most EQA use consensus values.
Precision	Closeness of agreement between indications or measured quantity values obtained by replicate measurements on the same or similar objects under specified conditions.	Comment: Measurement precision is usually expressed numerically by measures of imprecision, such as standard deviation, variance, or coefficient of variation under the specified conditions of measurement. Precision is inversely related to the random error of a measurement and covers several reasons of it. Thus, the precision is measured by evaluating its components (repeatability, intermediate precision and reproducibility). These components refer to specific conditions under which the experiments are performed. Thus, the definition of the conditions is essential for understanding the related precision component.	Intra-assay and inter-assay precision need to be assessed. Intra-assay precision is determined when same sample is measured repeatedly under the same conditions, and how close the results are. Accepted criteria for immunophenotyping are co-efficient variation (CV) of 10–25% (31). For rare events and dimly staining antigens higher CV values may be accepted. Inter-assay precision (reproducibility) is measured by obtaining the variability between the instruments, analysts, and different laboratories.
Repeatability	Measurement precision under a set of repeatability conditions of measurement with *repeatability* condition: condition of measurement, out of a set of conditions that includes the same measurement procedure, same operators, same measuring system, same operating conditions and same location, and replicate measurements on the same or similar objects over a short period of time	The most effective and sufficient experiment follows a hierarchical design. Within this design, several variance components (e.g., repeatability, operator-to-operator-variability and day-to-day variability) are evaluated together. A hierarchical design with nested factors (e. g., 3 operators investigate on 5 days 3 replicates (3 × 5 × 3 measurements). In case of 1 factor and repeatability, the analysis can be performed using simple Excel-Spreadsheets.	Repeatability can be measured by preparing 3–6 samples in at least three replicates. In one run all samples can be tested. This assay should be run on one instrument by one technical person. It should be measured on the most representative type of samples and the most representative cell subset, at different levels. Within the statistical analysis the results per sample are pooled. This analysis, however, requires the homogeneity of the results over the concentration range.

(Continued)

TABLE 3 | Continued

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
Intermediate precision	Measurement precision under a set of intermediate precision conditions of measurement with *intermediate precision* condition: condition of measurement, out of a set of conditions that includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period, but may include other conditions involving changes		This type of measurement can only be assessed with QC samples when available. Because of the sample shortage and the cost of the analysis, repeats cannot be done as many times as usually recommended in biochemistry. Dorn-Beineke et al. recommend higher numbers (17, 18). We believe that 11 repeats (47) would be safer as long as the sample volume makes it possible. We recommend hierarchical designs. **Supplement II** shows the example of an experiment investigating 1 factor together with repeatability.
Reproducibility	Measurement precision under reproducibility conditions of measurement with reproducibility condition: condition of measurement, out of a set of conditions that includes different locations, operators, measuring systems, and replicate measurements on the same or similar objects		Reproducibility measurements for instruments can be performed by two different technicians (one for each instrument). If there is an inconsistency between the results, then the technical person and the instrument need to be evaluated. Stabilized IQC if available can be analyzed daily, keeping in mind that the stabilization procedure alters cell shape and marker expression. Again, because of the sample limited volume and the cost of the analysis, we propose testing at least one IQC per level, per type of sample available, per operating day. Inter operator reproducibility can be estimated by comparing IQC analyses between different operators on different times. We recommend hierarchical designs. **Supplement II** shows the example of an experiment investigating 1 factor together with repeatability.
Accuracy (resulting from trueness and precision),	Closeness of agreement between a measured quantity value and a true quantity value of a measurand.	Accuracy is a conceptual term describing the agreement of a single measured value with the true quantity. Inaccurate measured values could be caused by systematic (bias)= and random (imprecision) errors. The "true quantity" is an ideal state. Accuracy is therefore not directly validated but is covered by validation of trueness and precision. *Systema*tic error: Component of measurement error that in replicate measurements remains constant or varies in predictable manner (7). *Ran*dom error: Component of measurement error that in replicate measurements varies in an unpredictable manner (7). A random error shows up when a measurement is repeated under the same conditions.	If bias could not be established, accuracy given by precision. Comparison of results from different laboratories may be used for calculation of accuracy. Participation to external QC/proficiency testing programs when available will provide the most useful information for systematic error. Systematic error = Mean of bias (48). Random error = Standard deviation of bias
Limits of detection	Measured quantity value, obtained by a given measurement procedure, for which the probability of falsely claiming the absence of a component in a material is β, given a probability α of falsely claiming its presence.	The LoD signals the presence of a measurand in the sample. Lowest measured quantity value at which it is statistically shown that "something" of the component is in the sample (qualitative statement). α and β are typically set to 5%.	MRD is a good example. There are different options for detection of LOD. FMO (fluorescence minus one) can be used as LOD tool, by omitting the antibody of interest. Using healthy donor samples is also possible. Rare results require high cell counts to be analyzed (Poisson challenge). Cell identification is based on a good separation of positive/negative labeling and the sensitivity of detection that is limited if the fluorescence of the conjugate is poor or if the antigen is expressed at low density on cells, e.g., below 1,000 molecules/cell (49). Antigen density can be quantitatively measured using FCM and reference values have been published by the European Working Group on Clinical Cell Analysis (49–51). As an example, B cell antigens have density varying from 12 ± 2 CD21 antigens per cells, 27 ± 3 CD19 up to 149 ± 29 CD20 (49).

(Continued)

TABLE 3 | Continued

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
Limit of quantitation	Lowest amount of measurand in a sample can be quantifiably determined with stated acceptable precision and trueness under stated experimental conditions		Similar tools used for obtaining LOD can be used for LOQ determination. Spiking leukemia samples with known dilutions into healthy donor samples can also provide data for determination of LOQ. This resolution allows to distinguish two populations in a mixture of particles that differ in mean signal intensity (52). It must be adapted to the medical need by adapting the number of total events to be acquired. For the lymphocyte count, a 10–50 cell/μL (10^{-3} of leukocytes) resolution is usually enough while high sensitivity detection, below 0.10–1 cell/μL require an acquisition of at least 10^{-4} to 10^{-5} of leukocytes) or even less (10^{-6} to 10^{-7}) for the assessment of minimal residual diseases.
Measuring range	Working interval set of values of quantities of the same kind that can be measured by a given measuring instrument or measuring system with specified instrumental measurement uncertainty, under defined conditions.		For fit for purpose validation, verification with a minimum of ten donors are recommended when validated IVD/CE assays are used (46). This is not the case for rapidly alternating tests in immune oncology. Purified subsets and depleted matrix close to the sample characteristics (e.g., whole blood) are not available for proper spiking tests. This should be repeated for each of the several subsets analyzed in one analysis. We propose that the linearity of the analysis can be approached, on ONE representative cell subset, by spiking a sample with high concentration of the subset (e.g., Lymphoproliferative syndrome) in one sample with a lymphopenia in the considered subset, as low as possible (e.g., patient treated with depleting biotherapy such as anti CD20 monoclonal antibody). We recommend performing 6 to 10 serial dilutions (1/3 or 1/4) of a sample with a subset at concentration from 10^4 to 10^5 cell/μL, in a sample with same subset at concentration <10 cell/μL as much as possible. Usual sensitivity for reliable routine T cell count requires an acquisition of at least 10,000 leukocytes.
Linearity	Assuming no constant bias, the ability (within a given range) to provide results that are directly proportional to the concentration (amount) of the measurand in the test sample.	According CLSI EP06 (19), the data are analyzed by linear, quadratic and cubic regression. If one of the quadratic or/and cubic regression parameters are significant, the deviation from linear model has to be checked whether they are relevant or not (by regarding them in view of the repeatability of the measurements)	Linearity can be achieved by use of standard calibrators to control the efficacy of fluorescence detectors on the measurement device. To achieve linearity measurement on biological samples can be possible by spiking healthy donor samples with known cells such as leukemia cells.
Cut-off	The cut-off refers to a specific measurement value which is used as a decision limit to distinguish between different categories of test results, typically between positive and negative test results.	Cut-off level is a test value or statistic that marks the upper (or lower) boundary between diagnostic categories, i.e., between negative (acceptable or unaffected) results and positive (unacceptable or affected) results (53).	Cut-off values are used for clinical performance determination and for qualitative tests as detection of allergen-specific basophil granulocytes. For quantitative analysis (expression strength), the minimal level of fluorescent intensity measured on each cell is directly dependent on (a) the antigen density (42, 49), (b) the optimal immuno-labeling (54) and (c) the fluorochrome properties. The use of calibration beads (55, 56) allows to check instrument performance over time and to provide direct comparison of data between different instruments (57, 58).
Determination of appropriate criteria for specimen collection and handling		Common criteria are defined in the pre-analytical handbook of laboratories.	For different matrix (bone marrow, peripheral blood, body fluids) and different analysis (such as platelets or activated platelets), appropriate specimen collection and handling instructions should be validated and be provided in written format. Clotting, contamination, or mucous must be avoided.

(Continued)

TABLE 3 | Continued

Term	Definition/explanation	Comments	Specific considerations for flow cytometry
Robustness	Show, that specific factors have no influence on measurement results	When the aim is to show no influence of the factor, the analysis with equivalence tests (TOST) is appropriate. To use criteria like "no statistical significance (p value >0.05)" as found with a conventional t-test are not correct from statistical point of view since imprecise measurements would lead to false negative results, whereas precise measurements could lead to significant but not relevant deviations and therefore to false positive results.	Robustness can be measured by measuring the tested parameters' impact on results.

PERFORMANCE TARGETS (TABLE 4)

For a validation, we must define acceptance criteria in advance as part of the validation plan. Performance targets must enable the reviewer of the validation data to state whether the determined performance capability is adequate for the intended use or not. In some cases, the assessment may lead to the conclusion that further investigation is necessary or that restrictions exist for the analytical procedure that need to be considered in routine diagnostics.

There are only few international recommendations for tolerated variability in flow cytometric diagnostics. As a rare example, references are proposed in Westgard data base for CD4+ T cells counts although no technical conditions are defined such as system used, internal standards, or even units that are critical in Quality Assurance of the technique as discussed before (15, 59).

EXPERIMENTAL SET-UP

The design of validation experiments must follow general rules but can be adapted if necessary. Especially, very often the small number of samples, the limited time in which the samples can be processed, and the small volume accessible are limiting factors. The best options to overcome this are multi-sample or multi-center approaches. The aspect of sample size as an important part of experimental design is mentioned below.

STATISTICS FOR VALIDATION EXPERIMENTS

There have been strong efforts to improve the quality of statistical approaches in design and analysis of method validation experiments in the last years. There are four principle features of statistical methodology which should be considered (**Box 3**).

In addition to statistical methodology for analysis of validation experiments, the following practical aspects of analysis should be discussed:

- Deviation from normal distribution: Statistical tests determining deviations from normal distribution are not useful for demonstrating a lack of normal distribution. One can apply visual inspection of histograms (no outliers, symmetrical gauss-shaped distribution, or QQ-plot presenting a straight line). Moreover, one can use the fact, that replicates of a measurement are very often normally distributed. Finally, a transformation of data could be useful (see below).
- Outliers or better "aberrant values": Statistical methods could help to identify whether an aberrant value is an outlier, however, the decision whether the outlier has to be incorporated in the data is not a statistical task, since an imperfection of the method, e.g., to handle matrix effects, could be the reason. Rules how to handle outliers must be defined in advance. An easy way to enlighten the situation is to perform the measurements in duplicates and in a random order: when both replicates are aberrant values although they were processed on different positions in the work flow, they cannot regarded as outliers but to be real values. When only one of the replicates is aberrant, it might be an outlier which can be handled according the internal SOP how to handle outliers.
- Counting data like single cells or particles, especially in the low range (1 … $\sim$ 20) follow the Poisson distribution. This distribution has some specific properties in that large imprecision is just given by the distribution and cannot be improved by experimental efforts. It is out of the scope of this report to address the specific approaches necessary for Poisson-distributed data, see (63–65) for further reading. Note that square root transformation of count data is helpful within statistical analysis (66) in the same sense as log-transformation is often applied.
- In case of low sample sizes one can statistically average (other term: pool) the results over the samples. An example are precision analyses: If only a small number of replicates are available per sample, a pooled precision can be calculated as the square root of the sum of squared standard deviations (or by specific methods related to variance components). We refer also to the next chapter, §4, and to **Supplement II**). However, homogeneity of the variances (standard deviations do not systematically depend on concentration) is a prerequisite for the pooling and—if not given—could be achieved by appropriate transformation of data (ln, square root).
- ln-transformation: In case of natural log (ln)-transformation, the standard deviations obtained for ln-transformed data can directly be read as CV in the originally scaled data (for instance: SD=0.1 in ln-transformed

TABLE 4 | Specific method validation and acceptance limits.

	Method specificities		Type of analyses			acceptance limits
Validation	**Dates, operators**	**Quantitative**	**High-sensitive**	**Qualitative**	**Functional**	
Risks	Sample, reagents operator, data analysis	+	+	+	+	
Sample type	Typical cite other accepted	+	+	+	+	
Repeatability	RSD (%)	11 repeats 2 levels. preferentially combined with reproducibility in a hierarchical precision experiment (**Supplement II**)	+	NA	7–10	<10%
Reproducibility	IQC Levey-Jennings, eventual interlaboratory comparison	18-24 tests 2 levels bias to mean of labs preferentially combined with repeatability in a hierarchical precision experiment (**Supplement II**)	NA	NA	NA	<10–15% Precision index < 2* repeatability
Trueness (bias)	EQC usual workflow	3–5/year 2 levels	+	NA	NA	<15%
Global uncertainty	Uncertainty ² = Precision² + Accuracy²/ √3	+	+	NA	NA	
Working range linearity	6–10 × 1/3 or 1/4 dil. At least one subset 1 test, 1 sample type	clinical relevance e.g., 5–5,000 cell/μL, generic form	+	NA	+	Set deviations from linearity in relationship with repeatability
LOQ (low)	% of leukocytes Event acquired	10^{-3} % (10 cell/μL) 2–5 × 10^4 events	10^{-4} –10^{-5}% for 10^5-10^6	Extrapolated		
Sample stability	10 fresh samples on 2-3 days	Subpopulations labeling MFI	+	+	+	<10%
Stability of pre-mixed reagents	2–3 fresh samples fresh/old mix 2 IQC one mix on time	Subpopulations (%) labeling MFI	+	+	+	<10%
Interferences	Atypical phenotype "alert gates"	Generic form	+	Extrapolated	Extrapolated	
Carry-over	3 (very) high, 3 low, 3–5 times	(L1-L3)/(meanH-L3) generic form	+	Extrapolated	Extrapolated	<1%
Method comparison	At least 30 double tests mean difference, slope	Multiple instruments change of technique	Few tests	–	–	Difference∼0, Slope∼1 95% CI within +/– 10...15%
Reference values	30 healthy donors (F/M) initially, to be verified by data from daily routine >100 healthy donors	Most representative Parametric analysis: Two sided: mean +/–2 SD, One sided: mean + or – 1.645 SD, presentation with 90%-confidence intervals non-parametric analysis: percentiles	–	–	–	
Special groups	literature	Children, elderly.	–	–	–	

data CV=10%in originally scaled data, valid up to 30% CV).

EVALUATION OF THE RESULTS

Validation is successful when the acceptance criteria are met. If these performance criteria are not met, this may be for the following reasons: (1) the estimated target value is outside of the criteria, (2) uncertainty of the target value is too high and does not allow a decision, or (3) representative samples are absent in the experiment (e.g., missing positive specimen). Whereas, in case 1 the method itself must be modified, in both latter cases, an extension of the validation process can be indicated. A common approach is a two-step clearance procedure with an extended sample collection phase that increases the

BOX 3 | Four principle features of statistical methodology

1) Stringent use of prospectively defined acceptance criteria, which are used as limits in later statistical tests.
2) Any result (statistical term: estimate) should be reported together with its uncertainty, typically expressed as a confidence interval (CI). Within the framework of statistical analyses, the location of the CI is considered in comparison to the acceptance criteria. If the confidence interval does not overlap with the acceptance limits, the validity is proven. It should be noted that conclusions can only be drawn in this direction: if an acceptance criterion is within confidence interval, no conclusion is possible.
3) We therefore recommend the application of equivalence tests: often, the aim is to show a difference of zero, e.g., in experiments evaluating robustness or selectivity, where the results of distorted measurements should be equivalent to results of an undistorted control experiment. After establishing acceptance criteria prospectively, the CI of the difference of distorted and undistorted measurement results should be within acceptance criteria around zero (**Figure 1**). The related statistical test is the TOST approach (two one sided *t*-tests, see **Supplement III** for details) (60).
4) Finally, sample sizes should be determined by power calculations. Statistical tests differ in their robustness to small numbers of cases. The user should know and estimate the behavior of the algorithms used. Procedures that are more reliable for small case numbers should be preferred. An example is given for robustness in **Table 5**. The sample sizes required for sufficient test power should be known before validation. The resulting test power should be included in the evaluation, especially if the sample size is smaller. Practically, the sample size is determined using software, formulas, statistically derived recommendations as CLSI-guidelines (19–21) and tabulations (see **Table 5** for TOST in this paper). We cannot recommend oversimplified so-called practical approaches ("<5 replicates were found adequate to validate assay imprecision levels below the 5–10% CV" (61). Here, simulations (62) performed on common spreadsheet software or R could be helpful, **Figure 2** shows such considerations for uncertainty of standard deviations one could achieve in simple repeat experiments when 3, 5, 10, 20, and 50 replicates are used.

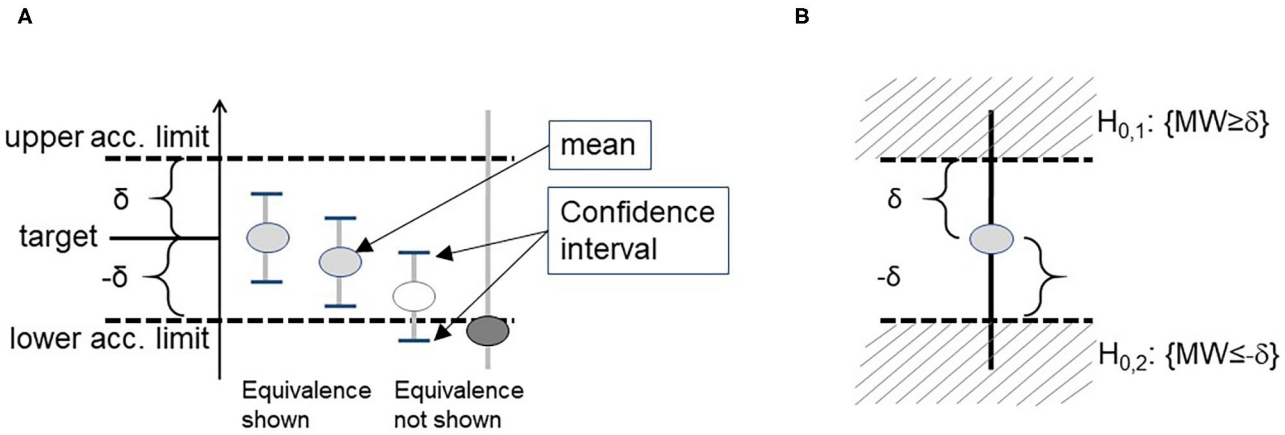

FIGURE 1 | Demonstration of a statistically proof using confidence intervals **(A)**. When this problem is formulated as a statistical test, it refers to the two 1-sided test approach (TOST) **(B)**.

sample size by continuously evaluating the results of measured patient samples and accompanying data on quality assurance. In such cases, the completion of the validation process should be declared preliminary and clear instructions should be given on the measures still to be taken. The reservations resulting from a preliminary clearance status should be formulated and reported to the customers.

OUR PROPOSAL FOR THE INTRODUCTION OF LABORATORY DEVELOPED TESTS IN ACCREDITED LABORATORIES

Considering all difficulties in the accreditation process of FCM analysis and all discussions in dedicated meetings, we propose a reasonable and pragmatic solution (**Table 4**). We also include the consideration that the majority of samples with pathological phenotypes are rare or only available in small volumes and cannot be tested too many times for repeatability and reproducibility.

1. New antibodies are often only available in research-only vials. They are not always labeled with the desired fluorochrome. To check the specific binding, it has proven to be best to use two different or differently labeled antibodies in the validation phase. In addition, Full Fluorescence Minus One control (FMO) must be used to ensure that there is no spill-over into other channels.
2. The reagent quality is guaranteed by the manufacturer, but some alteration can appear during the delivery from the provider to the laboratory according to the conditions. The basic requirement is a stable measuring instrument, which is ensured by daily checking with fluorescent beads. Furthermore, fluorescence intensity of novel antibody batched should be checked with antibody binding standard

TABLE 5 | Sample sizes necessary to demonstrate equivalence via TOST in a paired design when acceptance criteria cover the range (−1, 1), in dependence on standard deviation of the pairwise differences, real deviation, and power.

Sample sizes N for acceptance criteria (-1, 1)		Real deviation						
		0	0.1	0.2	0.25	0.3	0.4	0.5
StdDev	Power	N						
0.25	80%	4	4	4	4	4	4	4
0.5		4	4	5	5	5	6	8
0.75		7	7	8	8	9	12	16
1		11	11	12	13	15	19	27
1.25		15	16	18	19	22	29	41
1.5		21	22	25	27	30	41	58
1.75		28	29	33	36	41	54	78
2		36	37	42	47	53	71	101
0.25	90%	4	4	4	4	4	4	4
0.5		5	5	6	6	7	8	11
0.75		8	9	10	11	12	15	21
1		13	13	15	17	19	26	36
1.25		19	20	23	26	29	39	55
1.5		26	28	32	36	41	55	79
1.75		35	37	43	49	55	75	107
2		45	48	56	63	72	97	139

Overall alpha level is set to 5%. The proportional relationship between acceptance criteria, standard deviation and real deviation can directly be used to derive samples size for other scenarios. Example: Acceptance criteria: +/– 30%, CV of the differences = 15%, real deviation = 0%, power = 80% → sample size = 4 (achieved by using StdDev = 0.5, deviation = 0 and power = 80%). The CV of differences should be the precision of the single experiment multiplied with 1.4 (= square root of 2).

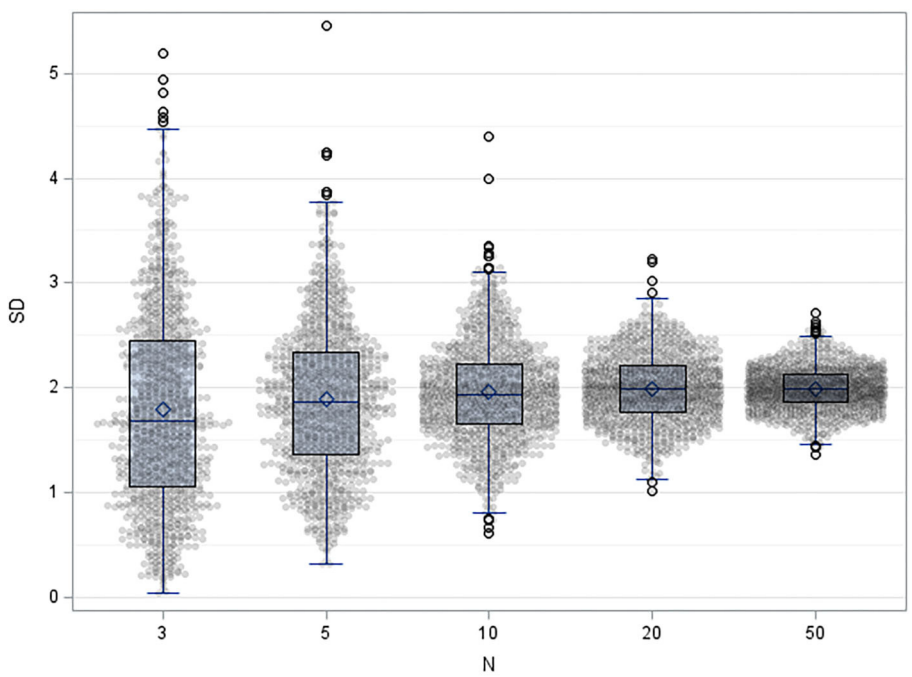

FIGURE 2 | Result of 1,000 simulation of results of repeatability experiment when 3, 5, 10, 20, and 50 replicates are used, with mean=10 and standard deviation =2, shown as dot-plots with overlying Box-whisker plots.

beads. It would be a huge endeavor to check each single vial before doing analysis but daily checks of the fluorescence intensity of control blood is a good way to validate not only the reagent quality but also the labeling process and the state of the sample. The proper labeling can be easily checked by using a pre-recorded template where each cell populations should fit into the gates positioned at the usual place. So, it is critical to validate each analysis with checking all dot plots graphs.

3. The premix stability must be compared to freshly mixed antibodies on a fresh sample or following IQC. Because labeling intensity may gradually decrease with time, not only population phenotypes but also median fluorescence intensity should be compared.
4. Cells are analyzed from different sample types. The analyses are similar to each other within prespecified acceptance criteria regarding the sample type excepting some minor adaptations for the sample preparation. We recommend doing the method validation on one of the most representative type of samples such as peripheral blood or bone marrow aspirate. Sample types which are explicitly unsuitable for the considered test, but which may arrive in the laboratory should be specified and the reasons leading to the rejection of the order should be described.
5. Several cell subsets are analyzed in one analysis (one analysis, several results). However, each subset cannot be fully tested individually. As all subsets are exposed to the same preparation and same risks of errors, we propose to consider that the performances observed for two representative subsets and one type of sample can be used as reference for Quality Assurance for the analysis of the other cell subsets and sample types. The selected sample type should correspond to the most frequently occurring ones. Subsets chosen should be of clinical relevance. Expected values should cover a wide measurement range or at least include both low and high measurement signals.
6. The effect of transportation and storage on sample stability must be tested typically on 10 samples for the acceptable storage duration (2–3 days, dependent on target cells). Again, TOST approaches are helpful for the analysis: the mean of deviations due to a possible instability should be within predefined limits around zero. Modern approaches include using a regression analysis and setting the confidence band of the regression line into relationship with prespecified acceptance criteria (67).
7. Carry-over can be evaluated by measuring consecutively 3 times the sample with the highest content (e.g., Lymphoproliferative disorder) and 3 times the sample with the lowest content (e.g., depleted sample in biotherapy) the day they are both available. The high values should be at least 100 times higher than the lower content. As the risk does not depend on the subset identification, it can be extrapolated to all other subsets. perform the experiment in at least 3 cycles and use non-inferiority testing (= one sided equivalence test) for statistical analysis (68).
8. **Bias estimation/method comparison:** When two or more instruments are used independently or as backup in case of instrument malfunction, assays should be performed repeatedly on both machines for comparison. In clinical FCM, number of repeats is often limited by the number of samples required for valid results, therefore alternative procedures must be found. Statisticians commonly recommend performing at least 30 assays on both systems and the CLSI EP 9 guidance (69) recommends using 40 samples for the laboratory and 100 samples for the manufacturer. When the TOST is used for analysis of difference plots (**Supplement III**), sample sizes provided in table 5 can be used. For analysis Bland-Altman plots (70, 71) as well as specific regression methods like Passing-Bablok regression (72) or Deming regression are recommended (73). Note that simple ordinal linear regression as well as the correlation coefficient r^2–although often used—are not appropriate (74, 75). Especially the r^2 does not detect proportional and constant biases, e.g., one could achieve a $r^2 = 1$ even when one method measures the double of the other method. For analysis the TOST or similar approaches are helpful. In the Bland-Altman plot the CI of the mean of sample-wise differences should be within predefined limits around zero. When regression methods are applied, the CI of the slope should be within predefined limits around 1 and the intercept within predefined limits around zero, or the CI of biases calculated from the regression line vs. line of equality at specific concentrations (typically 3 values within the measurement range) should be within predefined limits.
9. **Precision:** The most effective way to estimate several components of variability follows a hierarchical design with nested factors (e.g., 3 operators investigate on 5 days 5 replicates ($3 \times 5 \times 5$ measurements) (21). Within this design, several variance components (e.g., repeatability, operator-to-operator-variability, and day-to-day variability) are evaluated together (**Supplement II**). Especially repeatability is pooled over several experimental units. In case of one parameter and repeatability, the analysis can be performed using simple spreadsheet-software like MS Excel. It is also possible to pool the results over several samples and use fewer replicates within the factors, however, homogeneity of variances must be achieved for the analysis then, eg. by transformation of the measurement values (ln, square root). One should note that the CI-approach (which would use the one-sided upper confidence limit here) is not common in precision evaluations in the laboratory medicine community. It was shown that the level of variability was mainly related to the size of the population. Accordingly, Tosato et al. (76) described a CV of 2% for large T cell populations, 5.5% for B cells, and 12.5% for NK cells in 10 independent measurements of an IQC for clearly defined markers (Immuno-Trol Cell Control; Beckman Coulter).
10. In the absence of any international standard to validate EQA samples, accuracy can often be approached only by inter-laboratory comparisons in EQA. The targeted accuracy (EQC bias) should be below 15%.
11. Calculation of measurement uncertainty combines reproducibility and accuracy. Because of the rarity of EQA, we propose to use IQC for this calculation.

When investigating measurement uncertainty, it must be considered that the various cytometric stains used are not independent variables. This influences the propagation of errors in a positive way (25).

12. As discussed, the determination of the complete working range is not possible. We propose that the linearity of the analysis can be approached, on ONE representative cell subset, by spiking a representative cell line into one sample with a low count in the considered subset. We recommend performing 10 serial dilutions. The usual sensitivity for reliable routine T cell count requires an acquisition of at least 10 000 leukocytes.
13. Definition of limit of quantitation (LoQ) must be adapted to the medical need by adapting the number of total events to be acquired. For the lymphocyte count, a 10–50 cell/μL (10e-3 of leukocytes) resolution is usually enough while high sensitivity detection, below 0.10-1 cell/μL require an acquisition of at least 10e-4 to 10e-5 of leukocytes) or even less (10e-6 to 10e-7) for the assessment of minimal residual disease.
14. **Robustness, specificity:** When measurements of distorted and not-distorted samples must be compared, it is the aim to show a missing difference. As introduced and explained above, the TOST can be used to show the equivalence. Depending on the design, paired or unpaired measurements must be regarded, whereby a paired design is more powerful. Beside other software, free of cost MS Excel-tools are available (https://www.acomed-statistik.de/en-gb/statistical-tools-download.html#TOST). The sample size depends on width of interval included by acceptance criteria, the expected real difference and its standard deviation as well as on the assumed α (typically 5% and β errors (typically 10–20%). The following **Table 5** provides sample sizes for a paired design (all samples are measured under both conditions; the difference of both results is evaluated in analysis). **Supplement III** provides an example.
15. Reference ranges can be preliminarily calculated from 31 to 35 assays, however CLSI guideline EP28 (77) recommends 120 to 135 healthy donors. The CLSI recommendation refers to a non-parametric estimation of percentiles. Lower sample sizes require the application of complex parametric methods (78). As the reliability of reference ranges is limited if the proposed sample size used, the 90% confidence interval of both lower and upper reference interval limits should be calculated and critically reviewed (10, 11). By doing this, an inappropriate sample size becomes obvious. Even in case of recommended sample sizes the CI are surprisingly wide. More accurate determination specific to the population to be tested (e.g., babies/children, elderly over age 75, or gender) cannot be measured in each lab for practical, economical, and ethical reasons and can be taken from international data available although they are rarely standardized (79–83). Here, quantile regression for age groups is superior but not realistic for most laboratories. A simplified proposal has been described by Özcürümez et al. (84). For complex phenotypes, subset identification regarding antibody combination and gating strategy must be clearly described in the SOP. Gating strategy must be double-checked repeatedly. A simple tool is the control of the quality of the sample in FSC/SSC plots and each single labeling vs. SSC that gives information on the quality and specificity of the immunostaining (85–88).
16. As accreditation is a continuous process, we propose method validation should be repeated periodically. If established, an IQC program should be done every operating day. Precision, working range, and contamination should be checked repeatedly every 1 or 2 years. Normal ranges should be verified every 10 years.

DOCUMENT HIERARCHY

All method descriptions and characteristics must be reported in detail and continuously updated in the accreditation records, SOP, and LIMS. These reports must be easy to read and in a fixed layout.

Because of protocol flexibility and frequent evolution in FCM, details on the method description must be frequently updated. Typical examples would be:

- Removing or replacing an antibody or one clone or
- Adding a washing and red blood cell lysing step, if incomplete lysis was occasionally observed in some samples.

If the same information is cited at different positions along the accreditation forms or in the LIMS, there is a very high risk for discordance. Redundancy severely impairs readability and makes document maintenance risky and error-prone and consequently should be avoided as much as possible.

Lots of facts are common to several assays, e.g., environment, the instrument characteristics, the method principle, procedures on standardization, sample preparation, samples/reagents management, security, and risks. Results of different sub-populations are frequently complementary subsets of some parent populations. Several combinations of antibodies (panels) can have common features. As an example, a panel for diagnosis of leukemia can require 6–8 assays with a common backbone. Multiple results are produced and should then be considered together for interpretation. An accreditation report must combine multiple results (one analysis—several results) or possibly multiple assays as a panel (several analyses—one result), in the same file and preferentially lists of information are presented in a table for readability.

For efficacy and safety reasons, we propose organizing the documents on 4 different levels (**Figure 3**):

1. Any common information must be gathered ("factorized") in a common "generic" accreditation form as much as possible.
2. The specificities (reagents, method, performances) must be detailed in analysis-specific forms: One analysis "one analysis—several results" or "several analyses—one result" in one common accreditation form
3. The technical specificities required for daily practice at the bench and interpretation (gating strategy, reagents specificities, etc.) must be specified in the analysis-specific SOP.

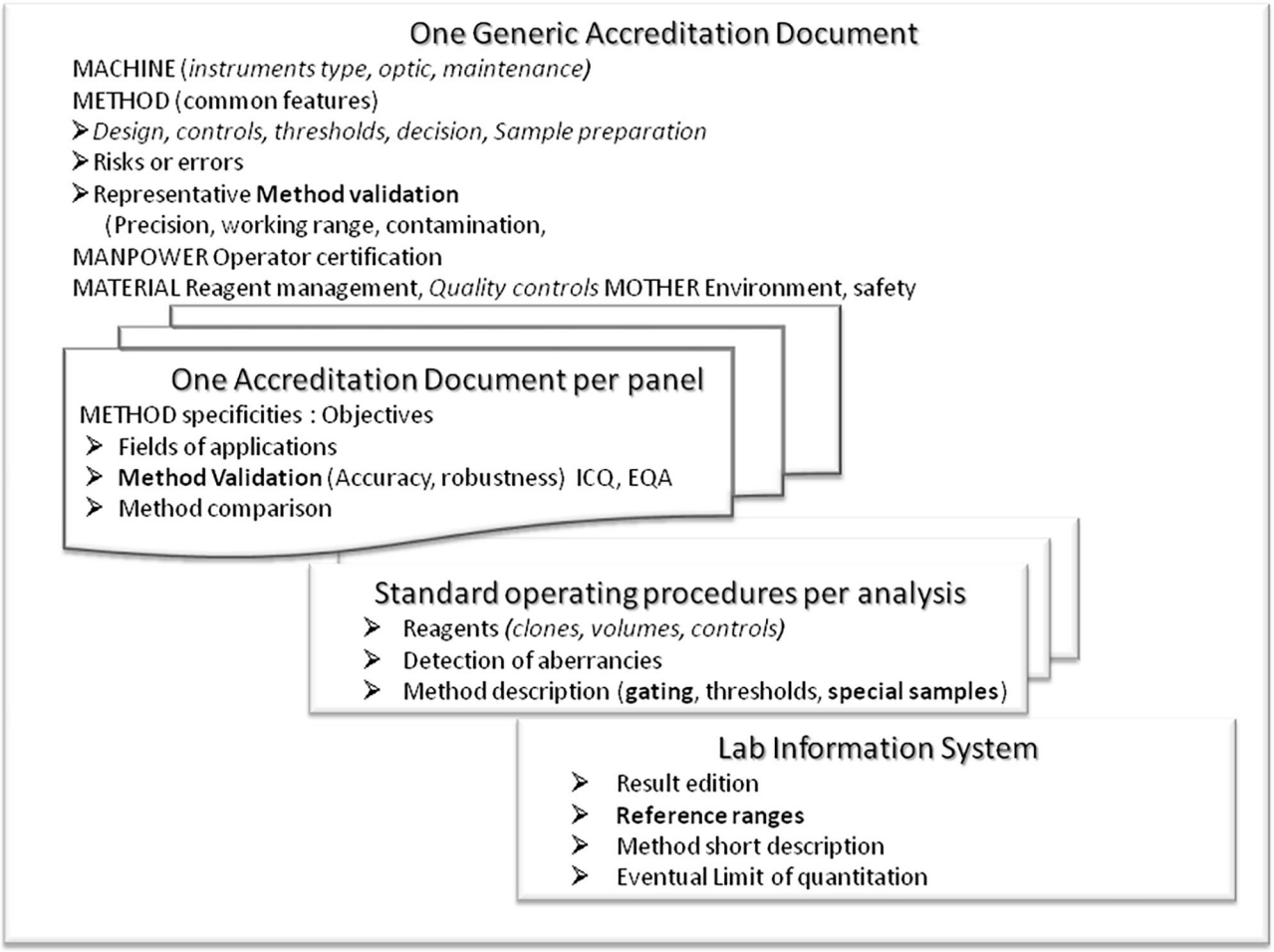

FIGURE 3 | Presentation of the structure proposed for the accreditation documents. A generic form is to record and report all common information (including environment, material, management, manpower) and method characteristics that cannot be tested for each panel. Then specific forms should be written individually per panel (several parameters, several assays). Technical details (antibodies, clones, conjugates, gating strategy, risks of error, and guidelines for interpretation) should be presented in an easy-to-update SOP. Results with technical and reference information should be managed by the laboratory informatics system to be published for correct interpretation. Any redundancy should be avoided for safety and management reasons.

4. The information necessary for interpretation and a report with the results (reference values, LoQ, units, etc.) must be collected in the LIMS.

The generic description must mention all common critical points; operators and supervisors (education, training, CPD/CME, information), environment (storing requirement; work space ergonomics, hygiene, air quality, humidity, room temperature), measurement principles, material management (reagents, standards and samples; conditioning, storing, transportation, label/identity, acceptability/rejection, registration, tracking); instrument characteristics including cytometer and accessory instruments, optical bench, instructions, daily checks for fluidic and optical stability, principles for settings, spectral overlap compensations, standardization of signal detection, check-up, maintenance. Some common components of method validation can also be gathered in this generic form such as sample preparation including process for immuno-labeling, washes, red blood cell lysis, fixation, storing, calibration, absolute counting strategy; units, standards, data acquisition, interpretation; reference to peer recommendations (ICSH), quality control management, risks of error, result validation, recording, transfer, and reporting. Part of the method characteristics is also common. Risks of Error (RoE, caused by pipetting errors of antibodies or internal standards, incomplete lysis of red blood cells, clots, centrifugation, cell loss), and effects on fluorochromes (between fluorochromes, energy transfer, steric hindrance, matrix effects such as bile salts or antibodies to fluorochromes), their detection (minimal count of cells, correct cell location in dot plots) and their prevention and correction must be listed. Most RoE are common to all FCM analyses and thus should be detailed in the generic form rather than in the panel-specific information. Lists of technical parameters/materials (antibodies, fluorescence dyes, clones, provider, concentration) must be presented in tables that are easier to read instead of text and attachments.

The analysis-specific records must include the specificities for the environmental conditions and method (lysis, washing steps, internal standards, dyes, templates, expected normal, and aberrant populations) and should be conceived according to clinical relevance (awareness for doublets or dead cells relevant, relevance of percentages of absolute values, delta check, limit of detection). If required, these forms can also merge data from different analyses like non-stimulated and stimulated cells or different panels for the distribution of T cell clonotypes. These analyses are usually closely related, sharing many features (sample type, incubation steps, lysis, washing buffers, centrifuge, incubation). Each detail that can be changed or adapted frequently should not be included here like reagent lots, pipetting, volumes respective cell numbers of cells, additional washing steps, rare sample types), but in the SOP. These specific forms (per analysis) should also contain as much as possible information on analysis characteristics. Some assays validation could be approached from a related analysis (working range, linearity, limit of quantitation) that cannot be done for all analysis but can be extrapolated from other analyses and described in the generic form (like absolute count linearity, limits of detection, or contamination. This is also true for common errors (like pipetting, reagents quality, centrifugation, red blood cell lysis, cell separation procedures, washing).

The SOP must detail all technical specificities, the method principles, specific reagents (references, isotypes, clones, providers, fluorochromes, and conjugated antibodies), concentrations (based on titration or manufacturer recommendations), calibration, specific requirements on sample preparation, acquisition parameters (delay, number of events to acquire), and expiration date. As phenotype definition is critical, each subset should be clearly described (antibody, gating strategy, population hierarchy) and be referred to peer literature when available. FSC/SSC plots provide valuable information on the sample quality and debris. Doublets and dead cells must be excluded from analysis. This is easily done for dead cells because a live/dead staining such as 7-Aminoactinomycin D or aggregation of dead cells helps to exclude them. Doublet exclusion can be done by gating scatter height vs. area. Population overlap (e.g., lymphocytes and monocytes) must be avoided by gating strategies such as Boolean gates. Backgating and use of color codes are good tools to check the quality of the gating. The template with typical results including dot plots, level of fluorescence intensity expected, and most common and atypical types (sub-populations) should be described. It is recommended that the template include "alert gates" for unexpected combinations to provide a signal in case of improbable phenotypes.

LIMS should include all information needed to interpret the results. Subset definitions, LoQ, reference values must be listed in the data management system (LIMS).

As discussed, operator competence in FCM directly relates to quality assurance. Different projects supporting education and certification at an international standard are under development by various international societies: ESCCA, ICCS, or ISAC. The educational sessions (courses, congresses, etc.) visited by staff members should be clearly described and competence should be tested. All documents must be archived.

EDUCATIONAL SOURCES

FCM technique is rarely formally taught in general biological fields and even less in diagnosis. Only a few countries grant certificates or have study programs in this specific technique like the French University Certificate on Cytometry. The International Society on Analytical Cytometry (ISAC) proposes an internationally recognized qualification in basic cytometry (International Cytometry Certification Exam (http://cytometrycertification.org/) with continuous follow up. The International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) offers courses and schools, organized by the working group flow cytometry WG-FC (http://www.ifcc.org/). The European Society for Clinical Cell Analysis (ESCCA) promotes continuous education and training in annual international schools and courses as well as professional development and evaluation on specific topics. In 2017, ESCCA has initiated an examination for their members to become an ESCCA-certified cytometrist. ESCCA European cytometry certification includes two levels of certification, one for cytometry operators and one for cytometry specialists (http://www.escca.eu).

CONCLUSION

We propose a "generic" accreditation method for all common steps (instrument settings, protocol design, and data analysis and decision strategy), a detailed description of each method (protocol, RoE), and quantitative validation of a few representative methods. More detailed and frequently updated data such as reagent characteristics, gating strategy, typical results, and reference data must be described in the SOP and, in part, also in the LIMS. The flow cytometry technique is entering a mature state with better-defined methodology for instrument settings, protocol design, standardization, and data analysis and interpretation. Nonetheless, because of its large scope and flexibility and for economic reasons, FCM accreditation procedures must be pragmatic, feasible, and efficient. Our proposal also defines several premises for further harmonization of the processes connected with the validation of FCM assays. In a next step, for instance, the community of laboratories that frequently perform such validation routines could now compile a collection of sample records and may develop "best practice" templates for the evaluation of validation data.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

CL, GY, TK, FP, KP, MS, MÖ, and US wrote parts of this manuscript, double-checked the submitted draft, and agree to be accountable for the content of the work.

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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.2020.02169/full#supplementary-material

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MINI REVIEW
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Natalizumab in Multiple Sclerosis Treatment: From Biological Effects to Immune Monitoring

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Multiple sclerosis is a chronic demyelinating disease of the central nervous system (CNS) with an autoimmune component. Among the recent disease-modifying treatments available, Natalizumab, a monoclonal antibody directed against the alpha chain of the VLA-4 integrin (CD49d), is a potent inhibitor of cell migration toward the tissues including CNS. It potently reduces relapses and active brain lesions in the relapsing remitting form of the disease. However, it has also been associated with a severe infectious complication, the progressive multifocal leukoencephalitis (PML). Using the standard protocol with an injection every 4 weeks it has been shown by a close monitoring of the drug that trough levels soon reach a plateau with an almost saturation of the target cell receptor as well as a down modulation of this receptor. In this review, mechanisms of action involved in therapeutic efficacy as well as in PML risk will be discussed. Furthermore the interest of a biological monitoring that may be helpful to rapidly adapt treatment is presented. Indeed, development of anti-NAT antibodies, although sometimes unapparent, can be detected indirectly by normalization of CD49d expression on circulating mononuclear cells and might require to switch to another drug. On the other hand a stable modulation of CD49d expression might be useful to follow the circulating NAT levels and apply an extended interval dose scheme that could contribute to limiting the risk of PML.

Keywords: multiple sclerosis, natalizumab, biotherapy, drug modifying therapy, Mab therapy monitoring, Integrin, neutralizing antibodies, PML

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INTRODUCTION

Multiple sclerosis (MS) is a chronic, inflammatory autoimmune disease leading to demyelination. It is a heterogeneous, multifactor disease with environment factors acting in a susceptibility genetic background, still only partially described. Following a silent phase, the most common clinical form of MS is the relapsing remitting MS (RRMS) with accumulation of lesions during relapse phases. With time the disease may evolve as a progressive phase without remission (secondary progressive MS, SPMS) although some patients may have a progressive disease from the onset called primary progressive MS (1). Although few treatments are active on the progressive forms of MS, the treatment of RRMS has been dramatically modified in the era of monoclonal antibodies and other disease modifying therapies (DMT).

Among them, Natalizumab (Tysabri® , NAT) is a humanized IgG4 antibody (Ab) that recognizes α4 chain (CD49d) of the VLA4 (Very Late Antigen 4) antigen, a component of the α4β1 integrin, and of the α4β7 integrin. It is the clinical achievement of the pioneer work of Yednok et al., who demonstrated the role of this adhesion molecule in the interaction of leukocytes with inflamed endothelium in the brain and had shown that the injection of an anti-α4 monoclonal antibody prevents EAE (experimental autoimmune encephalomyelitis) in a rat MS model (2). Consequently, a mouse anti-human α4 chain Ab able to block VLA4 interaction with its ligand VCAM1 (Vascular Cellular Adhesion Molecule1) was selected for humanization (3). Two phase III studies demonstrated the efficacy of NAT to improve the evolution of RRMS in terms of annual relapses or development of brain MRI lesions (4, 5). This success was not obtained in SPMS (6). In addition, a severe adverse effect was then reported with the appearance of progressive multifocal encephalopathy (PML) (7) which was usually occurring in immuno-compromised or immunosuppressed patients. The standardized protocol consists in a 300 mg dose every 4 weeks but many schemes extending interval dosing have been tested with similar efficacy (8–11).

MECHANISMS OF ACTION

The underlying MS pathological process involves both antigen specific and non specific inflammatory mechanisms. Part of the knowledge is coming from animal studies using the EAE model (12) but contradictory features concerning the human pathology have emerged from several therapeutic trials. For example, the central role of antigen specific T cells observed in EAE has been extended from CD4+ T cells in EAE to more numerous CD8+ T cells in human with autoreactivity against myelin derived peptides, and from a critical role of Th1 cells secreting IFNγ to the participation of Th17 cells producing GM-CSF (13, 14). The importance of B cells has long been recognized with the presence of oligoclonal bands in CSF, but the recent evidence of the efficacy of therapies depleting these cells without significant effects on immunoglobulins shifts their role toward their ability to present antigens to T cells (15). Furthermore a complex inflammatory infiltrate in central nervous system (CNS) and CSF is described including various innate cells that complete the role of the locally activated microglia. Whatever the mechanisms, the activation of antigen specific lymphocytes either in the periphery or not, and the secondary colonization of CNS need cell interactions and migration which are dependent on chemokines and adhesion molecules. The rationale for using anti-α4 Abs in EAE was their blocking effect on the adhesion of leukocytes leading to inhibition of inflammatory migration to CNS. Although a large body of results strengthen this strategy, some pre-clinical data suggest that according to the timing of monoclonal Ab (Mab) administration or the experimental model, anti-α4 Abs can be inefficient or deleterious despite VLA4 blockade (16, 17), possibly because of agonistic properties of anti-VLA4 Abs (17). Nevertheless the activating effect of anti-VLA4 Abs has not been described in NAT treated patients (18–20). In addition, in EAE models, infiltration of Th17 cells or GM-CSF-producing Th1/Th17 cells into the CNS has been shown to be mediated by lymphocyte function associated antigen 1 (LFA1) adhesion molecules and not VLA4 integrin, thereby suggesting more differential effects of anti-VLA4 blockade, at least, in animal models (21, 22).

By preventing the interaction of α4β1 integrin expressed on lymphocytes to its ligand VCAM1 on endothelial cells, NAT inhibits the migration through the brain blood barrier into the CNS parenchyma. There are two ways to confirm this effect: in the blood compartment, an increase of leukocytes has already been observed (23) whereas a decrease of infiltrating cells could be assessed in the CSF. Evidently, CNS infiltrating lymphocytes were decreased in patients treated with NAT as compared to untreated patients or pre-treatment levels. This was observed for T lymphocytes, mainly for CD4+ cells, and for B cells (24–28) and led to a diminished level of immunoglobulins (IgM, IgG) including oligoclonal bands, with a decrease of local production (24, 27–29). These effects were confirmed in longitudinal studies and disappeared – albeit slowly (within 6 months) – after treatment interruption (26). Monocytes were increased relatively to lymphocytes during treatment suggesting that their migration might be less VLA4-dependent (30, 31). Few reports analyzed the effects of NAT on antigen presenting cells, but a reduced number of dendritic cells (DCs) had been observed in perivascular spaces in post mortem samples of a NAT treated patient (32). Furthermore, in addition to a decreased expression of CD49d, both myeloid and plasmacytoid DCs had impaired capacities to stimulate T lymphocytes (33).

As a consequence of this extravasation blockade, mononuclear cells accumulate in the circulation. In addition, some haematopoietic precursors might be released from the bone marrow due to loss of VLA4-VCAM1 interactions with the stromal cells or altered homing (34, 35). The net result is an important lymphocytosis following the first injection which soon reached a stable plateau. The more altered cells were B lymphocytes (more than 3 times pre-treatment values), NK and T lymphocytes (2 and 1.8, respectively) without modification of the CD4+/CD8+ ratio (36–38). Cell numbers decreased after 8 weeks of treatment interruption and returned to basal levels around 16 weeks after this interruption (38). The phenotype and function of the circulating cells have been explored and inconstantly showed an increase of memory T cells which might reflect their higher CD49d expression, and of activated cells (18, 39, 40). Although Th17 or Th1/17 cell migration has been suggested to be partially VLA-4 dependent (31), it is mostly observed that under NAT treatment these cells also accumulate in the circulation (41, 42). Furthermore, the frequencies or proliferative capacities of potential encephalogenic myelin basic protein reactive cells were not modified under NAT treatment (39). Some variations in cytokine production merely pro-inflammatory were also observed, especially in the early phases of treatment (39, 43, 44). In contrast, no quantitative nor qualitative effect was noted on regulatory T cells (Tregs) (18, 45). These cells constantly showed a strong decrease of CD49d expression (46, 47) but their migration was still efficiently blocked and their suppressive effects preserved (47). B cells were the most impacted

circulating cells and also demonstrated a memory phenotype, prone to activation, and pro-inflammatory profile (25, 40, 48).

A direct activation role of natalizumab through CD49d has been excluded for all types of cells (25, 39) arguing for a mere accumulation in the circulation of cells potentially activated, due to the inhibition of migration. It might favor the recurrence of the disease after treatment interruption, observed in approximately one third of the cases, which needs a switch to another treatment (49). In some cases a more severe relapse is observed as compared to the pre-treatment status of the patient, described as a rebound effect (50) and can be related to the migration of autoreactive Th1, Th17, or Th1/17 cells accumulated in the circulation during NAT treatment (41, 42).

PML COMPLICATION

Progressive multifocal encephalopathy, a demyelinating disease caused by the John Cunningham virus (JCV), was soon observed in NAT treated patients although it was previously associated with immunodeficiency or immunosuppression (7). Despite a high incidence (1/1000) with an 18-month treatment, (51), a clear benefit/risk balance reinstated it after a short market withdrawal. In MS treatment, other drugs such as anti-B cell Mabs (anti-CD20 Mabs), dimethyl fumarate, or fingolimod had an increase of PML risk, but far less than NAT (52). Another anti-adhesion molecule, efalizumab (anti-LFA-1) used in psoriasis, has been withdrawn because of PML complications (53). The concept of altered immune surveillance to virus in CNS due to the cell circulation migration inhibition has long been the main argument described as the cause of this increased risk. However, some properties of NAT might facilitate this disease. The JCV infection is a very frequent asymptomatic disease usually occurring during childhood, then remaining latent until a possible reactivation, which remains a very rare event. Although the knowledge of JCV biology has greatly improved, some critical issues persist about the process of latency and reactivation (54). It has been suggested that the increase of circulating haematopoietic precursors and/or the accumulation of pre-B and B cells (34, 35, 55–58) might represent a potential virus reservoir for JCV (59, 60). Analysis of JCV in these cells showed some conflicting results (35, 60–63), probably depending on method sensitivity. Nevertheless, when detectable, it should be mentioned that the virus is detected at low levels or under inactive form; and sometimes in asymptomatic patients (60, 61, 64). These data are consistent with a latency phase of the virus. In addition, normal brain might also be another site of latent viral persistence (65).

It has been shown that NAT is able to upregulate transcription regulators POU2AF1 and Spi-B in B cells (59, 66). Consequently, transition from latent archetype to prototype virus variant, viral transcription and replication are suspected to be facilitated in lymphoid cells (60, 62, 67). Spreading to CNS through B cells or free virions is speculated but has not been proven (68). But, even if this hypothesis is true in immunocompetent people, it is likely that the spreading would be inhibited under NAT treatment. On the target cell side, NAT has not been shown to facilitate neural cell infection, at least *in vitro* (69). In the context of immune modulation induced by NAT, there is a decrease in antigen presenting cells in the CNS (32), and the trafficking of memory T cells is not selectively inhibited by NAT. It has also been shown that the anti-viral Th1 compartment is retained in the circulation hampering the JCV elimination (41). At this stage, the main parameters for susceptibility to JCV infection are NAT treatment longer than 2 years, prior immunosuppression and anti-JCV seropositivity.

DRUG MONITORING

Circulating and CSF Levels of NAT

As for most drugs, the measurement of concentrations is a tool to determine the best dosage. Various methods have been used to measure NAT concentrations. Due to its heterodimeric structure, cellular assays have been developed using cells expressing CD49d and FACS analysis with a standard curve of NAT (70, 71). Alternatively ELISA methods have been set up. A particular property of the IgG4 isotype that has been uncovered is that due to the absence of covalent links between the two heavy chains, "Fab arm exchange" occurs between IgG4, rendering them monovalent (72). In addition to potentially modifying NAT functional effect, it can directly interfere with detection assays. Accordingly, an alternative to classical bridging test has been developed (73) but no strict comparison measurements have been thoroughly published yet. The variable median results of NAT free circulating levels observed among studies (from 18 to 51 μg/ml) may be assay dependent, but a common characteristics noted within each study was the high variability among patients (less than 4 μg and up to 100 or 200 μg) (71, 74, 75). No clear relationship has been evidenced to identify factors involved in this heterogeneity although body weight might contribute (76, 77). Nevertheless, for a given patient, trough levels soon reach a plateau and remain stable whatever the number of infusions (9) and for more than 90% of them were over 10 μg/ml (78). In comparison, levels within CSF were a hundred times lower from 45 to 110 ng/ml (71, 74).

In the serum, free NAT was measured, but the cell bound part can also be determined. Cytometry allows determining the level of NAT bound to cells using a fluorescent anti-IgG4 antibody, as well as the free CD49d molecules on the cells that are not covered by the administered drug, using an additional incubation with an excess of NAT. This assay is suitable for determining the saturation level of CD49d on the cells which, although slightly different according to the circulating cell type analyzed, is around 70% (79, 80). Surprisingly, and despite the low levels of free NAT measured in CSF, nearly the same degree of saturation was observed in CSF (79).

These assays were performed during ongoing treatment but the disappearance of NAT was also evaluated in studies performed after interruption of treatment (38, 81). In the RESTORE study designed to evaluate the consequences of treatment interruption, NAT circulating levels after the last injection differed from patients still treated 8 weeks after interruption of treatment, and it takes 16 weeks for the NAT levels to become undetectable (38). In parallel, at the same time, the

saturation of circulating cells started to decrease (68% vs 87% for treated patients) but some antibody remains detectable on the cells between 16 and 28 weeks after interruption (38).

When the clearance of NAT needs to be very rapid, for instance because of PML, protocols of plasma exchange are used and allows almost 90% elimination of circulating NAT within 1 week. In these conditions, the saturation of the cells falls under 50% when NAT is <1 μg/ml, and partial restoration of migratory capacities is obtained 3 weeks after plasma exchange treatment (82). It should be mentioned that this strategy is not without risk. In addition to a potential reactivation of the disease, it may represent a worsening factor in PML, inducing an immune reconstitution inflammatory syndrome (IRIS) that leads to a poorer prognosis that in case of spontaneous NAT clearance (83).

Pharmacodynamic Analysis

These pharmacokinetics parameters have been completed by pharmacodynamic analysis checking some dose-dependent functional effect. Parallel to the receptor saturation, it could be noticed that CD49d expression, as determined using a fluorescent anti-CD49d antibody recognizing another epitope, was decreased around 50% of the pre-treatment level soon after treatment initiation (19, 70, 71, 84). It then remained stable all along treatment except in cases of immunization (cf infra). This diminished expression, associated with a decrease of CD29, the β1 chain of this heterodimer, (84) might contribute to the inhibition of VLA4/VCAM interactions. The recovery of the expression after treatment interruption is slower than the decrease of receptor occupancy (9).

Using fluorescent beads allowing quantification (Quantibrite, BD), a more precise evaluation has been performed to compare the number of membrane expressed CD49d molecules and the number of bound NAT molecules (85). It allows a direct estimation of the level of saturation in patients receiving standard protocol (Standard interval dosing SID, 4 weeks) or protocols with an extended interval (EID) between two injections. This schedule was evaluated in order to limit the risk of PML. Using a regular treatment, T CD4, CD8, B cells expressed, according to the cell type, around 1300–1400 CD49d molecules. In contrast with an interval of 6 weeks between injections, the number of CD49d was 2000–2400 molecules/cell. Nevertheless, the number of NAT bound molecules was not different between the 2 groups leading to decreased receptor occupancy (RO) from 76–84% to 54–62% (85). Using a simple measurement of the mean fluorescence intensity of an anti-CD49d antibody, a modest increase of CD49d expression was observed in EID (9%) as compared to SID, still at 60% of the pre-treatment levels, and it was associated with a decrease of NAT circulating levels from 36 to 18 μg/ml (9). These trough levels are still over the levels needed for an almost receptor saturation. With these EID protocols, no worsening of the clinical status was noticed suggesting that increasing the time between injections is not altering efficacy (10, 11).

So, biological parameters for monitoring the interval injection duration are available. As far as now, no studies have determined a critical level for saturation or modulation of CD49d required for clinical efficacy. These parameters might be useful for an adaptation of dose or timing on a case by case basis to limit the adverse biological effects of NAT.

Anti-drug Antibodies

Therapeutic strategies were greatly completed by introducing monoclonal antibodies but despite the molecular engineering of humanized molecules these proteins keep a potential immunogenicity especially when used as monotherapy. In the case of NAT, nearly 9% of the patients were identified with anti-NAT antibodies, and 6% are immunized permanently (4). For some patients the injection related side effects suggest immunization, that needs to be investigated, whereas for many of them the process is silent or relapses might occur by therapy inhibition. For these patients, a systematic screening for immunization has been suggested at 6 months. The presence of high titers of anti-NAT antibodies is suggestive of a permanent immunization. Depending on the test used, no clear cut-off has yet been defined (75, 86, 87). However, in our experience, transient anti-NAT Ab were detected at rather low levels (10 times less) as compared to patients with persistent neutralizing antibodies (70). The neutralizing effect of immunization can also be suggested by using the monitoring parameters previously discussed. Among them, the end of CD49d expression down-modulation is suggestive of the immunization (70) which can be either transient or permanent.

Immunization is also responsible of NAT clearance, and complete disappearance of circulating free NAT was observed in immunized patients with clinical relapse (75). Depending on the local laboratory practice, it can be easier and more flexible to measure modulation of CD49d for a given patient than to perform complete series of natalizumab and anti-natalizumab ELISA. The measurement of the lymphocytosis has also been suggested to be a potential biomarker of efficacy (88) but has not been related to NAT levels, saturation, or anti-NAT antibody appearance.

In-depth analysis of the immune response of two patients has allowed the characterisation of the B and T cell responses. In contrast to the large polyclonal anti-idiotypic B response, an immunodominant T cell epitope was identified in the FR2-CDR2 region of NAT light chain. In addition this epitope could be modified to avoid T cell recognition without loosing the binding to CD49d (89) providing a deimmunized antibody (90). Such a modified molecule could be an alternative for immunized patients.

In conclusion NAT is one of the recent therapies that have changed the evolution of RRMS. However, long term treatment has been associated with PML, a severe infectious complication. No specific biologic risk linked to NAT properties has been definitively identified in this susceptibility, which is also observed in other immunosuppression states either related to HIV or monoclonal antibody treatments or other DMT. In the context of NAT, no drug overdose was noticed at the time of infection (77) and risk evaluation remained to be assessed on treatment duration and anti JC antibody status. In order to limit the risk of PML, EID protocols seem to maintain a sufficient efficacy, although

the real benefit on large cohorts has not yet been reported, and the ongoing NOVA study might contribute to this evaluation (91). On the other side, inefficient treatment might not always be clinically detectable until new release. In both circumstances, to offer an optimized treatment with potential therapeutic switch and to improve the cost/benefit, it might be interesting to develop an adapted biological monitoring using an easy-to-measure parameter such as modulation of the expression of CD49d, which is a good and robust functional reflect of the circulating levels of NAT.

AUTHOR CONTRIBUTIONS

All authors contributed to manuscript revision, read and approved the submitted version.

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