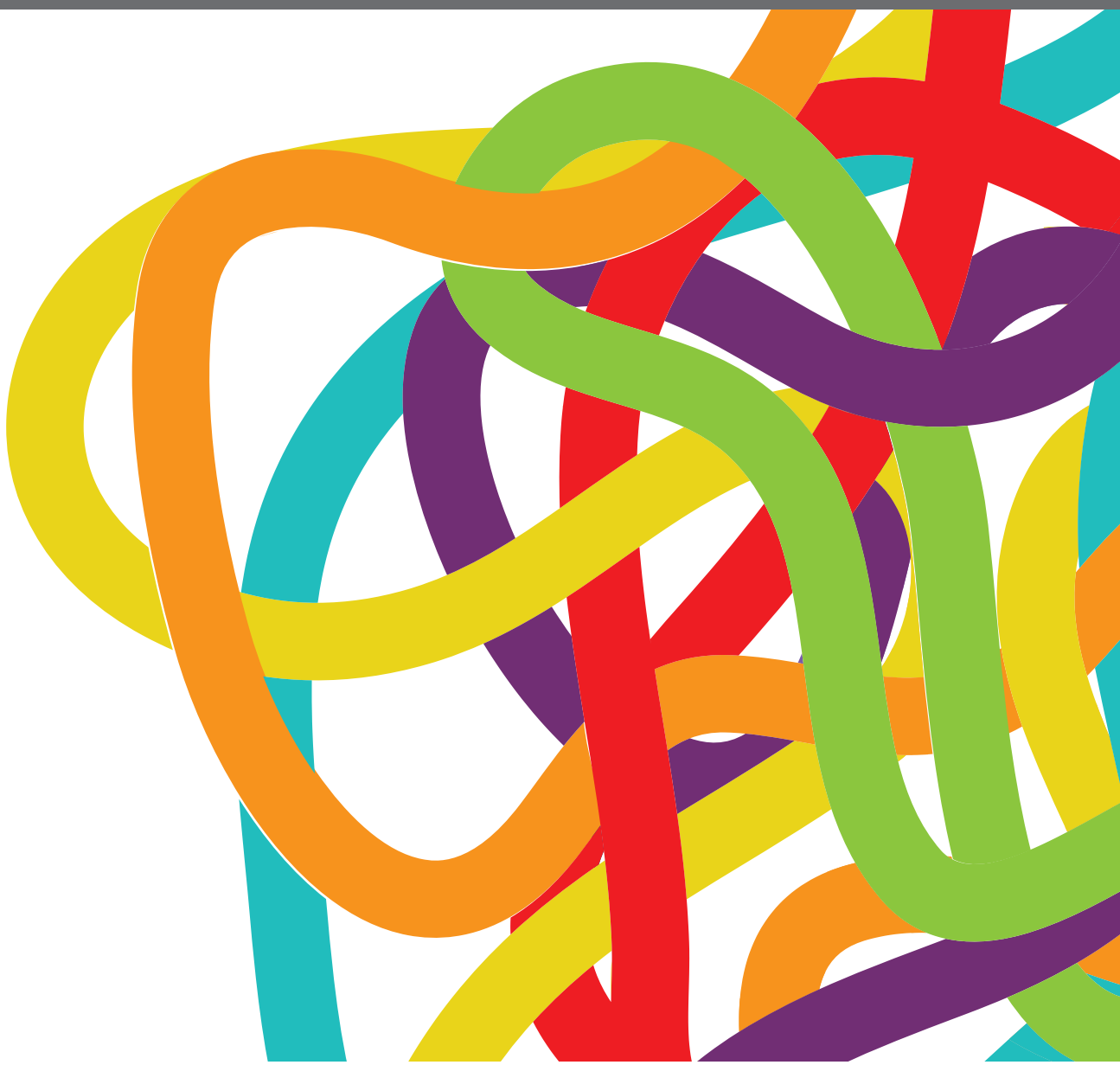
CURRENT PERSPECTIVES, CHALLENGES AND ADVANCES IN CELL BASED THERAPIES

EDITED BY: Prashant Trikha, Monica Thakar and Conrad Russell Cruz
PUBLISHED IN: Frontiers in Oncology and Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88963-564-1
DOI 10.3389/978-2-88963-564-1

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CURRENT PERSPECTIVES, CHALLENGES AND ADVANCES IN CELL BASED THERAPIES

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Citation: Trikha, P., Thakar, M., Cruz, C. R., eds. (2020). Current Perspectives, Challenges and Advances in Cell Based Therapies. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88963-564-1

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frontiers in Oncology

EDITORIAL
published: 06 February 2020
doi: 10.3389/fonc.2019.01061

Editorial: Current Perspectives, Challenges and Advances in Cell Based Therapies

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Keywords: immunotherapy, NK cell, tumor micoenvironment, CAR (chimeric antigen receptor) T cells, cell based therapy

Editorial on the Research Topic

Current Perspectives, Challenges and Advances in Cell Based Therapies

"Current Perspectives, Challenges and Advances in Cell Based Therapies," a special collection in Frontiers of Oncology/Immunology, focuses on new developments in the field of cellular immunotherapy. In this collection, we hope to capture the challenges of developing cellular therapy for different diseases, the emergence of new technologies, and the ways cell and immune based therapies can be made safer and more effective. Many of the newest cell-based immunotherapies in the clinic today have focused on chimeric antigen receptor (CAR)-T cells. These therapies have led to impressive clinical results for a subset of diseases with historically poor outcomes. However, while T cells have been effective at eradicating hematological malignancies (most specifically lymphoid diseases), they have so far had limited efficacy against solid tumors (1). While multiple groups continue work on enhancing efficacy of CAR-T cells for solid tumors, several laboratories have begun work on other immune cells. In addition to T cells, innate immune cells, especially natural killer (NK) cells, play a pivotal role at not only eradicating cancer cells but also in modulating the function of adaptive immune cells (2). These cells are emerging as important players in cellular immunotherapy. Concurrently, we have improved our knowledge of the role of the tumor microenvironment (TME) in the pathogenesis of cancer (3). This has increased our understanding of the interaction between cancer cells and immune cells, and the mechanisms by which cancer cells suppress the anti-tumor function of innate and adaptive immune cells.

Several challenges remain, which, when addressed, would dramatically improve efficacy of cell-based therapies, particularly against solid cancers. These include (1) identification of optimal tumor antigens, (2) enhancing trafficking of adoptively transferred cells to tumor and metastatic sites, and (3) neutralizing the immunosuppressive TME. Some of these challenges can be addressed by genetic modification of T/NK cells. Moreover, better characterization of immune cells using mass cytometry (CYTOF) and single cell RNA sequencing will increase our knowledge about their vast repertoire of receptors and genes, which will help decipher their immune functions. In turn, precise augmentation or inhibition of these receptors can potentially make cellular immunotherapy more effective. For example, knocking down inhibitory receptors on cells using CRISPR can augment their anti-tumor function.

We have come a long way since the early days of cell-based therapies, and still exciting new avenues are being explored (4). The development of new technologies to measure metabolic profiles now allows us to interrogate how cellular metabolism regulates immune cell physiology, and how this metabolism impacts the anti-tumor response of immune cells.

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *26 September 2019*
Accepted: *30 September 2019*
Published: *06 February 2020*

Citation:
Thakar MS, Cruz CR and Trikha P (2020) Editorial: Current Perspectives, Challenges and Advances in Cell Based Therapies. Front. Oncol. 9:1061. doi: 10.3389/fonc.2019.01061

There are now studies that investigate the fate of immune cells following infusion. This knowledge could then be applied to improving *in vivo* trafficking of effector and regulatory cells to the tumor and metastatic sites. Additionally, toxicities and side effects that develop following the infusion of CAR cells are being better understood, leading to improved strategies that focus on decreasing risks associated with this therapy. Finally, long term effects of these genetically modified cells can now be addressed.

It is our hope that we capture these ideas in the following articles in this collection. While they are understandably not all-encompassing, these articles are representative of current efforts in the field.

Du and Wei explore the role of NK cell immunotherapy in patients with gastric cancers, which the application of emerging immunotherapies to a set of diseases that has not typically been included in immune cellular-based strategies. Chen and Gao study the potential of using anti-LMP CAR-modified T cells against LMP^+ nasopharyngeal carcinoma. Nayyar et al. provide a detailed overview of challenges seen in using NK cell immunotherapy for solid tumors and provide a systematic overview of methods to improve NK cell function and potential. Ali et al. reviewed the development of CAR T cell therapies for pancreatic cancer, noting the progress in the field and current challenges in utilizing this therapy in this disease. Dwyer et al. talk about common gamma chain cytokines and their role in T cell survival and generation of memory, and how this understanding can pave the way for enhancing cell-based immunotherapies. Shah et al. review the concept of cancer immune evasion and the strategy of targeting multiple antigens as a method to overcome resistance in CAR-T therapies. Liu et al. investigate using CRISPR technology as an engineering method to create a potent and potentially universal CAR-T therapy. Patel et al. summarize non-CAR T gene-modified cell-based approaches—both T cells modified with other transgenes and non-T cell-based therapies. Qin et al. focus on their lab's specific pre-clinical studies evaluating the improved activation of cytotoxic T lymphocytes using an immortalized mouse embryonic cell line (NIH3T3)-conditioned medium as a method to augment adoptive cell therapy. Han et al. examine the mechanisms involved in regulatory T cell modulation of T cell function, looking at avenues to overcome them to enhance anti-tumor immunity. Xu et al. discuss methods to improve CAR T cells by looking more closely into metabolism, effectively improving the function of CAR T cell-based approaches. Wang et al. review innate immune cell-based therapies for osteosarcoma and enumerate strategies to enhance their efficacy. Thakar et al. reviewed the methods to improve the safety profile of CAR-based therapies, describing both pharmacological treatments and signaling pathways involved in cytokine release syndrome.

We anticipate that these articles will provide a state-of-the-art overview of the diversity and challenges faced in this growing arena, and open additional areas of future investigation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

CC received funding from the Alex's Lemonade Stand Foundation for this work. MT received funding from the NCI R01 CA179363, Midwest Athletes Against Childhood Cancer Fund, Rebecca Jean Slye Endowment.

ACKNOWLEDGMENTS

We will also like to thank all the authors in the collection for their valuable contribution.

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Conflict of Interest: CC is a co-founder of Mana Therapeutics, a biotechnology company that is developing cell therapies for cancer.

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

frontiers in Immunology

REVIEW
published: 21 January 2019
doi: 10.3389/fimmu.2018.03095

Therapeutic Potential of Natural Killer Cells in Gastric Cancer

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Gastric cancer (GC) is one of the most common cancers, with a high incidence of cancer death. Despite various therapeutic approaches, the cures and prognosis of advanced GC remain poor. Natural killer (NK) cells, which are known as important lymphocytes in innate immunity, play vital roles in suppressing GC initiation, progression, and metastases. A wide range of clinical settings shows that increasing the number of NK cells or improving NK cell antitumor activity is promising in GC patients. NK cell adoptive therapy (especially expanded NK cells) is a safe and well-tolerated method, which can enhance NK cell cytotoxicity against GC. Meanwhile, cytokines, immunomodulatory drugs, immune checkpoint blockades, antibodies, vaccines, and gene therapy have been found to directly or indirectly activate NK cells to improve their killing activity toward GC. In this review, we summarize recent advancements in the relationship between NK cells and GC and point out all the innovative strategies that can enhance NK cells' function to inhibit the growth of GC.

Keywords: natural killer cells, gastric cancer, immunity, adoptive therapy, checkpoint blockades

OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *02 September 2018*
Accepted: *13 December 2018*
Published: *21 January 2019*

Citation:
Du Y and Wei Y (2019) Therapeutic Potential of Natural Killer Cells in Gastric Cancer. Front. Immunol. 9:3095. doi: 10.3389/fimmu.2018.03095

INTRODUCTION

Gastric cancer (GC) was the world's third-leading cause of cancer death in 2012, resulting in 723,000 deaths (1). GC rates in men are nearly twice as high as those in women, and they vary widely across countries, with the highest incidence rates in Eastern Asia (particularly in China), Central and Eastern Europe, and Central and South America (1, 2). Although the incidence and mortality of GC have declined in recent years due to standardized surgical techniques, innovations in clinical diagnosis, and the development of new chemotherapy regimens, the survival rate for advanced GC remains low across the world. Therefore, investigating the molecular biology of GC to realize novel effective therapeutic approaches results in beneficial progress in the treatments of GC.

Natural killer (NK) cells are vital members of innate immunity and can recognize and kill tumors and infected cells as well as produce many cytokines to regulate adaptive immunity (3). Meanwhile, similar to cytotoxic T lymphocytes, NK cells also preserve specific memories after encountering a pathogen. Experienced NK cells show a robust protective response to reactivation by the initial pathogen but also by other pathogens (4, 5). However, the amount, subsets, cytokines, and cytotoxicity of NK cells are decreased in GC patients and impair the immune system severely (6). Immunotherapy, which has made some breakthroughs in many cancers, has been introduced as a modality to improve the function of the immune system in GC. Among immunotherapies, therapies targeting the activation of NK cells and enhancement of NK cell activity are under research, shedding light on the treatments of GC. Recent evidence shows that the signal transducer and activator of the transcription family 5 target CIS, interleukin (IL)-1R8, transforming growth factor (TGF)-β, and adenosine all function as inhibitors of NK cells. Better understanding of

these suppressive pathways is helpful for discovering effective candidates for the therapeutic manipulation of NK cells (7). Here, we discuss the relationship between GC and NK cells and review the research progress of NK cell immunotherapy for GC.

CHARACTERISTICS OF NK CELLS

NK cells play important roles in host innate immunity with high antitumor, antiviral, and antimicrobial activity and contribute to the activation and regulation of adaptive immune responses (8, 9). About 3–5% of human peripheral blood lymphocytes are NK cells. These cells react much faster than T cells upon stimulation, as they do not need previous sensitization, antibody binding, or pathogen presentation.

Phenotypically, NK cells are defined by the expression of CD56 and the lack of CD3. $CD56^{dim}$ $CD16^{bright}$ NK cells, which account for 90% of NK cells, are mature, which means they mediate antibody-dependent cellular cytotoxicity (ADCC), exhibiting high levels of perforin and enhanced killing. The remaining 10% of NK cells producing various cytokines are immature, which express $CD56^{bright}$ $CD16^{dim}$ or $CD56^{bright}$ $CD16^{-}$ (3, 10). The antitumor activity of NK cells is mostly determined by a set of inhibitory and activating receptors (11). Inhibitory receptors include killer-cell immunoglobulin-like receptors (KIRs) that bind to class-I human leukocyte antigen (HLA) molecules or inhibitory C-type lectins Ly49s and the heterodimer CD94/NKG2A-B, which recognize HLA-E molecules. Some activating receptors belong to the Ig-like family, such as CD16, NKp46, NKp30, and NKp44. Moreover, the C-type lectin receptors, including CD94/NKG2C-E (recognizing HLA-E) and NKG2D (recognizing non-classical HLA), also activate receptors (12–14). By interacting with target cells, NK cell activity is changed.

Emerging evidence shows that cancers develop multiple strategies to escape $CD8^{+}$ T cell recognition, but they can be preferentially attacked by NK cells (15). NK cells eliminate target cells through several different mechanisms (**Figure 1**). After adhering to the target cells, NK cells release many cytotoxic granules containing perforin and granzymes, which lead to cell lysis. NK cells express tumor necrosis factor (TNF)–related apoptosis-inducing ligand family (TRAIL) and Fas-Ligand (FASL) (CD95L), which interact with TRAIL receptors and FAS in target cells, respectively. The interaction leads to the formation of a death-inducing signaling complex and elicits apoptosis (10). NK cells can also secrete sufficient amounts of the cytokines interferon (IFN)-γ (16) and TNF-α (17) to increase cytotoxicity. In addition, one potent activating receptor, CD16 (FcγIIIA), can recognize Fc on human IgG1 antibodies and trigger ADCC (18). All these cytotoxicity mechanisms enable NK cells to eliminate different types of tumor cells.

Abbreviations: GC, gastric cancer; NK cells, natural killer cells; TGF, transforming growth factor; IL, interleukin; ADCC, antibody-dependent cellular cytotoxicity; KIRs, killer-cell immunoglobulin-like receptors; HLA, human leukocyte antigen; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis inducing ligand family; FASL, Fas-ligand; IFN, interferon; MDSCs, macrophages, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MICA, MHC class I chain-related A; GVHD, graft-vs-host disease; EBV-LCL, Epstein-Barr virus-transformed lymphoblastoid cell lines; GMP, good manufacturing practices; PBMC, peripheral blood mononuclear cell; PSK, polysaccharide krestin; PD-1, programmed death 1; PD-L1, programmed death ligand 1; CTLA-4, cytotoxic lymphocyte-associated antigen 4; GEJC, gastroesophageal junction cancer; mAb, monoclonal antibody; HER2, human epidermal growth factor receptor-2; EGFR, epidermal growth factor receptor; DCs, dendritic cells; ICAM, intercellular adhesion molecule; siRNA, small interfering RNA; CAR, chimeric antigen receptor; OS, overall survival; TAM, tumor-associated macrophage.

NK CELLS AND GC

Compared with tumor-specific cytolytic T cells, NK cells can kill tumors with low or absent HLA class I expression. Notably, NK cells could effectively be activated by cancer stem cells (CSCs). CSCs are responsible for tumor relapses, as they are resistant to chemo- and radiotherapy, because of their quiescent status. However, a study found that gastric CSCs can be killed by NK cells via CD133 in an NKG2D-dependent manner (19). An increasing amount of data show there is an important relationship between NK cells and the progression of GC. With the progression of GC, the number and the function of NK cells decrease sharply, which leads to the malignancy of GC in reverse. The interaction between NK cells and GC can be visualized in **Figure 2**.

NK Cell Dysfunction in GC Patients

A study demonstrated that the frequency of apoptotic NK cells in GC patients (21.3 ± 11.6%) was increased significantly compared with normal controls (11.2 ± 5.2%; $p = 0.0016$), and their frequencies were related to the progression of GC (20). NK cell infiltration in intratumoral regions is significantly decreased, which is associated with decreased survival and disease progression in GC patients (21, 22). Gulubova et al. elucidated that the number of NK cells was decreased in patients with gastric and colorectal cancer with liver metastases compared with those without liver metastases (10.1 ± 11.6% vs. 16.6 ± 8.9%, $p = 0.039$) (23). The percentages of NK cells in blood as well as NK cell activity were significantly increased after gastrectomy (24).

NK cell activity is damaged in GC patients. Data show that there is an evident association between NK cell activity and some clinicopathological parameters, including tumor volume, clinical stage, lymphatic and vascular invasion, and lymph node metastases in GC (25, 26). In GC patients, NK cells show a suppressive phenotype, with downregulated expression of activating receptors and upregulated expression of inhibitory receptors. In particular, NKG2D is a key receptor for NK cell activation and has multiple ligands, including MHC class I chain-related A (MICA), MICB, and several UL-16–binding proteins (27). Yoshimura et al. investigated 98 GC patients who underwent surgery from 2004 to 2008. They found that patients with NKG2D expression in tumors had significantly longer overall survival (OS) than patients without NKG2D expression in tumors ($p = 0.0217$), and the longest OS was observed in patients positive for ULBP1 and NKG2D (28, 29). Except for downregulated receptors of NKG2D, NKp30, and NKp46, NK cells also release fewer cytotoxic granules of perforin and granzyme B and are characterized by decreased IFN-γ, TNF-α,

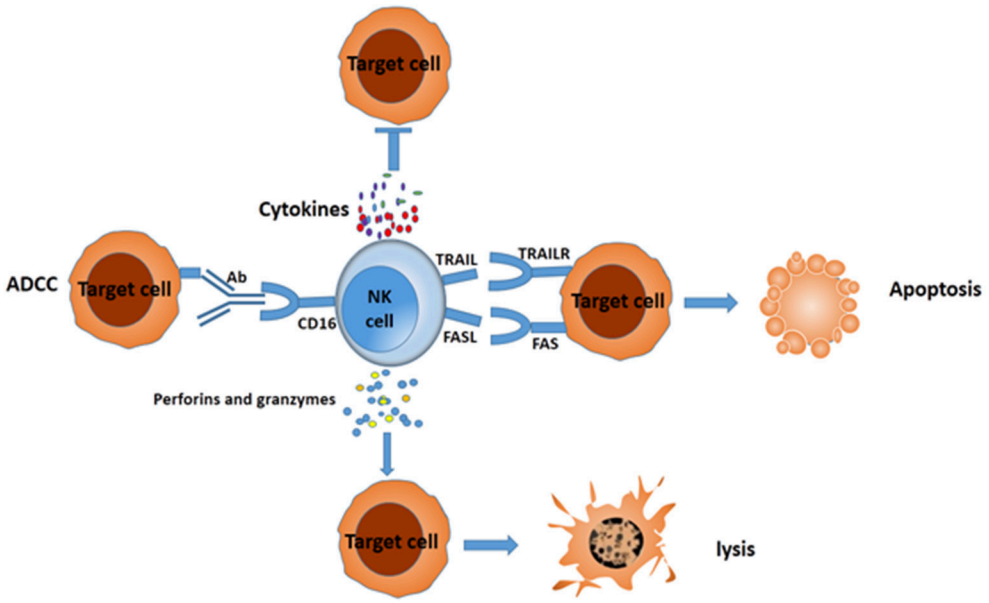

FIGURE 1 | Different mechanisms for NK cells to suppress target cells. NK cells can mediate the death of target cancer cells by ADCC, secreting IFN-γ and TNF-a, releasing perforin and granzymes, or eliciting apoptosis via formation of complex FAS/FASL and TRAIL/TRAILR.

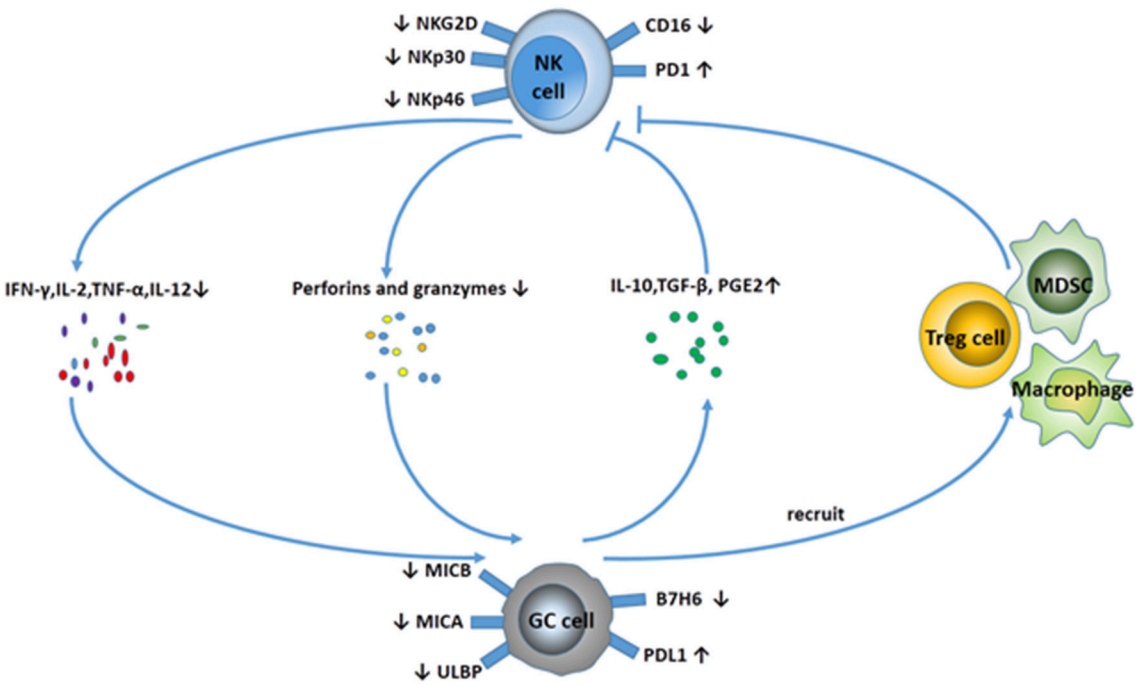

FIGURE 2 | Interaction between NK cells and GC. NK cells display a suppressive phenotype with fewer activating receptors (NKG2D, NKp30, NKp46) and higher expression of PD-1 in GC patients. In addition, NK cells secret fewer cytokines (IFN-γ, IL-2, TNF-α, IL-12), and the ability to release perforins and granzymes is decreased. Meanwhile, GC cells downregulate the expression of MICA/B, ULBP, and B7H6 to avoid NK cell-mediated innate immunity. GC cells can also release some cytokines, including IL-10, TGF-β, and PGE2, and recruit MDSCs and Treg cells to suppress NK cell activity.

and Ki-67 expression in GC patients (22, 30). In addition, TNF-α, IL-2, T-bet, and IL-15Rβ levels were decreased in NK cells from the GC tissue and peripheral blood in the GC patients, leading to a decrease in the function of NK (6). Moreover, Kono et al. discovered that NK cell dysfunction contributed to the impaired Herceptin-mediated ADCC in advanced GC patients,

which was correlated with the downregulation of CD16zeta expression (31).

Strategies for GC to Escape From NK Cell-Mediated Immunity

GC develops various measures to escape from innate immune response based on NK cells. NK cells play their roles mainly by the interaction between immunoregulating receptors and the ligands. Some GC cells express fewer NKG2D ligands to decrease NK cell sensitivity. The NKG2D ligand expression in GC patients is associated with favorable presenting features and a better OS (32). Patients with GC release higher levels of soluble MICA and MICB compared with healthy donors to downregulate NKG2D expression and dampen NK cell cytotoxicity (33). In addition, Xing et al. demonstrated that the sensitivity of GC cells to the cytotoxicity of NK cells was determined by copy number variations of HLA-I and activation of the NKp30 pathway (34). B7-H6, a human receptor, alerts innate immunity to cellular transformation via its interaction with the NKp30 (35). Chen et al. discovered that B7-H6–positive carcinomas were significantly associated with a higher differentiation, whereas there was no significant difference between B7-H6 expression and prognosis of GC patients (36). In addition, as a non-classical MHC-I antigen, HLA-G is expressed in most of GC tissues. The overexpression of HLA-G in GC cell lines inhibits the cell proliferation and cytotoxic activity of NK-92MI cells and reduces the secretion of IFN-γ and TNF-α through immunoglobulin-like transcript 2 (37).

In addition to ligand expression, GC achieves immunosuppression through suppressive cytokines and cells in its tumor microenvironment. Development of GC is accompanied by augmented levels of serum IL-10 and TGF-β1, which result in a remarkable decrease in cytotoxic activity of NK cells (38). Recently, TGF-β was discovered to convert NK cells into intermediate type 1 innate lymphoid cells (intILC1s) and ILC1s to help tumor escape immunosurveillance (39), whereas the signal transducer SMAD4 impedes the conversion by curtailing non-canonical TGF-β signaling (40). A study suggested that the production of prostaglandin E2 by GC cells may play a primary role in suppressing NK cell proliferation and inducing apoptosis (21). Midkine, a heparin-binding growth factor overexpressed in various human cancers, upregulates MICA/B serum levels of GC patients and inhibits CD107a and granzyme B expression, thereby suppressing NK cell cytotoxicity (33). The neoplastic cells can also evade immune surveillance via generation of regulatory cells, such as Tregs and macrophages, myeloid-derived suppressor cells (MDSCs) (41). A study by Choi et al. showed that an increased proportion of MDSCs was an adverse independent prognostic factor in GC (42). Moreover, the tumor-associated macrophages (TAMs) also play important roles in immune suppression in GC (43). They differentiate into M1 or M2 subtypes according to the stimulus present in the tumor microenvironment (44). M1 TAMs exert antitumor activities by releasing proinflammatory cytokines, whereas M2 TAMs may drive local immune suppression through production of IL-10 and TGF-β. Peng et al. demonstrated that TAMs were physically close to NK cells, and they could impair NK cell expression of IFN-γ, TNF-α, and Ki-67 via producing TGF-β1 (22).

NK CELL ADOPTIVE THERAPY AGAINST GC

Although different treatment strategies have been evaluated in recent years, such as adjuvant chemotherapy, adjuvant chemoradiotherapy, and perioperative chemotherapy, the outcome of GC is still not good. Therefore, investigating innovative therapeutic strategies is of great importance. Adoptive cellular immunotherapy has made some achievements in the treatment of GC (45). NK cells are considered to be promising effector cells in the adoptive immunotherapy of cancer (46) and have been used as an effective treatment modality for hematological malignant diseases and solid tumors (47).

Unlike T-cell infusion, donor-vs.-recipient NK cells reduce leukemia relapse and do not induce graft-vs.-host disease (GVHD) at the same time, as the inhibitory KIR (donor)–HLA-I (patient) mismatch leads to alloreactivity, and then NK cells lyse leukemia blasts, recipient dendritic cells (DCs), and recipient T cells (48–50). Re et al. reported that cancer cells from a GC patient who did not possess at least one of the major HLA class I allele groups were killed efficiently by NK cells (51). Although all published studies agree that NK cell infusion is a safe and well-tolerated procedure and is not associated with GVHD, results of NK cell infusion are not optimal (10, 52). One of the obstacles for NK cell-based treatments is that the exiguous amount of NK cells in blood cannot overcome the large tumor burden; hence, expanding NK cells *in vitro* to obtain a high number of NK cells with high cytotoxicity in compliance with good manufacturing practices (GMP) could be an active measure (10).

Clinical-grade NK cells can be produced from various sources, including peripheral blood, cord blood, bone marrow, and embryonic stem cells (10, 53). Effective amplification of NK cells was achieved by short-term culture with cytokines alone or by co-culture with cytokines and different feeder cells (54–57). In 2008, Alici et al. added anti-CD3 antibody for the first 5 days and IL-2 for the remaining days to peripheral blood mononuclear cells (PBMCs) from healthy individuals and managed to obtain a good quantity of activated NK cells without the need for feeder cells (58). Intriguingly, Sutlu et al. optimized the expansion of clinical-grade NK cells from PBMCs of healthy individuals using an automated bioreactor. The end product of the expansion protocol had a median of 38% NK cells, ensuring that a clinically relevant cell dose was reached (mean 9.8×10^9 NK cells) (59). Moreover, with repeated stimulation of irradiated Epstein-Barr virus-transformed lymphoblastoid cell lines and IL-2 as well as addition of IL-21 at the initiation of the culture, NK cells obtained a 10^{11}-fold expansion after 6 weeks. The expanded NK cells upregulate TRAIL, NKG2D, and DNAM-1 and have superior cytotoxicity against tumor cell lines *in vitro* (60). Interestingly, with osteoclasts as feeder cells, highly potent NK cells can be obtained in a considerable frequency (61, 62). Several studies suggested that using an artificial antigen-presenting cell K562-based system to expand NK cells from core blood units or

PBMCs was another efficient and safe technique, which enabled us to get plenty of NK cells off the shelf (56, 63–66). While clinical studies are ongoing using elutriation-derived monocytes for large-scale generation of DCs to treat a variety of metastatic cancers, Voskens et al. demonstrated that cytolytic NK cells could be generated from lymphocyte-enriched fractions obtained by GMP-compliant countercurrent elutriation from PBMCs (56). NK cells can also be generated from hematopoietic stem cells (67). In addition to donor-derived primary NK cells, cytotoxic cell lines, such as NK-92 have also been developed for clinical applications. Continuously expanding NK-92 cells do not require laborious isolation from blood, and sufficient NK cells with unlimited availability can be obtained (68). Sakamoto et al. successfully generated large numbers of activated NK cells by stimulating PBMCs of patients with digestive cancer, including GC with OK432, IL-2, and modified FN-CH296-induced T cells. The expanded cells were safe to administer in a monotherapy (69). Different methods of obtaining a large quantity of NK cells are shown in **Figure 3**.

In most instances, the expanded NK cells alter the balance of receptor expression and cytotoxicity, restoring cytotoxicity against both various allogeneic tumor targets and, more importantly, against autologous-derived gastric tumor targets. After expansion, NK cells significantly upregulate activating receptors DNAM-1, NKp46, NKp44, NKp30, and NKG2D and express high levels of CD16 as well as TRAIL and FasL. In addition, they rapidly release large amounts of IFN-γ and TNF-α after stimulation and efficiently kill tumor cells (13, 54, 56). Mimura et al. found that resting NK cells from GC patients showed negligible cytotoxicities against all GC cell lines. IL-2–stimulated NK cells showed variable cytotoxicities, which remained below 30% for most cell lines tested, and NK cells expanded by coculture with K562-mb15-4.1BBL cells were markedly cytotoxic, with the mean cytotoxicity exceeding 65% in three of the eight GC cell lines tested (32). These findings suggest that expanded NK cell-adoptive therapy could be used to augment the antitumor effects of endogenous NK cells.

THERAPIES TO IMPROVE NK CELL FUNCTION

So far, different immunotherapy approaches including vaccines, monoclonal antibodies, cytokines, and cellular adoptive therapy have been proven to directly stimulate and activate immunity and raise the number of effective cells or cytokines to strengthen the immune response or increase the immunogenicity or susceptibility of cancer cells. Besides NK cell-adoptive therapy, other immunotherapies that can manipulate NK cells and enhance NK cell activity to improve immune responses hold great promise for GC (**Figures 4A,B**).

Cytokines Reverse NK Cell Dysfunction in GC

With the progression of tumors, NK cells often become anergic due to downregulation of activating receptor signaling and upregulation of inhibitory receptors as well as suppressive regulatory cells or soluble factors in the microenvironment. Therefore, avoiding NK cell exhaustion would be an effective modality to cure cancer. Treatment with activating cytokines or blocking the signaling of suppressive cytokines might reverse NK cell exhaustion in tumors and chronic infections (14).

Treatments with NK-activating cytokines, IL-12, IL-18, or a mutant form of IL-2 (the "superkine" called H9), restored effector functions of MHC-I-deficient tumor-infiltrating NK cells with impaired signaling downstream of activating receptors. Finally, the survival of MHC-I-deficient tumor-bearing mice was increased (70). In addition, a human gastric carcinoma cell line HR, transduced with the IL-2 gene, could secrete sufficient quantities of bioactive IL-2. Thus, it became more susceptible than parental tumor cells to NK cells, and hepatic metastases in tumor-bearing mice regressed due to the recruitment of NK cells to the tumor site (71). Garcia-Lora et al. found that compared with unstimulated NKL cells, IL-2-stimulated NK cells obtained sustained growth and cytolytic activity by regulating different nuclear transcription factors, protein kinase C isoenzymes and mitogen-activated protein kinases (72, 73). In addition, IL-2 *ex vivo* treatment of NK cells could restore the impairment of Herceptin-mediated ADCC in patients with GC, concomitant to the normalization of the expression of CD16zeta molecules (31). Another cytokine, IL-15, can promote the survival and expansion of NK cells mainly through various STAT5 species. NK cells expressing membrane-bound IL-15 achieved autonomous growth and increased cytotoxicity (7, 74). Moreover, IL-15 fused with the extracellular domain of NKG2D (dsNKG2D–IL-15) exhibited enhanced NK cell tumor infiltration and higher efficiency than IL-15 in suppressing xenografted GC growth in nude mice (75). On the other hand, fractalkine (CX3CL1), a CX3C chemokine, could enhance the recruitment of NK cells and induce both innate and adaptive immunity, thereby yielding a better prognosis in gastric adenocarcinoma (76). Cytokines that reverse NK cell dysfunction are illustrated in **Table 1**.

Immunomodulatory Drugs Tune the NK Cells Immunologic Function

Immunomodulatory drugs have made great progress in the treatment of cancer in recent years. Combined treatment of recombinant macrophage inflammatory protein-1 alpha and signaling bacterium acnes could recruit a large number of NK cells to both tumor sites and regional lymph nodes. Moreover, it induced a strong T-helper 1 immunity at an early time, which later led to improved survival of tumor-bearing mice (77). Alternatively, polysaccharide krestin (PSK), a mushroom extract, is a specific TLR2 agonist and could offer significant advantages in survival over chemotherapy alone for patients with curative resections of GCs (78). PSK was previously reported to mediate induction of the NKL cell proliferation and activation, and it was found to induce apoptosis in the AGS cell line as well (79). Of interest, a neutral polysaccharide fraction (SMPA) from salvia miltiorrhiza significantly promoted the production of anti-inflammatory cytokines (IL-2, IL-4, and IL-10) and augmented the killing activity of NK cells in GC rats (80). Lupeol, a triterpene found in various vegetables, increases the proliferation and killing

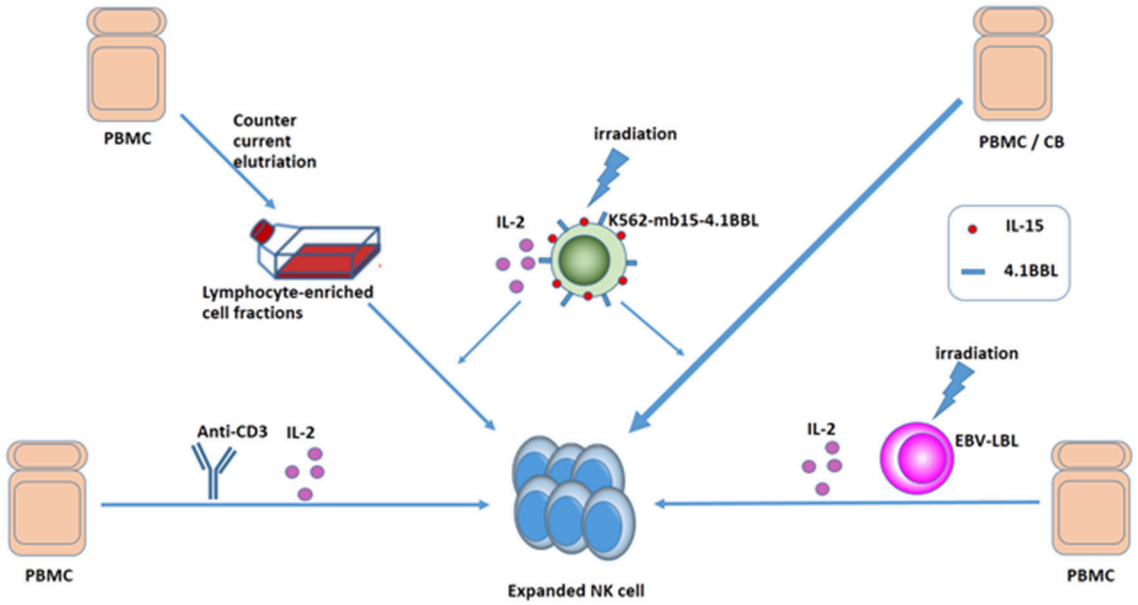

FIGURE 3 | Ways to expand NK cells. Expanded NK cells can be generated from PMBC or CB: (1) culture with cytokines and anti-CD3 without feeder cells, (2) culture with cytokines and irradiated EBV-LCL, and (3) GMP-compliant countercurrent elutriation, and (4) using an APC K562-based system.

effect of NK cells on GC cell lines BGC823, N87, and HGC27 by increasing the expression of perforin, IFN-γ, and CD107a via the activation of the PI3K/Akt and Wnt/β-catenin signaling pathways (81). Subsequently, Qu et al. unveiled a synthetic analog of double-stranded RNA intracellular poly(I:C), which not only triggered gastric adenocarcinoma cell apoptosis but also induced type I IFN production by gastric adenocarcinoma cells (82). In addition, the histone deacetylase inhibitor valproic acid could increase sensitivity to expanded NK cells by upregulating the expression of MICA/B (32).

Immune Checkpoint Blockades Augment NK Cell-Mediated Lysis

Immune checkpoints are molecules that can provide either activating or inhibitory signals to the immune system. Stimulators CD28, OX40, CD58, CD40L, CD80, CD86, and CD137 can promote immune activation, whereas inhibitors programmed death-1 (PD-1), cytotoxic lymphocyte associated antigen-4 (CTLA-4), lymphocyte activation gene 3, T-cell immunoreceptor with Ig and ITIM domains, T-cell immunoglobulin, and mucin-domain containing-3 suppress immune activation (47, 83). Blockades targeting these checkpoints are being tested for the potential to treat cancer.

Two main checkpoints are PD-1 and CTLA-4, and therapeutic blockades of them have become a paradigm-shifting treatment in solid tumor oncology (85). Engagement of PD-1 with programmed death ligand 1 (PD-L1) expressed on cancer cells results in the suppression of T-cell proliferation and response, which eventually leads to tumor immune evasion (86). Accumulating data show that the expression of PD-L1 is upregulated in tumor cells from patients with GC, especially in mismatch repair-deficient and Epstein-Barr virus-positive GC, which suggests that the PD-1/PD-L1 pathway plays a critical role in the immune evasion of GCs (87–90). In addition, Liu et al. detected PD-1 expression on peripheral NK cells in patients with GC by flow cytometry. Compared with that in healthy controls, a significant increase in PD-1 expression on NK cells was observed in GC patients. More importantly, PD-1/PD-L1 blockades significantly augmented degranulation and IFN-γ secretion and suppressed apoptosis of NK cells by enhancing the activation of the PI3K/AKT signaling pathway in NK cells (91). Many clinical trials on the anti-PD-1 antibody pembrolizumab have been explored in the treatment of GC and were proven to be safe in the setting of design. In a phase 1b trial involving the use of pembrolizumab in patients with PD-L1-positive advanced GC, pembrolizumab had a manageable toxicity profile and promising antitumor activity, eliciting sustained antitumor responses in 22% of patients according to a central review (92). As a fully human anti-PD-L1 IgG1 antibody, avelumab obtained an acceptable safety profile but did not result in an improvement in OS or progression-free survival in patients with gastric or gastroesophageal junction cancer (GEJC) with single-agent avelumab in the third-line setting (93). Apart from anti-PD1/PD-L1 antibodies, ipilimumab and tremelimumab (which target CTLA-4) are also under clinical investigation in the treatment of GC. A recent study employing ipilimumab in unresectable locally advanced/metastatic GC/GEJC did not prove ipilimumab efficacy as monotherapy, whereas a comparable median OS of ∼1 year and a favorable safety profile supported the investigation of ipilimumab in combination with other therapies for advanced GC (94). Tremelimumab as a second-line treatment for metastatic esophageal and gastric adenocarcinomas achieved

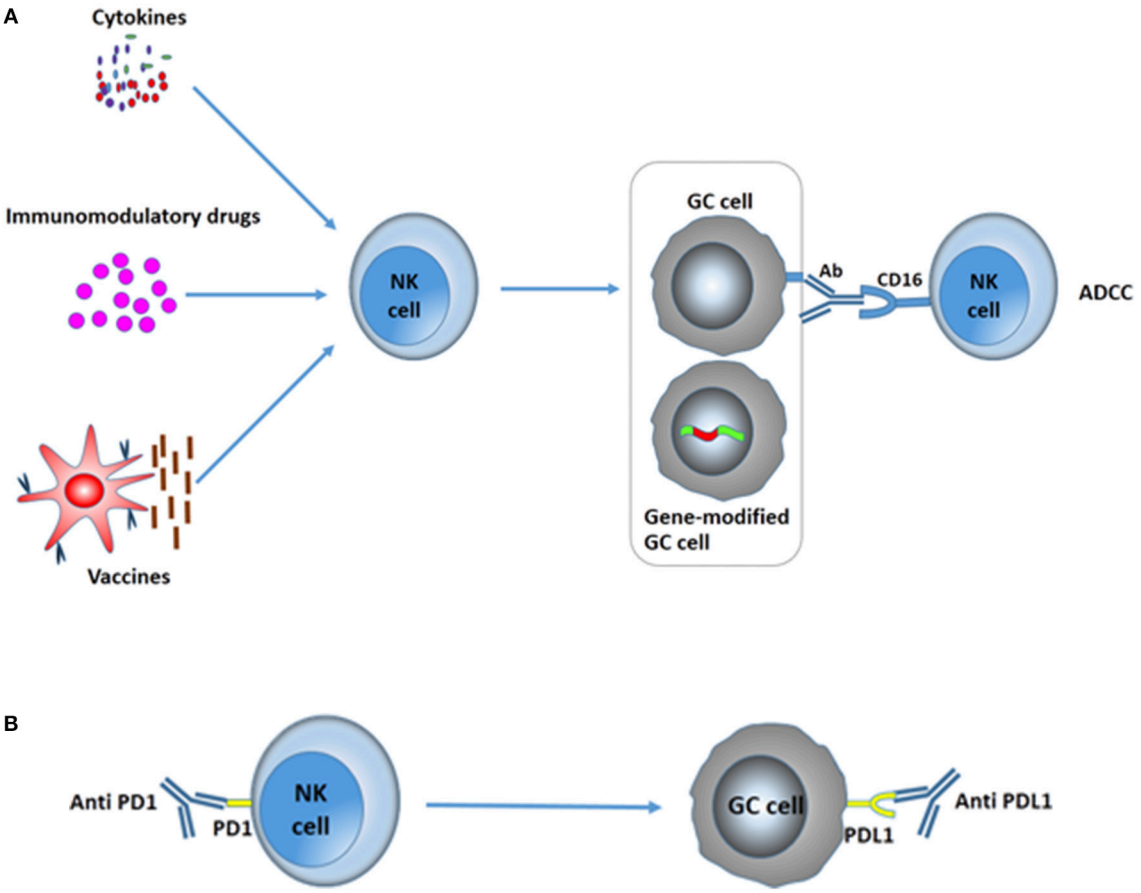

FIGURE 4 | Various immunotherapies in the treatment of GC to improve NK activity. **(A)** Cytokines, immunomodulatory drugs, and vaccines can increase the cytotoxicity of NK cells to kill GC cells. Moreover, antibodies can markedly increase NK cell-mediated ADCC, resulting in the death of GC cells. Gene therapy toward GC cells could improve the immunogenicity or susceptibility of gastric tumor cells to NK cells. **(B)** Immune checkpoint blockades. PD-1/PD-L1 blockades significantly augment degranulation and IFN-γ secretion of NK cells and suppress the evasion of GC.

only a 5% objective response rate in a phase II study. However, a small cohort of patients (4 of 18) achieved disease control, as assessed by stable computed tomography scan (95). Nevertheless, effects of anti-PD-1/PD-L1 antibodies on NK cells in patients with GC need further evaluation.

Antibodies Increase NK Cell Cytotoxicity to GC via ADCC

Immunotherapy of tumors with specific antibodies has achieved great success in the past 20 years. Herceptin is a humanized monoclonal antibody (mAb) that specifically targets human epidermal growth factor receptor-2 (HER2)/neu and exhibits growth inhibitory activity against HER2/neu-overexpressing tumors. Research has suggested that HER2/neu-expressing GC cells could be killed by Herceptin-mediated ADCC and depend on the degree of HER2/neu expression on the GC cells. However, Herceptin-mediated ADCC was significantly impaired because of its NK cell dysfunction in patients with advanced disease. Interestingly, IL-2 *ex vivo* treatment of NK cells could restore CD16zeta expression, contributing to restoration of Herceptin-mediated ADCC (31). Furthermore, Mimura et al. discovered that the presence of Herceptin markedly increased the cytotoxicity of expanded NK cells against the HER2-positive GC cell lines MKN7 and NCI-N87. Meanwhile, lapatinib, which targets both HER2 and epidermal growth factor receptor (EGFR), could upregulate HER2 cell surface expression on both MKN7 and NCI-N87, resulting in an increase in Herceptin-mediated ADCC by expanded NK cells (32). Another antibody, cetuximab, is a chimeric mAb to EGFR. Hara et al. demonstrated that cetuximab showed moderate antitumor activity to MKN-28 cells by slightly inhibiting ligand-induced phosphorylation of protein kinase B and extracellular signal-regulated kinase, but cetuximab in combination with IL-2 significantly inhibited subcutaneous and intraperitoneal tumor growth of MKN-28 cells in nude mice by NK cell-mediated ADCC rather than the blockade of the intracellular signaling pathway (96). Besides, Hasegawa et al. produced an afucosylated humanized anti-EPHA2 mAb DS-8895a, which could recognize and bind to EPHA2 that was anchored to cell membranes. DS-8895a markedly enhanced NK cell-mediated ADCC *in vitro* and also inhibited tumor growth

TABLE 1 | Cytokines reverse NK cell anergy in GC.

Cytokine	Function	Molecular mechanisms	Trials	References
IL-2	Improve NK cell–mediated ADCC Increase IL-2 gene–transduced HR susceptibility Recruit NK cells	Restore CD16zeta expression of NK cells Lower Lower expression of HLA-I molecules on tumor cells	Patients, cell lines Tumor-bearing mice, cell lines	(31) (71)
IL-5	Activate NK cells Promote proliferation and cytotoxicity of NK cells	Increase CD69 and CD107a expression Elevate IFN-γ production production	Tumor-bearing mice, cell lines	(75)
dsNKG2D–IL-15	Activate NK cells Promote proliferation and cytotoxicity of NK cells	Efficiently bind to MICA Increase CD69 and CD107a expression Elevate Elevate IFN-γ production	Tumor-bearing mice, cell lines	(75)
Recombinant mouse IL-15	Induce NK cell proliferation Increase cytotoxic activity of NK cells	Increase IFN-γ secretion	Tumor-bearing mice	(84)
Fractalkine (CX3CL1)	Increase NK cell infiltration Induce innate and adoptive immunity	Chemoattract NK cells	Human resections of gastric adenocarcinoma	(76)

in EPHA2-positive human GC SNU-16 xenograft mouse models (97). Moreover, an FGFR2b-specific humanized monoclonal antibody, FPA144, has been investigated to treat patients with GC overexpression of the FGFR2b as a single agent in clinical trials (NCT02318329). FPA144 not only blocks ligand binding and induces FGFR2b internalization but also enhances ADCC. Notably, FPA144 increased PD-L1-expressing cells in the tumor microenvironment, and the combination of FPA144 and RPM1-14, a PD-1 blockade, inhibited tumor growth by 49% ($p < 0.001$) (98). All the above studies suggest that using mAb to increase NK cell activity would be a promising alternative approach for a subset of GC patients, even though most are not yet available for routine use in GC.

Vaccines Stimulate Antitumor Immune Responses Against GC

Vaccines have been a useful tool to stimulate both adaptive and innate antitumor immune responses to improve cancer immunotherapy. As DCs are professional antigen-presenting cells that can capture and process tumor-associated antigens, DC-based vaccines evolved as promising vaccination protocols in cancer therapy. Schmitz et al. revealed that M-DC8$^+$ DCs could stimulate proliferation, IFN-γ secretion, and tumor-directed cytotoxicity of NK cells depending on cell-to-cell contact (99). Liu et al. pulsed DCs with total RNA from MFC GC cells as vaccine and discovered that it stimulated and upregulated NK cells and tumor-specific CTL activity in mice with GC xenograft, highlighting that the use of DC-based tumor vaccine could provide a glimmer of hope for patients with GC (100). HSP-gp96 is a heat shock protein glycoprotein named according to its molecular weight of 96 kDa. Lu et al. infected human GC cell lines KATOIII, MKN-28, and SGC-7901 with adenovirus gp96 at a multiplicity of infection of 100 and purified gp96-GC antigen peptide complexes. Compared with GC-derived peptide, gp96-GC antigen peptide complexes markedly improved NK cell activity at different concentrations (101).

Gene Therapy Improves the Immunogenicity or Susceptibility of Gastric Tumor Cells to NK Cells

Gene therapy offers a new measure for GC treatment. Intercellular adhesion molecule (ICAM)-2 is a second ligand of leukocyte function-associated antigen-1 (CD11a/CD18). The interaction between CD11a/CD18 and ICAM-2 can mediate many leukocyte functions, including Ig production and the cytotoxicity of NK cells. Tanaka et al. gave mice with peritoneal dissemination of scirrhous gastric carcinoma an injection of an adenovirus vector, AdICAM-2, that encoded the full-length human ICAM-2 gene. The tumor-bearing mice survived for a significantly longer time, and many NK cells filtrated the peritoneal metastatic lesions, indicating ICAM-2 transfection might be an effective form of gene therapy for peritoneal metastasis of GC (102). Moreover, Li et al. transfected the MGC GC cell line with small interfering RNA (siRNA) that could silence the expression of heavy chain genes of all immunoglobulin isotypes consistently. The siRNA could knock down cancerous Ig, which inhibited ADCC by competitively binding to the Fc receptor on NK cells. As a result, it enhanced ADCC induced by an EGFR antibody in a dose-dependent manner and inhibited the growth of MGC GC cells (103).

CONCLUSION

As essential effectors in host immunity, NK cells can mediate the death of GC cells by ADCC, releasing perforin and granzymes, secreting IFN-γ and TNF-α, or eliciting apoptosis via formation of complex FAS/FASL and TRAIL/TRAILR. NK cells' activity is correlated to clinical stage, lymphatic and vascular invasion,

lymph node metastases, and prognosis in GC patients. Gastric tumors could escape NK cell surveillance via downregulating ligands of activating receptors, secreting suppressive cytokines, and attracting suppressive cells. With the progression of GC, both the number and activity of NK cells are decreased. Thus, reversing NK cell dysfunction may be an effective treatment for GC.

As mentioned earlier, NK cell adoptive therapy is a safe and well-tolerated procedure, but it showed limited value because of the limited number of available NK cells. Expanded NK cells for adoptive treatment has become a promising measure to solve the problem. However, there is little research investigating whether expanded NK cells for adoptive treatment is effective for GC patients. A phase I clinical trial determined that autologous expanded NK cell therapy was safe and well-tolerated in patients with advanced digestive cancer, including GC. Although NK cell transfer as a monotherapy did not result in a clinical response in patients, transferred NK cells persisted in the peripheral circulation of patients and exerted cytotoxicity *in vitro*, providing the potential of efficacious combination treatment with other reagents (69). Combination therapy of adoptive NK cell therapy and IgG1 monoclonal antibodies shows good tolerability and preliminary antitumor activity in patients with unresectable advanced gastric or colorectal cancer along with induced Th1-type immune response and reduced peripheral Tregs (104). Simultaneously, some cytokines, immunomodulatory drugs, immune checkpoint blockades, antibodies, vaccines, and immunogene therapies can enhance NK cell function through different mechanisms and have made some achievements in inhibiting the growth of GC in some studies. But further research is still needed to optimize NK cell-based therapy. A combination of different treatment strategies may make promising outcomes. In recent years, chimeric antigen receptor (CAR)-modified NK cells have appeared as a revolutionary immunotherapy option for the treatment of many malignancies (105, 106). CARs consist of an extracellular single-chain variable fragment capable of recognizing a cancer antigen and intracellular activation motifs that activate NK cytotoxicity upon antigen recognition. With the addition of a CAR, NK cells might add a new method of redirecting target cells to increase the number of NK cells in tumor regions. It is specifically useful in patients with downregulated activating receptors. CAR-NK may provide a new research direction for GC. CAR-NK can be obtained from umbilical cord blood gene-modified human hematopoietic stem cells using co-culture with a feeder stroma of murine OP9-DL1 cells in the presence of human recombinant cytokines or using insulin-like growth factor 1 alone (67). Recognizing the low transfection efficiency of blood NK cells, investigators are trying to generate a clonal NK-cell line. At present, only the NK-92 cell line displays a consistent and high cytotoxicity to cancer targets. NK-92 cells can be easily engineered by non-viral transfection methods to express CARs that can retarget them toward malignant cells. In addition, the preparation and administration of NK-92 cost significantly less compared with autologous or allogeneic NK cells and, particularly, compared with CAR-T cells (107). CAR-NK cells specific for CD19, CD20, EGFR, and HER2 have made promising progress in killing of target cells. Of note, CAR designs with 4-1BB co-stimulation led to a higher cytolytic capacity and cytokine production (108). Given this, HER2+ GC may benefit from treatment of CAR-NK cells. Nevertheless, our review suggests that NK cell-based therapy is expected to offer a promising prospect to GC patients and deserves more study.

AUTHOR CONTRIBUTIONS

YW conceived this study and YD wrote the manuscript. All authors revised the manuscript.

FUNDING

This study was supported by the National Natural Science Foundation of China (grant No. 81673033 to YW).

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

frontiers in Immunology

MINI REVIEW
published: 08 February 2019
doi: 10.3389/fimmu.2019.00187

Th17 Cells Paradoxical Roles in Melanoma and Potential Application in Immunotherapy

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The progressive infiltration of immune cells is associated with the progression of melanoma. Specifically, Th17 cells in melanoma microenvironment have both antitumor and protumor effects. It is now necessary to understand the contradictory data associated with how Th17 cells play a role in melanoma. This review will summarize the current knowledge regarding the potential mechanisms that may be involved in the effects of Th17 cells in melanoma progression. Currently, since adoptive transferring Th17 cells has been successful in eradicating melanoma in mice, it offers promise for next-generation adoptive cell transfer, as *ex vivo* expanded stemness-like memory Th17 cells which are induced by distinct cytokines or pharmacologic reagents may be infused into melanoma patients to potentiate treatment outcome.

Keywords: Th17 cell, melanoma, tumor microenvironment, immunotherapy, adoptive cell transfer

OPEN ACCESS

Edited by:
Prashant Trikha, Nationwide Children's Hospital, United States

Reviewed by:
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Yared Hailemichael, University of Texas MD Anderson Cancer Center, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *20 November 2018*
Accepted: *22 January 2019*
Published: *08 February 2019*

Citation:
Chen C and Gao F-H (2019) Th17 Cells Paradoxical Roles in Melanoma and Potential Application in Immunotherapy. Front. Immunol. 10:187. doi: 10.3389/fimmu.2019.00187

INTRODUCTION

Melanoma is a tumor originated from melanocytes, specialized pigmented cells that are mainly resident in the skin (1, 2). Worldwide, it is estimated that around 287,723 (1.6%) cases of all newly diagnosed cancers are cases of melanoma of the skin, and about 60,712 cancer deaths are due to melanoma of the skin, based on cancer statistic carried out in 2018 (3). Melanoma can be cured by surgical resection if it is diagnosed early, and the 5-year survival rate is over 89% (4). However, once melanoma has spread, it rapidly becomes life-threatening (5).The treatment options for patients with advanced, unresectable, or metastatic melanoma, especially BRAF-V600E/K mutant melanoma which comprises ∼50% of the cases, have changed dramatically over a short period of time (6). BRAF inhibitors (Vemurafenib and Dabrafenib) and MEK inhibitors (Trametinib and Cobimetinib) have been approved by the FDA for treatment of advanced-stage melanoma patients with BRAF-V600-mutant (7, 8). In patients with BRAF-mutant, BRAF-inhibitor-refractory disease checkpoint inhibitors against PD-1 (pembrolizumab and nivolumab) and CTLA4 (ipilimumab) have demonstrated efficacy (6, 7, 9, 10). Nevertheless, 10–15% of patients treated with ipilimumab experienced a range of inflammatory side effects, also called immune-related adverse events (irAE) that even lead to death sometimes (4, 11, 12). So, development of new therapeutic approaches and optimization of current therapeutic approaches would be of great importance in the field of melanoma therapy research.

Extensive literature describes how immune cells recruited to the tumor microenvironment can exert critical functions in tumor development and progress (13). Specifically, the roles of T cells in cancer are of great interest. Apart from $CD8^+$ cytotoxic T lymphocytes, $CD4^+$ T cells are essential components of cell-mediate immunity. Th17 cells, as a third subset of $CD4^+$ T cells, are developmentally distinct from Th1 and Th2 lineages(14, 15). IL-6,

TGF-β, and IL-1β are required for Th17 cells differentiation from naïve $CD4^+$ T cells and IL-21, IL-23 contribute to their maintenance (16–18). These cytokines induce signal transducer and activator of transcription 3 (STAT3) activation and expression of the major transcription factors of Th17 cells retinoic acid-related orphan receptor (ROR)γt, RORα as well as other transcriptional factors (19, 20), and then promote expression of IL-17, IL-21, IL-22 (21). It is well known that Th17 cells play an important role in inflammation disease (22, 23). And accumulating evidence suggest that Th17 cells present in tumors, including melanoma. Moreover, progressively greater number of infiltrating Th17 cells were appreciated during the development of melanoma from common melanocyte nevi to dysplastic nevi to malignant melanoma (24). Currently, many research groups have made great efforts to reveal the effects of Th17 in melanoma. Herein, we focus on the mechanisms of Th17 cells accumulation and function in melanoma and discuss ways to manipulate Th17 via cytokines and pharmaceutics to potentiate treatment outcomes in patients.

MAJOR MECHANISMS REGULATING TH17 CELL ACCUMULATION AND EXPANSION

Mechanisms of Th17 Cells Recruitment in Melanoma Microenvironment

Recent studies have suggested the potential mechanisms responsible for the recruitment of Th17 cells in melanoma microenvironment. Chemokines secreted by melanoma cells and the cells within melanoma microenvironment play an important role in recruiting immune cells with corresponding receptors to melanoma microenvironment. Th17 cells express both Th1-associated (CCR2, CXCR3, CCR5, and CXCR6) and Th2-associated (CCR4) trafficking receptors. In addition, Th17 cells express non-lymphoid tissue trafficking receptors (CCR4, CCR5, CCR6, CXCR3, and CXCR6) as well as homeostatic chemokine receptors (CD62L, CCR6, CCR7, CXCR4, and CXCR5) that are involved in T cell migration to lymphoid tissues (25). However, tumor-infiltrating Th17 cells may have different chemokine receptors in different tumor contexts. In melanoma, tumor-infiltrating Th17 cells express CCR2, CCR4, CCR5, CCR6, CCR7, and CXCR3 (26).

Work by Peng's group showed that tumor-derived fibroblasts secreted MCP-1 (known as CCL2), the ligand for CCR2 or CCR4, and RANTES (known as CCL5), the ligand for CCR1, CCR3, or CCR5. And they both mediated the recruitment of Th17 cells in tumor microenvironment (26). When triggering TLR and Nod signaling to increase the expression of MCP-1 and RANTES by melanoma cells and tumor-derived fibroblasts, they found that the chemotactic activity of Th17 cells was enhanced. This result suggests that chemokines, involved in TLR and NOD signaling, secreted by melanoma cells and tumor-derived fibroblasts could lead Th17 cells recruiting to melanoma sites (**Figure 1**) (26).

Tumor-associated macrophages (TAMs) are also involved in Th17 cells infiltration. Researchers used modified melanoma-condition (MCM) to differentiate human monocytes to macrophages and they found that these MCM-induced macrophages strikingly increased CCL2 expression (27). Furthermore, tumor-infiltrating monocytic myeloid-derived suppressor cells (MO-MDSC) and granulocytic myeloid-derived suppressor cells (PMN-MDSC) from B16-bearing mice could produce higher levels of CCL3, CCL4, CCL5 than that in control mice at melanoma sites, especially MO-MDSCs (28). And CCL4/MIP-1β, which is produced by immature myeloid cells, could recruit Th17 cells to tumor sites (**Figure 1**) (29).

Th17 Cells Development, Differentiation, and Expansion in Melanoma Microenvironment

Tumor cells and tumor-derived fibroblasts promote expansion of human Th17 cells within melanoma by producing proinflammatory cytokines and providing cell-cell contact. When cocultured naïve $CD4^+$ T cells isolated from human peripheral blood mononuclear cell with melanoma cells and tumor-derived fibroblasts, the percentage of Th17 cells was higher than in the medium alone. Instead, if naïve $CD4^+$ T cells were separated from melanoma cells or tumor-derived fibroblasts using the transwell system, the generation of Th17 cells from naïve $CD4^+$ T cells was significantly decreased compared to coculture (26). This indicated that melanoma cells and melanoma associated fibroblast could provide cell-cell contact mechanism to promote the expansion of Th17 cells, but the underlying mechanism remains to be defined.

Cytokines engaged in Th17 cells differentiation and maintenance also contribute to Th17 cells expansion in melanoma. Melanoma cells and tumor-derived fibroblasts could express high levels of IL-1β, IL-6, TGF-β, and IL-23, which provide an optimal proinflammatory cytokine milieu for Th17 cells expansion (26). Apart from melanoma cells and tumor-derived fibroblasts, dendritic cells in melanoma sites could also produce IL-6, TNF-α, IL-12p70, and IL-23 (30). In addition, macrophages are abundant leukocytes in melanoma lesions. They express high levels of IL-1β and IL-6, which may also contribute to Th17 cells expansion in melanoma (**Figure 1**) (31).

PARADOX OF TH17 CELLS FUNCTIONS IN MELANOMA

Although Th17 cells are prevalent in melanoma microenvironment, the relationship between Th17 cells and tumor immunopathology remains controversial with both antitumor and protumor effects depicted in melanoma (32).

Antitumor Effect of Th17 Cells in Melanoma

Th17 cells do not express neither granzyme B nor perforin and have no ability to inhibit tumor cells proliferation directly (33, 34). However, increasing evidence suggests that Th17 cells have potent antitumor effects in melanoma. Firstly, the conversion of Th17 cells toward Th1 cells may contribute to the antitumor effect of Th17 cells in melanoma. Expression IFN-γ is the main character of these Th17 cells. In metastatic melanoma

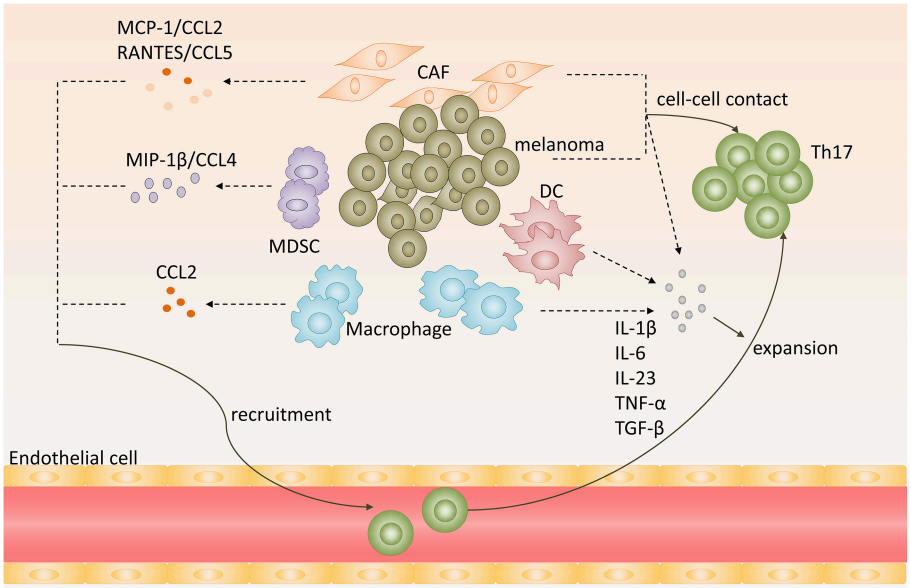

FIGURE 1 | Recruitment, expansion of Th17 cells in melanoma microenvironment. Chemokines, including CCL2, CCL5, CCL4, secreted by melanoma, cancer-associated-fibroblasts (CAF), myeloid-derived suppressor cells (MDSC) or tumor-associated macrophages (TAM), promote Th17 cells recruitment to melanoma. Melanoma cells, CAFs, TAMs, and DCs produce proinflammatory cytokines, such as IL-1β, IL-6, IL-23, TNF-α, and TGF-β, and provide cell-cell contact that promote expansion of Th17 cells.

patients characterized by high frequency of Th17 cells and IFNγ-secreting Th17 cells in peripheral blood before being vaccinated with therapeutic survivin-derived peptide epitopes were more likely to develop survival-specific T-cell reactivity and have higher survival rate than patients with lower frequency of these cells (35). Muranski and coworkers created a transgenic mouse expressing MHC class II-restricted T cell receptor, in which $CD4^+$ T cells recognize tyrosinase-related protein 1 (TRP-1), an antigen present both in normal melanocytes and B16 melanoma cells. They found that by adoptively transferring tumor specific Th17-polarized cells into large, established B16 melanoma mice, Th17-polarized cells-mediated destruction of advanced melanoma was more effective than that of Th1 cells. And this therapeutic effect was strictly dependent on interferon-γ (IFN-γ) and IL-17 production (36). Deficient IFN-γ or IL-17A impaired Th17 cell-mediated melanoma eradication effect (37). These data suggest that Th17 cells plasticity toward Th1-like effector cells may be responsible for Th17 cells antitumor efficiency.

In addition, Martin-Orozco reported that Th17 cells within melanoma microenvironment enhance antitumor effect through recruiting other leukocytes into tumor (38). They found the expression of CCL2/CCL20 was significantly increased in lung cell fraction containing both tumor and lung cells. Further analysis revealed that $CD11c^+$ DC, $CD4^+$, and $CD8^+$ T cells were greatly increased in Th17-treated mouse compared to control mice with metastatic melanoma in lungs (38). These results suggested that tumor-infiltrating Th17 cells stimulated tumor tissues to express CCL2/20 for recruiting various inflammatory leukocytes, such as DCs, $CD4^+$, and $CD8^+$ T cells to induce antitumorimmunity (34, 38).

Moreover, Th17 cells can exert an antitumor effect by augmenting $CD8^+$ T cells. Martin-Orozco and coworkers found that IL-17A-deficient mice were more likely to develop lung melanoma. Adoptive transferring tumor-specific Th17 cells prevents tumor development. The same group also found that therapy using Th17 cells elicited a remarkable activation of tumor-specific CD8+T cells, which were indispensable for the antitumor effect (38). In a recent study, researchers used RORγ agonist to prime TRP-1 transgenic Th17 cells and Pmel-1 TCR transgenic CD8+ T cells *ex vivo* and found these cells could effectively regress melanoma compared with those untreated Th17 cells. When co-infused with equal numbers of TRP-1, Th17 cells, and Pmel-1 Tc17 cells in mice with established melanoma, the antitumor effect was greatly enhanced. These data are consistent with previous reports, further confirming that Th17 cells can exert antitumor function by augmenting $CD8^+$ T cells (39). The underlying mechanism of antitumor immunity and CTL activated by Th17 cells may be that Th17 cells stimulated CTL response via IL-2 and peptide/major histocompatibility complex (pMHC)-I, which can be recognized by $CD8^+$ T cells and induce $CD8^+$ T activation, based on the fact that $IL2^{-/-}$ Th17 cells and $K^{b-/-}$ (without MHC I) Th17 cells lost their antitumor immunity (**Figure 2**) (34).

Protumor Effect of Th17 Cells in Melanoma

Despite some studies demonstrating an antitumor role of Th17 cells in melanoma, several lines of evidence suggest that Th17 cells can also have potent protumor effect in melanoma. BRAF mutation has been attributed to a reduced apoptosis, increased invasiveness and increased metastatic behavior (40). And

emerging data is revealing the existence of at least two divergent immune phenotypes in melanoma. One type is the Th17 immune phenotype (Class A) with prevalent expression of cancer testis antigens, over-expression of WNT5A, enhanced cyclin activity and poor prognosis. The second class (B) Th1 immune phenotype is associated with a more differentiated status, a higher responsiveness to immune cytokines and better prognosis (41). The question whether these two different phenotypes depend upon the genetic background had been explored by Francesco M Marincola' group. When performing class comparison between BRAF mutant and wild-type metastatic melanoma samples, metastases showing a Th17 phenotype were preferentially BRAF mutated. Moreover, some genes differentially expressed between BRAF mutant and wild-type samples were related to IL-17 pathway. So Th17 cells may also have a potent protumor effect in malignant melanoma (42, 43).

Firstly, the expression of IL-17 by Th17 cells has been reported to be associated with tumor angiogenesis in melanoma. In IFN-γ deficient mice, the expression levels of vascular endothelial growth factor (VEGF) and MMP9 were up-regulated in melanoma cells. The expression of both VEGF and MMP9 were reduced in IFN-$\gamma^{-/-}$IL-$17^{-/-}$ mice (37). These data suggested that IL-17 may promote angiogenesis in melanoma. This has also been confirmed by Yan's laboratory. They found that expression levels of CD31 and MMP9 were strikingly lower in tumor tissues treated with Ad-si-IL17 than control. In addition, VEGF was down regulated when inhibiting IL-17A in tumor tissue (44). The underlying mechanism may be that IL-17 promote STAT3 activity via increasing its phosphorylation in melanoma cells and epithelial cells (45).

Secondly, Th17 cells promote tumor proliferation and survival. Lin Wang group reported that IL-17 enhanced melanoma growth due to its direct effects on IL-17 receptors expressing cells, such as melanoma cells, fibroblasts, endothelial cells, and DCs, via promoting their secretion of IL-6. And then IL-6 activated oncogenic STAT3 in melanoma cells and increased expression of prosurvival genes, such as Bcl-2, Bcl-xl. Therefore, Th17 cells can promote melanoma growth via IL-6-Stat3 pathway (45).

In addition, another mechanism involved in the Th17 cells protumor effect in melanoma may be the Th17/Tregs plasticity in melanoma microenvironment. Th17 cells can function as regulatory cells with the ability to suppress antitumor immunity. Th17 cells undergo lineage conversion into Tregs (46, 47). And this conversion results in the intermediate phenotypes that coexpress transcript factors Foxp3 and RORγt (47, 48). Tumor infiltrating Th17 cells could secrete moderate amounts of IL-10 and TGF-β1 after CD3 Ab stimulation and express Treg cell markers Foxp3, CD25, and CTLA4 (26). These results suggested that tumor-infiltrating Th17 cells may have a dual function performing both effector and regulatory roles in melanoma microenvironment. Thus, Th17 cells may contribute to immunopathogenesis of melanoma. The underlying mechanism may involve tumor-infiltrating myeloid-derived suppressor cells, which contribute to the Th17-to-Treg conversion via secretion of TGF-β and retinoic acid (**Figure 2**) (49).

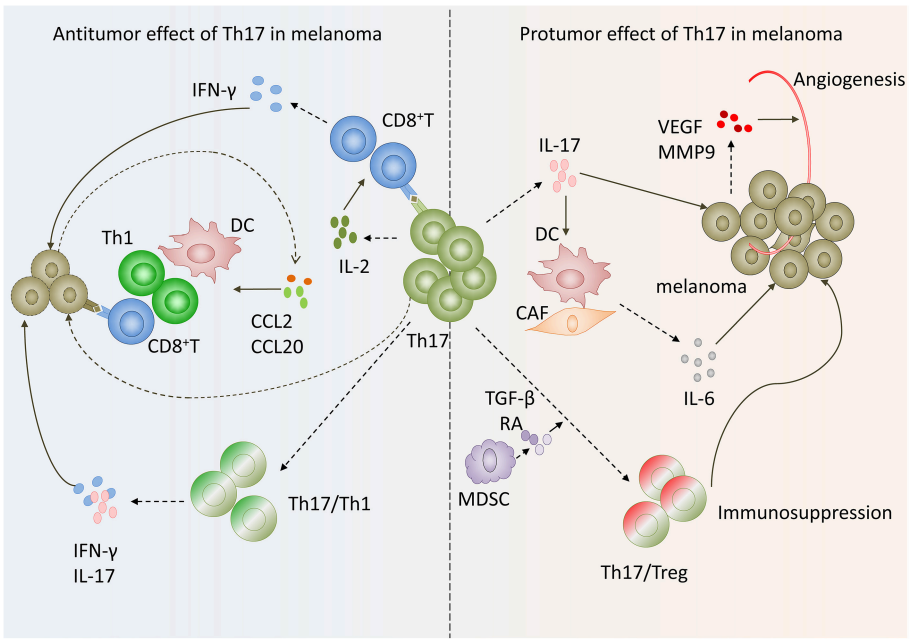

FIGURE 2 | Paradox of Th17 cells functions in melanoma. On the one hand, Th17 cells in melanoma exert antitumoral function via inducing effector cells recruitment and activating tumor-specific cytotoxic CD8+T cells as well as transform to Th1 phenotype. On the other hand, Th17 cells exhibit protumor function by promoting angiogenesis, melanoma cells proliferation and phenotype change toward Tregs.

BASIC STRATEGIES OF ADOPTIVE TRANSFERRING TH17 CELLS IN MELANOMA

Immunotherapy is a cornerstone in melanoma treatment. Adoptive cell transfer therapy (ACT) is a powerful way of improving patients' antitumor immunity via administration of *ex vivo* activated, expanded and selected autologous tumor-reactive T cells (50, 51). Until today, tumor-infiltrating lymphocyte (TIL) ACT was shown to elicit an objective response of 54% and complete remission of 24% in the population of melanoma patients with extremely advanced disease who have failed multiple standard therapeutic treatments (52–54).Currently, a key problem that prevents the adoption of TIL therapy is the need to infuse a vast number of cells to generate durable results in patients (53, 55, 56).To achieve consistent clinical responses, ACT requires administration of at least 40 to 60 billion tumor-reactive T cells and in some cases (57–59), up to 100 billion cells or even more (59–62), while most methods for generating vast T cells require 2 months or even longer (53). However, enhanced *in vitro* IFN-γ releasing and cytolysis $CD8^+$T clones did not induce an objective clinical response upon adoptive transfer because the cytotoxic $CD8^+$T cells lose their antitumor efficacy when expansively expanded *ex vivo* (52, 63, 64). This is because CD8+T cells entry into a proapoptotic and replicative senescent state and have a reduced capacity to persist *in vivo* once they reach terminal differentiation (51, 52, 65). Moreover, less differentiated $CD8^+$T cells may undergo incomplete maturation (66) or even be tolerized once encountered with the tumor specific antigen (67). Therefore, investigators focus on developing potential methods to circumvent these disadvantages by using a T cell subset that is refractory to senescence (52).

Chrystal M. Paulo' group found that three-week-expanded Th17 cells experienced robust growth *in vitro* and retained long-term eliminating melanoma efficacy with the effect of memory phenotype of $CD44^{hi}CD62L^{low}$ *in vivo*, which proved to be more efficient than Th1 and 1-week-expanded Th17 cells. Furthermore, mice transferred with long-term-expanded Th17 cells were protected from melanoma rechallenge as well as lung metastasis (52). The properties that robust expansion *ex vivo* and persist long term *in vivo* of Th17 cells may partially owe to Tcf7, as it is an essential protein in the Wnt/β-catenin pathway that is critical for stem memory T cells self-renewal and the formation of memory daughter cells (37). Group found that the transcription factor Tcf7 was constantly present in nucleus of tumor-specific Th17 cells during expansion *ex vivo* (52).

Many researchers also generate Th17 cells with stemness phenotype by adding distinct cytokines or pharmacologic reagents *ex vivo* to enhance their antitumor efficacy. When cultured in the presence of IL-1β, Th17 cells express high levels of IFN-γ and exhibit enhanced antitumor effect in mice with melanoma. In addition, low doses of TGF-β could induce stemness property of IL-1β-cultured Th17 cells (68).

In more recent studies, Chrystal M. Paulo' laboratory demonstrated that using RORγ agonist LYC-55716 *ex vivo* or β-catenin and p110δ inhibitors augments the antitumor activity of murine tumor-specific Th17 cells (39, 69). And these cells produced elevated levels of IL-17A and IFN-γ and developed a distinct memory phenotype with elevated expression of CD44 and CD62L during response to established melanoma while untreated cells mainly presented effector

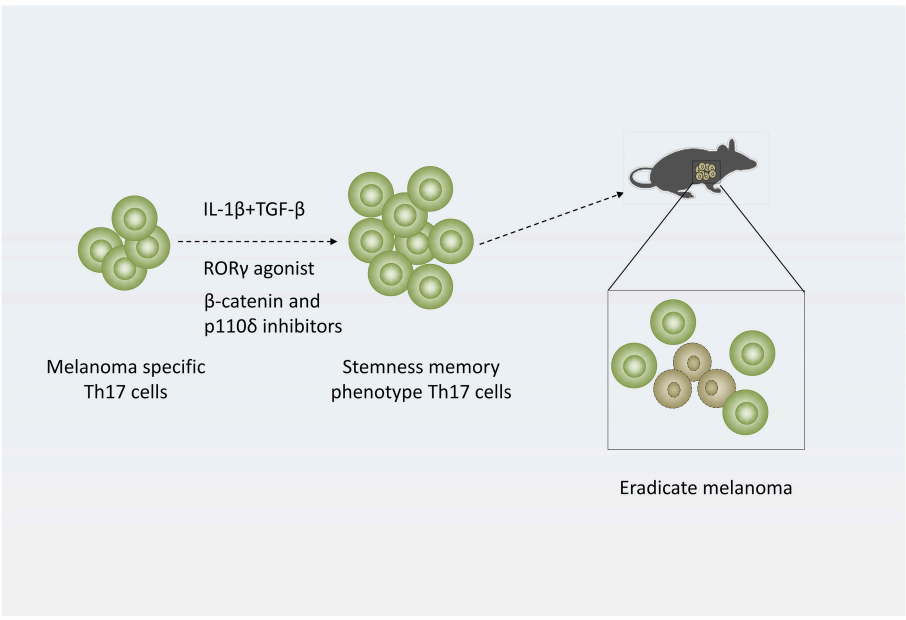

FIGURE 3 | Strategy of treating melanoma with Th17 cells. Adding distinct cytokines (IL-1β and TGF-β simultaneously) or pharmacologic reagents (RORγ agonist or β-catenin and p110δ inhibitors) to Th17-polarized culture medium to generate stemness or memory Th17 cells, which are more efficient to eradicate melanoma.

phenotypes in melanoma (69). Furthermore, mice previously infused with agonist-primed Th17 and Tc17 cells were protected from melanoma rechallenge (39). Their work suggests tumor specific Th17 cells treated with RORγ agonist or β-catenin and p110δ inhibitors *ex vivo* generate potent antitumor effects and persist as long-lived memory cells. This finding implicated that adoptively transferred RORγ agonist or β-catenin and p110δ inhibitors primed Th17 cells mediate long-lived memory response protecting against melanoma (**Figure 3**) (39, 69).

CONCLUSIONS

Based on current studies, Th17 cells in a melanoma microenvironment have both antitumor and protumor effects. Whereas, adoptive transfer of tumor-specific Th17 cells into melanoma-bearing mice has been successful in eradicating established melanoma in mice, there still remains potential issues concerning the fact that Th17 may also contribute to tumor growth. So further studies are required to fully explore the mechanistic effect of Th17 cells in melanoma and their therapeutic value in an adjuvant therapy approach in ACT clinical trials.

AUTHOR CONTRIBUTIONS

F-HG and CC drafted the outline of the manuscript and conducted the literature review. CC assessed the articles and wrote the manuscript. F-HG revised the manuscript. All authors have read and approved the final version of this manuscript.

FUNDING

This work was supported in part by the National Natural Science Foundation of China (81172322, 81302006, and 81572796), Shanghai Municipal Education Committee (13ZZ089), Science and Technology Committee of Shanghai (14401901500), Science and Technology Committee of Baoshan District (12-E-2).

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

frontiers in Oncology

REVIEW
published: 11 February 2019
doi: 10.3389/fonc.2019.00051

Overcoming Resistance to Natural Killer Cell Based Immunotherapies for Solid Tumors

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Despite advances in the diagnostic and therapeutic modalities, the prognosis of several solid tumor malignancies remains poor. Different factors associated with solid tumors including a varied genetic signature, complex molecular signaling pathways, defective cross talk between the tumor cells and immune cells, hypoxic and immunosuppressive effects of tumor microenvironment result in a treatment resistant and metastatic phenotype. Over the past several years, immunotherapy has emerged as an attractive therapeutic option against multiple malignancies. The unique ability of natural killer (NK) cells to target cancer cells without antigen specificity makes them an ideal candidate for use against solid tumors. However, the outcomes of adoptive NK cell infusions into patients with solid tumors have been disappointing. Extensive studies have been done to investigate different strategies to improve the NK cell function, trafficking and tumor targeting. Use of cytokines and cytokine analogs has been well described and utilized to enhance the proliferation, stimulation and persistence of NK cells. Other techniques like blocking the human leukocyte antigen-killer cell receptors (KIR) interactions with anti-KIR monoclonal antibodies, preventing CD16 receptor shedding, increasing the expression of activating NK cell receptors like NKG2D, and use of immunocytokines and immune checkpoint inhibitors can enhance NK cell mediated cytotoxicity. Using genetically modified NK cells with chimeric antigen receptors and bispecific and trispecific NK cell engagers, NK cells can be effectively redirected to the tumor cells improving their cytotoxic potential. In this review, we have described these strategies and highlighted the need to further optimize these strategies to improve the clinical outcome of NK cell based immunotherapy against solid tumors.

Keywords: natural killer cell, chimeric antigen receptor, immunotherapy, solid tumor, cytokines, tumor microenvironment, checkpoint inhibitors, bispecific antibody

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Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *15 November 2018*
Accepted: *18 January 2019*
Published: *11 February 2019*

Citation:
Nayyar G, Chu Y and Cairo MS (2019) Overcoming Resistance to Natural Killer Cell Based Immunotherapies for Solid Tumors. Front. Oncol. 9:51. doi: 10.3389/fonc.2019.00051

INTRODUCTION

Natural Killer (NK) cells are the effector cells that constitute a key part of the innate immune system. They have emerged as a promising option for immunotherapy of a variety of malignancies due to their ability to identify and kill cancer cells without any prior sensitization. NK cells have unique ability to differentiate between the normal and transformed cells. They possess a variety of

activating and inhibitory receptors, and their net functional outcome is a complex integration of signals between these activating and inhibitory receptors. Over the past few decades, significant advances have been made in successfully targeting hematologic malignancies with the use of novel immunotherapeutic strategies. However, solid tumors continue to pose unique therapeutic challenges, and the conventional cytoreductive therapies have proven to be of limited efficacy. NK cell based therapeutic strategies have been applied against solid tumor with only modest success. The ability of solid tumor cells to escape the immune-surveillance, proliferate rapidly and metastasize when coupled with the abnormalities in the NK cells like decreased expression of activating receptors or overexpression of inhibitory receptors, decreased activation and persistence, defective cytokine production, abnormal intracellular signaling molecules, inefficient trafficking to the tumor site, and senescence resulting in a defective cytolytic response are likely the major contributors to the poor response of NK cells based strategies against solid tumors. In this review, we have attempted to address the unique characteristics of solid tumors and their microenvironment, mechanisms contributing to the NK cell resistance and describe the various applications that could be applied in an attempt to enhance the therapeutic potential of NK cells against solid tumors.

CHALLENGES IN TREATING SOLID TUMORS

NK cell based immunotherapies have been used widely and successfully for different hematologic malignancies, particularly acute myeloid leukemia. One of the early studies in patients with relapsed acute myeloid leukemia, showed haploidentical NK cell infusion in combination with high dose fludarbine and cyclophosphamide caused expansion of donor NK cells, significantly increased endogenous Interleukin (IL)-15 and achieved a complete hematologic remission in 5 of 19 (26%) patients (1). More recently, NK cell based therapies have emerged as an attractive strategy for targeting solid tumors. However, there are some considerable challenges in use of NK cell based therapies against solid tumors. Solid tumors are a very heterogeneous group of malignancies that have historically been more difficult to treat even with the use of multimodal approaches. This heterogeneity could be due to differences in evolution of these tumors caused by varying gene profile signature, different mutations and involvement of different cell signaling pathways (2, 3). One of the major challenges with NK cell based therapies against solid tumors is the trafficking of these immune cells to the tumor location and infiltration into the tumor. Multiple studies have shown that the tumor progression and outcomes correlate with the presence of NK cells at the tumor site (4–6). The density of NK cells infiltrating into the tumor has been shown to be an independent predictor of the progression free survival in gastrointestinal stromal tumors, and in pulmonary adenocarcinoma (4, 7). The chemokines expressed on the surface of NK cells, and the ones secreted by the tumor cells play a central role in NK cell infiltration into the tumor (8).

It has been well established that tumor microenvironment plays a key role in the proliferation and survival of the cancerous cells. Tumor microenvironment consists of a variety of cells including tumor associated fibroblasts, tumor associated macrophages, dendritic cells, neutrophils, regulatory T cells (Tregs), myeloid derived suppressor cells (9), that provide a constant chronic inflammatory milieu leading to angiogenesis, tumor cell survival and proliferation. The presence of inhibitory signals in the tumor microenvironment and altered immunogenicity of tumor cells also leads to poor infiltration and activation of NK cells into the tumor. Furthermore, rapidly growing solid tumors create an environment of localized hypoxia (10). The low oxygen tension in the solid tumor tissue not only creates metabolic disturbances in the tumor microenvironment but also leads to generation of reactive oxygen species. This cellular environment of hypoxia is mediated by a variety of transcriptional regulators primarily, hypoxia inducible factor-1 (11). Poorly oxygenated tumor cells undergo adaptive changes at the proteomic level leading to transcriptional activity resulting in inhibition of apoptosis and promoting angiogenesis and upregulation of the tumor growth factors (12). Net result is the continued survival and proliferation of the tumor cells with an aggressive phenotype, that frequently metastasize to distant tissues, and are relatively resistance to treatment (11).

Recently, a lot of advances have been made in targeting hematologic malignancies using novel immunotherapeutic strategies like chimeric antigen receptors (CAR). However, the success stories have been less exciting against solid tumors, particularly due to lack of appropriate immunologic targets, that are highly expressed on surface of tumor tissue with relative absence on the non-vital tissues to avoid "on-target/off-tumor" effects (13). In addition, these genetically modified effector cells have to overcome the challenges posed by the physical barriers preventing infiltration into the tumor tissues and hostile tumor microenvironment (14, 15). Antigen escape phenomenon due to downregulation or loss of targetable antigen happens frequently in solid tumors rendering these CAR based therapies less effective (14, 16). However, unlike CAR T cells, NK cell based therapies have the advantage of overcoming the limitation posed by the antigen escape mechanism to a certain extent due

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; aNK, activated natural killer; CAR, chimeric antigen receptors; CEA, carcinoembryonic antigen; CB, cord blood; EGFR, epidermal growth factor receptor; EPCAM, epithelial cell adhesion molecule; FBP, folate binding protein; HER2, human epidermal growth factor receptor 2; HLA, human leucocyte antigen; HSCT, hematopoietic stem cell transplant; Hu, humanized; IgG, immunoglobulin g; IL, interleukin; IL1R, interleukin-1 receptor; INF-γ, interferon gamma; KIR, killer cell receptor; mAB, monoclonal antibody; MHC1, major histology antibody complex 1; MICA/B, MHC class I chain-related protein A/B; mRNA, messenger ribonucleic acid; NCAM, neural cell adhesion molecule; NK, natural killer cell; NKG2A, natural killer cell lectin-like receptor subfamily A; NKG2D, natural killer group protein 2 family member D; NKT, natural killer T cell; NSCLC, small lung cell carcinoma; PBMC, peripheral blood mononuclear cells; PD-1, programmed cell death 1; PDFGR, platelet-derived growth factor receptor; RCC, renal cell carcinoma; SABR, stereotactic ablative body radiotherapy; SCFv, single chain fragment variable region; SCT, stem cell transplantation; STAT, signal transducer and activator of transcription, TGF-β, transforming growth factor beta; TME, tumor microenviroment; TNF, tumor necrosis factor; Tregs, regulatory T cells; Trikes, trispecific killer cell engagers.

to their inherent ability to recognize and kill tumor cells without prior sensitization. Furthermore, NK cell alloreactivity following haploidentical SCT is protective against graft vs. host disease while producing a robust graft vs. tumor/leukemia effect (17, 18).

NATURAL KILLER CELL BIOLOGY AND TARGET RECOGNITION

Natural killer cells represent human body's first line of defense against tumor cells and infectious pathogens and play a key role in tumor immune surveillance. NK cells were initially identified in mice when investigators noticed a large granular subtype of lymphocytes distinct from T and B lymphocytes, and possessed cytotoxic activity against mouse tumor cell lines (19, 20). Phenotypically, NK cells lack B and T cells markers CD19/TCR/CD3 on their cell surface but they express CD16 and CD56 surface antigens. NK cells are further characterized by the degree of CD56 expression into dim and bright subsets where the subtypes have significant differences in terms of cytokine production, response to cytokines and their killing potential. Around 90% of the NK cells, including the alloreactive NK cells, express low levels of CD56 but have high expression of CD16 ($CD16^{bright}$ $CD56^{dim}$), and are generally found in the peripheral circulation. These cells are considered to be the "mature" NK cells and have higher cytotoxic potential. The remaining 10% of the NK cells are $CD16^{dim}CD56^{bright}$ with higher levels of CD56 expression, and they are considered "immature" NK cells (21, 22). These immature or unlicensed NK cells generally reside in the lymphoid tissues but they are more responsive to stimulation and respond readily by secreting a variety of cytokines including interferon γ (IFN-γ), tumor necrosis factor (TNF)-α, IL-5, IL-10, and IL-13 (4, 23). All subsets of NK cells express intermediate affinity heterodimeric IL-2 receptor. However, high affinity receptors and c-kit tyrosine kinase is only expressed by $CD56^{bright}$ NK cells, which gives them the unique ability to proliferate when exposed to very small concentrations of IL-2 (24, 25). In addition, there is also a differential expression of adhesion molecules between the two NK cell subsets. $CD56^{bright}$ cells have higher levels of expression of chemokine receptor type-7 and L-selectin which likely helps these cells to traffic to secondary lymphoid organs, whereas $CD56^{dim}$ cells have a higher level of expression of Leucocyte function-associated antigen-1, providing them the unique migratory properties in response to foreign pathogens (26). Therefore, $CD56^{dim}$ NK cells appear to have a predominantly cytotoxic function naturally, and $CD56^{bright}$ cells play a more immunomodulatory role. However, it is still unclear if these subsets represent just different stages in maturation of NK cells or if they are completely different cells emerging from a common hematopoietic precursor (3). Besides the circulation system, distinct subsets of NK cells also reside in tissues and organs (22). NK cells in lymph nodes, tonsils and spleen differ from NK cell subsets in peripheral blood by phenotypes and functions (27, 28).

NK cells do not require prior sensitization to target the transformed cells (4). NK cell receptor can play a stimulatory or an inhibitory role and has the unique ability to recognize major histocompatibility complex−1 (MHC-1) or MHC-1 like molecules on the target cells. The balance between the inhibitory signals received from the killer inhibitory receptors and natural killer group protein 2 family member A (NKG2A) and killer cell lectin-like receptor subfamily G member 1; and the stimulatory receptors including natural cytotoxicity receptors, NKp30, NKp44, NKp46, natural killer group protein 2 family member D (NKG2D) defines the net functional outcome of the NK cells.

NK cells recognize autologous cells that express human leucocyte antigen (HLA) Class I molecules that prevent them from attacking the host tissue, known as "tolerance to self." During viral infections or malignant transformation, there is decreased expression of MHC class I antigens on cell surface in order to avoid recognition by the antitumor T cells. NK cells that are surveilling the tissues for a normal level of MHC class I expression, recognize this as "altered self" resulting in decreased engagement of the killer inhibitory receptors and increase expression of the stimulatory receptors resulting in effector response and cytotoxic killing of the transformed cells (29). The mechanism of target recognition by NK cells is depicted in **Figure 1**. There are several mechanisms by which NK cells can kill the target cells without any prior sensitization. They can exert direct cytotoxicity through release of granules containing perforin and granzyme (31). NK cells also have the unique ability exert antibody-dependent cell mediated cytotoxicity (ADCC) due to presence of Fc receptor FcγRIIIa that recognizes the Fc portion of the antibodies. In addition, they can mediate cytotoxicity via apoptotic pathways involving fas ligand or TNF-related apoptosis-inducing ligand (32, 33).

NK RESISTANCE MECHANISMS

NK cells have shown significant alloreactive anti-leukemic effects against liquid tumor cells especially following haploidentical SCT (stem cell transplantation) (17) and the higher NK cell immune reconstitution in the early post allogeneic SCT period has also been demonstrated to be associated with significantly improved survival and lower leukemia relapse rates (34, 35). However, the adoptive transfer of autologous NK cells showed no clinical response in patients with progressive stage IV melanoma or renal cell carcinoma (RCC) (36). In solid tumor clinical trials, NK cells often display impaired functions in patients and impaired NK cell-function is related to high disease stages and poor prognosis (37, 38). The NK resistance, on NK side, is mainly due to the small numbers of active NK cells, the short lifespan of NK cells, poor persistence and trafficking, and lack of specific tumor targeting (39). On the tumor side, tumor cells make up a microenvironment that inhibits NK cell activity by altering the balance between NK activating and inhibitory receptors such as reducing NK activating receptor NKG2D and CD16, secreting inhibitory factors such as transforming growth factor beta (TGF-β), IL-6 and IL-10, shedding NKG2D ligands such as MHC class I chain-related protein A (MICA) and MHC class I chain-related protein B (MICB), and recruiting suppressive immune cells such as Tregs and myeloid derived suppressor cells (40).

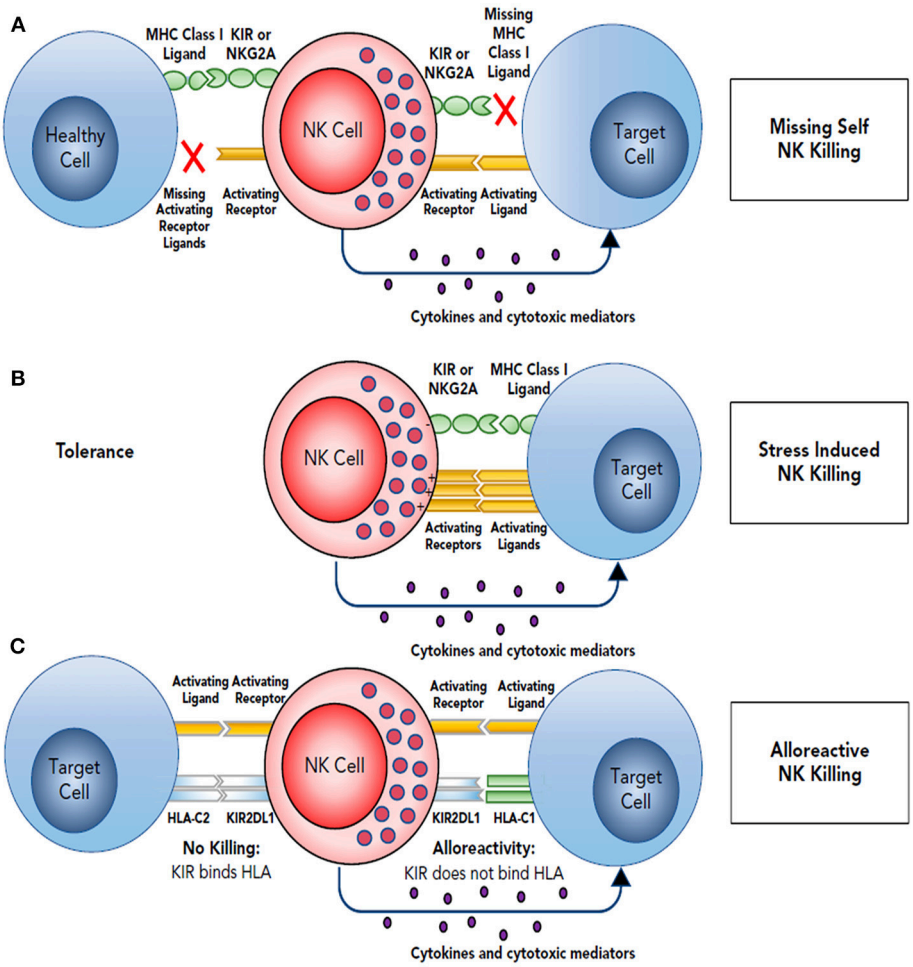

FIGURE 1 | Target recognition, tolerance, missing self. **(A)** NK cells recognize and kill their targets by an integrated balance of inhibitory and activating signals to discriminate between healthy cells (tolerance) vs. elimination of transformed or virally infected targets (killing). NK-cell tolerance depends on several MHC class I inhibitory signals (either classical, HLA-A, -B, or -C, or nonclassical, HLA-E) expressed by healthy cells that engage KIR or NKG2A with minimal activation signals resulting in tolerance. Malignant transformation or viral infection promotes target cell killing by downregulation of MHC class I expression and an upregulation of signals from activating NK-cell receptors. **(B)** Although in some cases, MHC downregulation is variable or incomplete, target cell killing can still occur by changing the balance with activating signals upregulated by stress-induced activating receptor ligands. **(C)** This balance between inhibition and activation can be uniquely manipulated in the hematopoietic transplant setting by selection of donors who will respond to apparent missing self HLA class I in the HLA-mismatched recipients. For example, reconstitution with a high frequency of donor KIR2DL1$^+$ NK cells would not be inhibited in a HLA-C1 (C1$^+$-HLA-C) recipient (KIR ligand mismatch). Here, NK-cell alloreactivity would kill the recipient's tumor. In contrast, when the same KIR2DL1$^+$ NK cells reconstitute in an HLA-C2 recipient (C2$^+$-HLA-C) (KIR ligand match), the recipient's tumor would be seen as having self HLA class I and would not provoke an alloreactive NK-cell response. Reproduced with permission from Cooley et al. (30).

Table 1 summarizes the potential mechanisms of resistance to NK cell based therapy of solid tumors.

NK RESOURCES FOR ADOPTIVE THERAPY

Four sources of active NK cells for adoptive transfer have been reported: autologous NK cells, allogeneic NK cells from donors, NK cell lines and embryo stem cell-derived/induced pluripotent stem cells -derived NK cells. Rosenberg et al. evaluated the efficacy of adoptively transferred IL-2 *ex vivo* activated autologous NK cells to patients with metastatic renal carcinoma and melanomas (36). Even the adoptively transferred NK cells persisted for long time, no significant clinical benefit was observed (36), indicating the limitation of utilizing patients' autologous NK cells alone as a therapeutic strategy. Due to the KIR mismatch to kill tumor cells, the adoptive transfer of allogeneic NK cells may have a superior antitumor effect compared with the approaches utilizing autologous NK cells (44). To overcome the limitation of small number of active NK cells in peripheral blood, our group and others have successfully expanded active NK cells *in vitro* by short term culture with cytokines alone, using cytokines and co-culture

TABLE 1 | NK cell resistance mechanisms against solid tumors.

Mechanism	References
Abnormal NK cell trafficking to the tumor location	(4, 41)
Poor NK cell infiltration into the tumor	(6–8)
Abnormal NK cell function/activity in patients with malignancy	(37)
Lack of unique targetable tumor antigens	(13, 42)
Antigen escape/downregulation following targeted therapies	(16)
Increased angiogenesis and upregulation of tumor growth factors by hypoxic microenvironment	(11, 12)
Hypoxia induced shedding of MHC class I chain-related (MIC) on tumor cells and downregulation of NKG2D on effector cells	(43)
Chronic immunosuppressive signals in the tumor microenvironment inhibiting NK cell function and activity	(4)

with irradiated Epstein-Barr virus-transformed lymphoblastoid cell lines as feeder cells, or cytokines and co-culture with K562 cells expressing transfected cell-membrane bound IL-15 and 4-1BBL (45–48). Lee and colleagues have developed a novel method of *ex-vivo* expansion of NK cells by stimulating peripheral blood mononuclear cells (PBMC) with a genetically-engineered feeder cell line, K562-mbIL21-41BBL, resulting in over 35,000-fold increase in NK cells and significant increase in NK cell functional activation (**Figure 2**) (49). Recently, Lee et al. used an anti-CD16 monoclonal antibody (mAb) for potent activation of resting NK cells and irradiated autologous PBMC (upregulated NKG2D ligand and CD48) for providing a suitable environment (activating receptor-ligand interactions and soluble growth factors) instead of cancer cell-based feeder cells for large-scale expansion of highly purified cytotoxic NK cells (50). These expanded NK cells showed potent cytotoxicity against various cancer cells *in vitro* and efficiently controlled cancer progression in severe combined immunodeficiency mouse models of human colon and lung cancer (50). Allogeneic expanded NK cells, which were expanded using CD3+ T-cell–depletion PBMCs from healthy donors with irradiated autologous PBMCs, mAb to CD3, and 500 IU/mL of IL2, were evaluated in a phase I study of adoptive transfer of these cells into patients with advanced, recurrent solid tumors besides malignant lymphoma (51). The results showed that the repetitive administration of *ex-vivo* expanded allogeneic NK cells was safe without any sign of graft vs. host disease or serious adverse event (51). Further studies are needed to enhance the persistence of these NK cells. Recently Jewett's group successfully expanded super-charged NK using PB-derived osteoclasts as feeder cells (52–54). These super-charged NK had superior cytotoxicity and IFN-γ secretion, survived for a longer period, and efficiently eliminated tumor growth in humanized xenografted mice (52–54). Considering more than 600,000 banked cord blood (CB) units worldwide (55), CB represents a unique opportunity as a readily available donor source with greater flexibility for the identification of HLA-compatible and KIR-mismatched lines. CB NK cells can be easily expanded with K562-mbIL21-41BBL feeder cells (18, 56) using CB mononuclear cells or they can be expanded to high log-scale with a cytokine cocktail from CD34+ CB progenitor cells (57, 58). NK cells derived from human CD34+ hematopoietic stem and progenitor cells showed efficient infiltration and killing of human ovarian cancer spheroids using an *in vivo*-like model system and reduced tumor progression in mice xenografted with ovarian carcinoma (59). NK cell lines also provide an unlimited source of effector cells and hold potential for development as standardized off-the-shelf therapeutics for adoptive cancer immunotherapy (60). Among different NK cell lines, NK-92 cells have been thoroughly investigated in preclinical studies and also been applied in clinical trials (61). The activated NK-92 based therapy from NanKwest was granted as the orphan drug designation against Merkel cell carcinoma. However, these NK-92 cell lines are aneuploid and must be irradiated before being administered to patients, which will limit the survival and proliferation of NK cells (62). In order to produce homogeneous and well-defined NK cells, a lot of effort has been put into generating NK cells derived from human embryonic stem cells or human induced pluripotent stem cells (iPSCs) (63–65). Most of these induced pluripotent stem cells-derived NK cells expressed no killer immunoglobulin (Ig)-like receptors (KIRs), which renders them unrestricted by recipients' HLA genotypes, and therefore they may serve as a universal "off-the-shelf" NK cell source for many recipients (64, 65).

STIMULATION OF NK CELLS EFFECTOR FUNCTION USING CYTOKINES AND CYTOKINE ANALOGS

One of the earliest and most common approaches in using stimulating and activating NK cells for cancer immunotherapy has been the use of cytokines. Multiple cytokines and other novel soluble factors have been described in the literature to enhance the number, function and persistence of NK cells *in vivo*. IL-2, IL-15, IL-12, IL-18, and IL-21 have all been described in regulating NK cell function, particularly their activation, maturation and survival. Two most commonly employed strategies have been either pretreatment of NK cells with cytokines before the adoptive transfer, or administration of cytokines *in vivo.*

IL-2

IL-2 is an immunostimulatory molecule first discovered in 1970s, and was initially described as a T cell growth factor (66). Further characterization showed the impaired NK cell function in IL-2 deficient mice, but NK cells were present in normal numbers in *IL-2* null mice suggesting that IL-2 is required for modulating NK cell function but is not essential for the development and maturation (67). To date, IL-2 has been the mostly commonly used cytokine in an attempt to boost NK cells *in vivo*. It was also the first every cytokine approved for clinical use (68). Earlier approaches using IL-2 in patient's involved using high dose IL-2 in conjunction with adoptive transfer of autologous NK cells. However, there was severe toxicity due to capillary leak syndrome related to IL-2 with no significant improvement in clinical outcomes (69). Subsequently, IL-2 was used to generate lymphokine activated killer cells, that were infused into the patients with melanoma or advanced RCC

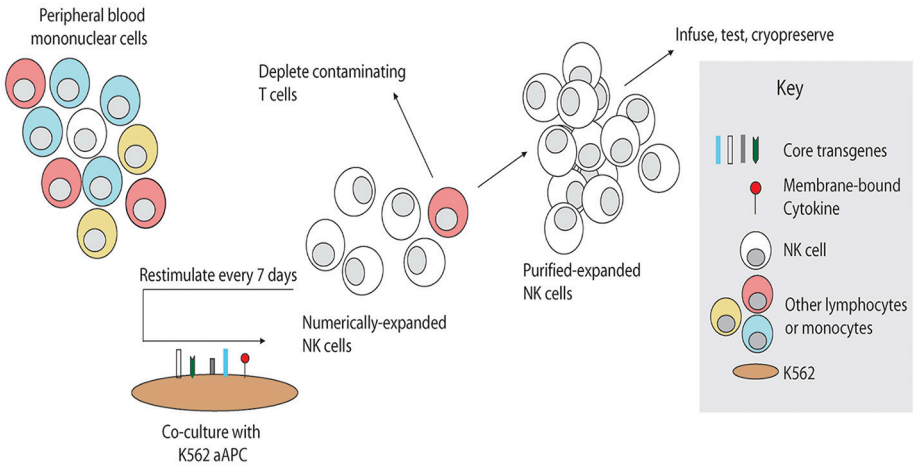

FIGURE 2 | Schema for NK cell manufacturing with artificial antigen-presenting cells. Artificial antigen-presenting cells were produced by genetic modification of K562 to express costimulatory molecules and membrane-bound cytokines. To expand NK cells *ex vivo*, unfractionated PBMC are stimulated weekly with irradiated PBMC, inducing rapid proliferation of NK cells and in some cases non-specific expansion of T cells. Contaminating T cells may be depleted, and the remaining purified NK cells may be stimulated weekly by the artificial antigen-presenting cells as needed to obtain sufficient numbers. Expanded NK cells may be used directly or cryopreserved for future use. Reproduced with permission from Denman et al. (49).

in combination with subcutaneous doses of IL-2 (70). This approach was not only well tolerated, and but clinical response showed a trend toward improved survival in melanoma patients who received a combination of lymphokine activated killer cells with IL-2 (5). In a phase I clinical trial involving patients with metastatic/unresectable digestive tract tumors, autologous NK cells were expanded *ex vivo* using IL-2, OK432, and modified recombinant human fibronectin fragment FN-CH296 induced T cells, and safely infused into the patients. However, no clinical responses were observed in these patients (71). Similarly, no improvement in clinical outcomes was observed in metastatic breast cancer patients who received IL-2 with autologous NK cell infusions following autologous SCT (72). Unfortunately, despite the compelling body of evidence suggesting that successful adoptive transfer and *in vivo* expansion of autologous NK cells when combined with IL-2 is safe and feasible, the clinical response against solid tumors has been minimal. It is likely that these impaired responses are related to the poor functional activation of the NK cells from cancer patients (73). Another potential explanation could be that IL-2 primed NK cells are sensitized to apoptosis upon coming in contact with the vascular endothelium likely causing a reduction in migration of these cells to the tumor site and infiltration into the tumor (74). Allogeneic or haploidentical NK cells infusions supplemented with IL-2 have been shown to produce significant clinical responses in hematological malignancies (75–77). However, such studies in field of solid tumors are lacking. In patients with advanced solid tumors, infusion of irradiated NK-92 cells that were *ex-vivo* expanded using IL-2 resulted in clinical responses in about three fourth of the patients with advanced lung cancer (78). Another phase II clinical trial using CD3 depleted, IL-2 stimulated haploidentical PBMC infusion in patients with recurrent ovarian and breast cancer showed a partial response in 20%, and stable disease in 60% of the patients (79). One of the major limitations of using IL-2 for modulating NK cell effector function, in addition to cytokine release with high dose IL-2 infusions, has been its ability to stimulate CD25 expressing Tregs (9). These Tregs have high affinity to IL-2 receptor, and diminish the effector response to NK cells by competing with NK cells for IL-2 and via TGFβ pathway (80). In a preclinical model, researchers have developed a mutant IL-2 molecule, "super-2," that has increased affinity to IL-2Rβ and has been shown to have superior NK cell activation and proliferation compared to wild type IL-2. Additionally, it caused selective proliferation of cytotoxic T cells but not Tregs (81). Similarly, novel fusion protein molecules that combine NK cell activating receptor ligand with IL-2 are being developed to selectively promote the *in vivo* expansion and activation of NK without affecting Tregs (82). It is critical for future trials using IL-2 to adopt strategies that can circumvent these inhibitory elements like Tregs and myeloid suppressor cells to improve clinical responses.

IL-15

Due to several potential drawbacks of IL-2 (as mentioned previously), IL-15 has emerged as an attractive alternative in cancer immunotherapy. IL-15 is a 15 kDa, gamma chain cytokine that possesses structural and functional similarities to IL-2, and is active in both cis- and trans- conformations (83). IL-15 receptor complex, which includes IL-15Rα/β/γ, wherein the β and γ chain receptor subunits are common to IL-2 and IL-15, and only difference exists at the α subunit. The relative high affinity between IL-15 and IL-15Rα, compared to IL-2 and IL-2Rα, results in NK cell activation at relatively lower doses. IL15Rα is expressed by a variety of immune cells like T cells, NK cells, natural killer T (NKT) cells, macrophages, and dendritic cells, and non-immune cells like skeletal muscle and endothelial cells (84, 85). *IL-15* deficient mice lack NK cells, NKT cells, memory CD8+ T highlighting that IL-15 is essential for development

of these immune effector cells (86). In comparison to IL-2, IL-15 has a more potent effect on NK cell expansion, and it does not upregulated the gene expressions of type 2 cytokines like IL-6, IL-10, and IL-13 (87). Similarly when compared to IL-2, soluble IL-15 does not appear to expand Tregs (88). IL-15 has been shown improve functional abilities of NK cells by inducing granzyme and perforin through mTOR pathway, resulting in enhanced cytotoxicity (89–91). The antitumor effects of IL-15 have been well established in different preclinical studies (89, 92), and in part are mediated through the activating NK cells receptor NKG2D (93). IL-15 has been shown to enhance ADCC in a murine model of colon cancer, when given in combination with anti CD40 antibody (94). Higher levels of IL-15 on Day 15 post-autologous hematopoietic stem cell transplant (HSCT) have been shown to directly correlate with improved overall survival (OS) in patients with relapsed non-Hodgkin lymphoma (NHL) (95). Early patient studies with IL-15 were done in post HSCT setting or in patients with relapsed/refractory hematologic malignancies. A phase I dose escalation study in patients with acute myeloid leukemia using recombinant IL-15 with adoptively transferred NK cells showed that it was safe and feasible to administer IL-15, and it resulted in persistence and proliferation of NK cells *in vivo* (96). Patients with metastatic melanoma and metastatic RCC receiving *E coli* derived recombinant human IL-15 for 12 consecutive days showed 10-fold expansions of NK cells and a significant efflux of NK cells and memory CD8 T cells from peripheral blood further established that IL-15 infusions are safe and feasible (97). Another phase I study established the safety and clinical efficacy of allogeneic NK cell infusions cultured with IL-15 and hydrocortisone in patients with advanced non-small cell lung cancer (98). In a different phase I/II trial, four out of six patients with refractory pediatric solid tumor who received IL-15-stimulated NK cell infusion at 30 days after haploidentical-HSCT showed a clinical response (99). Other clinical trials using IL-15 alone or in combination with other immunotherapeutic agents targeting solid tumors are currently ongoing (NCT01572493, NCT03388632).

IL-21

IL-21 is a type I cytokine synthesized by CD4+ T cells including NKT cells, T follicular helper and Th17 cells (100). It has been described to modulate both innate and adaptive immune responses, and is known to cause the lymphoid proliferation, particularly of CD8+ T cells and NK cells, and maturation of B cells (72). In addition to activating immune effector cells, IL-21 also plays a crucial role in mediating autoimmunity (101, 102). Binding of IL-21 to IL-21R primarily leads to activation of JAK1/JAK3 with subsequent phosphorylation of signal transducer and activator of transcription (STAT) (STAT3 and STAT1) signaling pathway resulting in upregulation of IFN-γ expression (103). However, IL-21 mediated activation can also occur via mitogen-activated protein kinase and phosphoinositide-3-kinase/serine/threonine kinase pathway. Combination of IL-21 and IL-15 has been shown to selectively promote the expansion of cytolytic CD56+CD16+ subtype of NK cells from human bone marrow (104). Using a K562 based antigen presenting cells genetically modified to express membrane bound IL-21 (mbIL-21), several thousand fold *ex vivo* expansion of NK cells can be achieved (49). Furthermore, these *ex vivo* expanded NK cells using mbIL-21 were found to have longer telomeres and higher expression of activating NK cell receptors. Multiple preclinical studies have established the powerful antitumor efficacy of IL-21 against solid tumors in mouse models. It has been shown to decrease tumor burden in mice bearing metastatic melanoma and RCC (105), melanoma and MethA fibrosarcoma (106), and head and neck squamous cell carcinoma (107). Several clinical trials have evaluated the safety, feasibility and antitumor effects of IL-21. Administration of recombinant IL-21 (rIL-21) has been shown to be safe with most common adverse event reporting grade1-2 toxicity, and severe toxicities requiring discontinuation being rare. A phase I study in patients with metastatic melanoma and RCC, rIL-21 at 30 μg/kg was well tolerated and shown to have antitumor activity, with about 70% patients showing some response or stable disease. One patient with melanoma achieved a complete remission (108), in a phase II study evaluating the efficacy and safety profile of IL-21 in patients, with metastatic melanoma, IL-21 was deemed safe and active against metastatic melanoma, with overall response rate being 22.5% and a favorable progression free and OS (109). Attempts to combine rIL-21 with targeted therapies have yielded mixed results. Combination of rIL-21 with sunitinib caused severe dose limiting toxicities with no clinical response resulting in early termination of the study (110). However, combining rIL-21 with sorafenib was shown to be relatively safe with mostly grade 1–2 toxicities, and was shown to have antitumor activity with objective response rate of 21% against metastatic RCC (111). Results are awaited from clinical trials evaluating the safety and efficacy of combining IL-21 with other immunotherapeutic agents (IL-21/Anti programmed cell death 1 [PD-1] against solid tumors/NCT01629758, IL-21/ipilimumab against melanoma, NCT01489059).

IL-12

IL-12 is a heterodimeric, pro-inflammatory, type I cytokine that has been shown to elicit T-helper type-1 immune responses against infectious agents and cancer cells. It is mainly secreted by antigen presenting cells (macrophages and dendritic cells) and has been shown to promote the differentiation of CD4+Th^0 cells into Th1 cells. It has been shown to increase cytokine production by NK cells and T cells, particularly IFN-γ (112). IL-12 does not appear to have any direct cytotoxic properties but exerts it's effects by stimulating NK and T cell proliferation and cytolytic properties (113), and by improving ADCC (114, 115). The antitumor efficacy of IL-12 has been well established in murine models in multiple preclinical studies (116–119). Despite the initial dose escalation phase I trial using recombinant human IL-12 (rhIL-12) establishing the safety of IL-12 administration in humans (120), subsequent phase II study had to be temporarily stopped due to severe toxicities, and deaths of the 2 patients (121). Subsequent studies have focused on establishing a safe dosing regimen for IL-12 administration to optimize the dose and frequency of IL-12 in patients. It was shown that a priming dose of IL-12 2 weeks prior can significantly decrease the toxicity of subsequent relatively high doses. Intratumoral injections of

rhIL-12 have been attempted in patients with head and neck squamous cell carcinoma with activation of B cell compartment, and presence of tumor infiltrating B cells, that correlated with OS (122). Other delivery methods that have been tried are electroporation of plasmid DNA coding for IL-12 in patients with melanoma (123), and PEGylated IL-12 plasmid formulations in patients with gynecologic malignancies (124). To date, clinical benefits of IL-12 administration have been modest. However, significant clinical responses with IL-12 have been reported in patients with cutaneous T cell lymphoma (125) and in patients with acquired immune deficiency syndrome associated Kaposi sarcoma (126).

IL-18

Similar to IL-12, IL-18 is another immunostimulatory cytokine belonging to IL-1 family that regulates both innate and adoptive immune responses. IL-18 is produced by monocytes, macrophages, neutrophils and dendritic cells, and is initially secreted in an inactive form pro-IL-18 which becomes biologically active upon cleavage by caspase-1 (127). IL-18 plays a key role in stimulating IFN-γ production from NK cells (128), and mice deficient in IL-18 have impaired cytotoxic responses, and decreases IFN-γ production (129). IL-18 has been shown to enhance TNF signaling in NK cells, prolonging the messenger ribonucleic acid (mRNA) expression of c-apoptosis inhibitor 2 and TNF receptor-associated factor 1 which inhibits NK cell death (130). *In vivo* antitumor efficacy of IL-18 has been well established in preclinical studies (131–133). However, there have only been few clinical studies evaluating its safety and efficacy in human subjects. Different phase I studies in patients with cancer have established the safety of rhIL-18 administration (134, 135). However, a subsequent study in patients with metastatic melanoma did not show any significant clinical responses as a monotherapy (136). Further studies evaluating its efficacy in combination with other cytokines and immunotherapeutic agents are required.

CYTOKINE ANALOGS

IL-15 Superagonist—ALT-803

Cytokine agonists have been well described in the literature, particularly for IL-15 (137). To further improve the biological activity and pharmacokinetics of a previously described IL-15 superagonist (IL-15N72D), investigators designed a novel molecule where IL-15N72D was fused with a dimeric IL-15 receptor a complex–(IL-15Rα/Fc). This redesigned IL-15 superagonist, ALT-803 has been shown to promote NK cell proliferation has been shown to possesses superior biological activity, higher potency and a much longer half-life (25 h vs. <40 min) compared to wild type IL-15 (138). Early preclinical studies showed that ALT-803 could upregulate the expression of NKG2D, promoted IFN-γ secretion and promoted the expansion of $CD8^{+}CD44^{high}$ memory T cells *in vivo* in a murine multiple myeloma model (139). Several other preclinical studies have established its efficacy in animal models against bladder cancer (140), B cell lymphomas (141), glioblastoma (142), breast, and colon cancer (143), and ovarian cancer (144). These antitumor effects have been attributed to increase in specific subpopulations of NK and memory CD8+ T cells, increased IFN-γ secretion and improvement in NK cell functionality. Early successes in preclinical studies have led to further investigation of ALT-803 in multiple clinical trials. A phase I trial in relapsed hematologic malignancies following SCT, ALT-803 induced clinical responses in 19% of the patients with one patient achieving complete remission. ALT-803 also induced proliferation and expansion of NK and CD8+ T cells in these patients (145). Another phase I trial in patients with advance solid tumors has established the safety and tolerability of ALT-803 administration (146). Combination of ALT-803 with nivolumab in patients with metastatic non-small cell lung cancer showed an objective response in 29% of the patients with 76% of the patients experiencing disease control. No dose limiting toxicities were seen in this trial (147). Several other clinical trials evaluating the antitumor effects of ALT-803 are currently ongoing (NCT03228667, NCT03127098, NCT03022825, NCT02384954, NCT02138734, NCT02890758, NCT02559674, NCT03520686).

NKTR-255

NKTR-255 is another novel IL-15 analog that is currently undergoing preclinical development. NKTR-255 consists of a polymer-engineered IL-15 molecule that has been designed to optimally engage IL-15 receptor complex. In preclinical studies, it has been shown to have superior binding affinity to IL-15Ra and lower *in vivo* clearance (22 h vs. 1 h) in comparison to IL-15. It was also shown to induce phosphorylation of STAT5, decrease tumor burden in metastatic lung cancer mouse model and enhance the activation and proliferation of NK cells (148). The early results are exciting, and highlight its role as a promising immunotherapeutic agent. However, further studies are required at this time.

OPTIMIZING NK CELL MEDIATED ADCC

One of the principle ways NK cells exert their antitumor effects is through ADCC, where Fc portion of the antitumor antibody binds to FcγRIIIA and/or FcγRIIC expressed on NK cells, leading to the NK cell activation, and initiation of a series of events like transduction of death signals via TNF family death receptor signaling, release of cytotoxic granules from NK cells, and production of inflammatory cytokines like IFN-γ causing target cell killing (149). There are wide differences in the expression of activating and inhibitory receptors profile of NK cells amongst individuals. It is also well documented that polymorphisms between FcγRIIIA and FcγRIIC can influence the Fc receptor function. These polymorphisms result in a differential activation upon binding with an antitumor antibody. Patients with higher affinity polymorphisms have been shown to have superior outcomes with mAb treatment (150, 151). In order to augment the polymorphonuclear cell mediated ADCC, investigators have attempted to design an anti-human epidermal growth factor receptor-2 with tandem IgG1/IgA2 Fc that retains IgG1 FcγR binding but also provides the benefits of FcαRI/IgA Fc interactions. Their results showed that the tandem

IgG1/IgA2 approach was superior in recruiting and engaging cytotoxic polymorphonuclear cells than either the parental IgG1 or IgA2 (152). Investigators have also attempted to improve the binding affinity of mAbs to maximize the ADCC. Obinutuzumab, a glycoengineered humanized anti-CD20 antibody has been shown to be superior to chimeric anti-CD20 mAb Rituximab in preclinical studies (153). By modifying the antibody backbone, it is possible to create chimeric antibodies (Ch14.18) with significantly longer half-life compared to the murine (mouse hybridoma 3F8), and avoid the human-mouse antibody response (154). An increasing number of humanized and fully human mAbs are currently being investigated in preclinical and clinical studies. Different combination strategies have been tried to improve antitumor ADCC of mAbs. NK cells have been shown to downregulate FcγRIIIA upon activation, and this downregulation is believed to be caused by activation of matrix metalloproteinases by the target cells (155). Preclinical studies have shown that ADAM17 inhibitor inhibits FcγRIIIA shedding and increased NK cell degranulation and IFNγ production (156). Strategies to increase the target antigen density on tumor cells for more efficient targeting by mAbs have been explored. Ionizing radiation (157) and Toll like receptor-9 agonists (158) have been shown to increase the expression of certain tumor target antigens. Currently there is limited preclinical data available about the clinical efficacy of these combinations and further studies are required.

PREVENTING CD16 SHEDDING AND EXPRESSING HIGH AFFINITY OF CD16

CD16, also known as the human IgG Fc receptor III (FCγRIII), consists of two isoforms (CD16A and CD16B) (159). CD16A is a transmembrane protein and the only FcγR expressed by NK cells (159). It binds to IgG of an antibody and is essential for ADCC, which is a key mechanism of NK cells to lyse tumor cells (149). CD16B is mainly expressed on neutrophil cells (159). Both CD16A and CD16B are cleaved rapidly on neutrophil and NK cell activation after mitogen stimulation and co-culturing with tumor targets and the cleavage is mediated by a metalloprotease, ADAM17 (a disintegrin and metallopeptidase domain 17) (160, 161). The plasma levels of CD16 were significantly reduced in patients treated with an ADAM17 inhibitor (160, 161). The recent preclinical study demonstrated that the ADAM17 inhibitor BMS566394 significantly enhanced the expression of CD16 on NK cells and more importantly, it enhanced the cytotoxic activity and IFN-γ production of treated NK cells combined with trastuzumab against breast cancer cell lines (162). MEDI3622 is a human mAb of ADAM17 with high specificity and a potent inhibitory activity (163). The combination of MEDI3622 with anti-human epidermal growth factor receptor 2 (HER2) antibody trastuzumab greatly augmented the production of IFNγ by NK cells against ovarian cancer cell by blocking the shedding of CD16A on NK cells (164). Engineering NK cells with a CD16 mutant which has mutation(s) in the cleavage domain can also disrupt cleavage and prevent CD16 shedding. Expression high affinity CD16 FcγRIIIa in NK cells is another attractive choice. The insertion of the high affinity CD16 FcγRIIIa (158V) allele and IL-2 into NK-92 cells render NK-mediated ADCC using cetuximab, trastuzumab and pertuzumab against a variety of solid tumor cells (165). Additional strategies include engineering NK cells with chimeric receptors CD16-BB-ζ and CD64-BB-ζ (166). These engineered NK cells significantly improved cytotoxicity against CD20-positive NHL cells in the presence of rituximab (166) but their anti-tumor effects need to be evaluated for solid tumor cells with targeted antibodies.

ROLE OF IMMUNOCYTOKINES IN IMPROVING NK CELL MEDIATED CYTOTOXICITY

As previously described in this review, a variety of cytokines have been utilized in an attempt to improve NK cell function and stimulation. Early clinical trials have demonstrated the improvement in outcomes in pediatric patients with neuroblastoma that received immunotherapy with anti-GD2 ch14.18 antibody in combination with IL-2 and granulocyte-macrophage colony-stimulating factor (167), whereas no clear benefit of antibody treatment without cytokine support was observed in a similar study performed by a German group suggesting a beneficial role for combining antibody therapy with cytokines (168). However, this approach has had mixed responses with limited clinical success against solid tumors. This is partly due to the challenges with systemic administration of these cytokines. Systemic cytokines have a narrow therapeutic window limiting their efficacy and they can cause severe toxicities by increasing the vascular permeability from a cytokine storm. These limitations have fueled the development of immunocytokines that are novel fusion proteins created by linking tumor specific mAbs to cytokines. The antibody component directs the cytokine molecule to the tumor location with selective activation of cytokine molecules at the site of antitumor activity. Studies have shown that treatment with immunocytokines leads to the targeted increase in the density of NK cells and lymphocytes in the tumor extracellular matrix (169, 170). Several immunocytokines molecules have shown promise in preclinical studies. Anti-GD2-IL2 fusion immunocytokine has been shown to have superior antitumor efficacy against neuroblastoma compared to both molecules administered separately at the same time. The mechanism was reported to be exclusively NK cell mediated (171). Similarly, anti-GD2-RLI (an IL-15 superagonist) fusion showed improved half-life of RLI and was effective against metastatic NXS2 neuroblastoma in a syngeneic mouse model (172). A fusion protein between tumor necrosis-targeting human IgG1 NHS76 and IL-12 (NHS-IL12) had longer half-life *in vivo*, stimulated lower IFN-γ release by immune cells thereby limiting the IL-12 mediated toxicity, and had superior antitumor efficacy in mouse models (173). Further modifications of IL-2 based immunocytokines have been attempted, e.g., single IL-2 variant (IL2v) moiety with loss of CD25 binding, to avoid Treg stimulation and improve the targeted biological activity (174). Several of these molecules have been tested in clinical trials. In

a phase II clinical trial of hu14.18-IL2, complete resolution of bone marrow disease and metaiodobenzylguanidine avid disease was seen in 5 out of 24 Stratum-2 patients with relapsed-refractory neuroblastoma (175). Phase I/II clinical trials have established the safety of intravenous administration of TNF-IL2 fusion protein (L19-TNF) in patients with advanced solid tumors (176), and it was shown to have clinical efficacy in patients with advanced localized melanoma in combination with melphalan and mild hyperthermia (177). More recently, phase I trial of NHL-IL12 established safety in patients with metastatic solid tumors. Evaluation of peripheral immune cell subset showed an increase in activated and mature NK and NKT cells in these patients (178). These agents have shown a great promise in stimulating immune cells like NK cells and cytotoxic T cells locally at the tumor site with cytokine component while maintaining the targeted effector antibody response. Multiple ongoing clinical trials are evaluating the safety and efficacy of several other immunocytokines alone, and in combination with other therapeutic modalities like immune checkpoint inhibitors (NCT03209869, NCT03386721, NCT02627274, NCT02350673). **Table 2** provides a comprehensive list of past and current clinical trials evaluating the safety and efficacy of immunocytokines against solid tumors.

ANIT-KIR ANTIBODIES FOR IMPROVNG NK CELL CYTOTOXICITY

As mentioned previously, NK cells remain tolerant to cells expressing HLA class I ligands but trigger cytotoxicity against altered cells that have a decreased level of HLA expression. This distinction between self and altered cells is mediated through inhibitory KIRs on NK cell surface. KIRs can recognize HLA molecules triggering inhibitory signals and resulting in decreased ADCC by NK cells. NK cells herald the immune recovery of lymphocyte subsets following allogeneic HSCT, and have been implicated in early graft vs. malignancy effects (35). This concept has been exploited clinically in allogeneic HSCTs for hematologic malignancies where donor KIR is mismatched with recipient's tumor creating a KIR-ligand incompatibility in order to create graft vs. leukemia effect (179). Similar to the KIR-ligand mismatch concept, investigators have designed mAbs that block the HLA-KIR interactions to prevent the NK cell inhibition and trigger cytotoxicity. Phase I clinical trial with IPH2101, the first in class anti-KIR antibody that inhibits KIR2DL-1, L-2, and L-3, in patients with relapsed/refractory multiple myeloma established the safety at dose that achieve full inhibitory KIR saturation (180). In another phase I study for relapsed multiple myeloma, a combination of IPH2101 with lenalidomide resulted in objective responses in five out of 15 patients, with median progression free survival being 24 months (181). In preclinical studies, the second generation fully human IgG4 anti-KIR2DL1, -L2, -L3, -S1, -S2 antibody (IPH2102/Lirilumab) was shown to potentiate the spontaneous cytotoxicity of NK cells against lymphoma cells lines. It was also shown to augment the NK cells mediated ADCC with Rituximab against CD20 lymphoma cells, *in vitro* and *in vivo* (182). Very few studies have looked at the efficacy of these anti-KIR antibodies against solid tumors. IPH2102 was well tolerated in patients with solid tumors and hematologic malignancies, with patients experiencing only mild and transient side effects (183). A recently published study established a correlation between the expression of inhibitory KIR and PD-1 on tumor cells in patients with non-small cell lung cancer suggesting a potential benefit of combining anti-KIR antibodies with anti-PD-1 treatment to circumvent the immune escape in these patients (184). Several active studies are currently evaluating these anti-KIR antibodies against solid tumors in combination with other immune therapies (NCT03341936, NCT03203876, NCT03347123).

RE-DIRECTING NK CELLS WITH CHIMERIC ANTIGEN RECEPTOR (CAR)

The adoptive transfer of T cells engineered to express an artificial CAR to target a specific antigen on tumor cell surface is an exciting approach for cancer immunotherapy. CARs usually include a single-chain variable fragment from a mAb, a transmembrane hinge region, and a signaling co-stimulatory domain such as CD28, CD3-zeta, 4-1BB (CD137), or 2B4 (CD244) endodimers (185–187). The co-stimulatory components attribute greater strength of signaling, and longer *in vivo* T-cell persistence (39). Four generations of CAR have been developed and evaluated pre-clinically and clinically (39) (**Figure 3**). The advantage of the CAR strategy is that no HLA expression on the target cell is required for the epitope to be accessible to CAR^+ immune cells. Thus, CAR^+ immune cell application is not limited to only a subset of patients with a specific HLA type (185–187). To increase the targeting specificity of expanded NK cells, our group has investigated functional activities of peripheral blood natural killer cells modified by mRNA nucleofection with anti-CD20 CAR against CD20+ B-NHL *in vitro* and in xenografted NSG mice (188). Lentiviral transduced methods had been used to generate CAR expressing NK cell lines targeting solid tumor cells. The CARs have been developed and engineered in NK cells lines against several antigens for solid tumors which include epidermal growth factor receptor (EGFR), HER2, EGFRvIII, GD2, epithelial cell adhesion molecule (EpCAM) (**Table 3**) with efficiency in preclinical studies. Schönfeld et al. generated a stable NK92 cell line expressing a humanized anti-HER2 CAR containing CD28 and CD3ζ signaling domains and these CAR NK cells efficiently lysed HER2+ tumor cells *in vitro* and the specific recognition of tumor cells resulted in selective enrichment of anti-HER2 CAR NK-92 cells in orthotopic breast carcinoma xenografts and reduction of pulmonary metastasis in a RCC model, respectively (189). In another study, the repeated stereotactic injection of anti-HER2 CAR NK-92 cells improved the symptom-free survival in glioblastoma xenografted mice (190). NK-92 cells and primary NK cells were engineered to express the second generation of EGFR-CAR to target breast cancer cells (191). *In vitro*, compared with mock-transduced

TABLE 2 | Clinical development of immunocytokines/fusion proteins against solid tumors.

Immunocytokine	Malignancy	Combination	Phase	Registry
Hu14.18-IL2	Neuroblastoma/GD2+ Tumors	Monotherapy	I	NCT00003750
Hu14.18-IL2	Relapsed/refractory neuroblastoma	*Ex vivo* expanded NK Cells	I	NCT03209869
Hu14.18-IL2	Recurrent neuroblastoma	Sargramostim and Isotretinoin	II	NCT01334515
Hu14.18-IL2	Melanoma	Monotherapy	II	NCT00109863
Hu14.18-IL2	Residual/refractory neuroblastoma	Monotherapy	II	NCT00082758
L19-TNFα	Stage III/IV limb melanoma	Melphalan	I	NCT01213732
L19-TNFα	Advanced solid tumors	Doxorubicin	I	NCT02076620
L19-TNFα	Solid tumors, colorectal cancer	Monotherapy	I/II	NCT01253837
L19-TNFα	Unresectable or metastatic soft tissue sarcoma	Doxorubicin	I	NCT03420014
F16-IL2	Solid tumors	Doxorubicin	I/II	NCT01131364
F16-IL2	Solid tumor, breast cancer, melanoma, NSCLC	Paclitaxel	I/II	NCT01134250
L19-IL2	Advanced solid tumors, RCC	Monotherapy	I/II	NCT01058538
L19-IL2	Oligometastatic solid tumor	SABR	I	NCT02086721
L19-IL2	Metastatic melanoma	Dacarbazine	I/II	NCT02076646
L19-IL2	Advanced/metastatic pancreatic cancer	Gemcitabine	I	NCT01198522
NHS-IL2LT	Metastatic or locally advanced solid tumors	Cyclophosphamide	I	NCT01032681
NHS-IL2LT	NSCLC	Local Tumor Irradiation	I	NCT00879866
NHS-IL12	Epithelial/malignant mesenchymal tumors	Monotherapy	I	NCT01417546
NHS-IL12	Advanced solid tumors	Avelumab	I	NCT02994953
CEA-IL2v (RO6895882)	Advanced/metastatic solid tumors	Monotherapy	I	NCT02004106
CEA-IL2v (RO6895882)	Locally advanced/metastatic solid tumors	Atezolizumab	I	NCT02350673
FAP-IL2v (RO6874281)	Solid tumors, breast cancer, head and neck cancer	Trastuzumab Cetuximab	I	NCT02627274
FAP-IL2v (RO6874281)	Advanced and metastatic solid tumors.	Atezolizumab Gemcitabine Vinorelbine	II	NCT03386721
huKS-IL-2 (EMD273066)	Refractory epithelial carcinoma	Monotherapy	I	NCT00016237
huKS-IL-2 (EMD273066)	Ovarian, prostate, colorectal, NSCLC	Cyclophosphamide	I	NCT00132522
BC1-IL12 (AS1409)	Metastatic malignant melanoma, RCC	Monotherapy	I	NCT00625768
LAG3-Ig (IMP321)	NSCLC head and neck cancer	Pembrolizumab	II	NCT03625323
VEGF-Ig (HB002.1T)	Solid tumors	Monotherapy	I	NCT03636750
PAP-GM-CSF (APC8015)	Prostate cancer	Bevacizumab	II	NCT00027599

NK-92 cells or primary NK cells, EGFR-CAR-engineered NK-92 cells and primary NK cells displayed enhanced cytotoxicity and IFN-γ production when co-cultured with breast cancer cell lines (191). In the mice intracranially pre-inoculated with EGFR-expressing breast cancer cells, intratumoral administration of EGFR-CAR-transduced NK-92 cells mitigated tumor growth compared to mock NK cells (191). A human NK cell line KHYG-1 expressing anti- EGFRvIII CAR was established and exhibited the inhibition of glioblastoma cell-growth via apoptosis in an EGFRvIII-expression specific manner (192). Another group engineered NK-92 to stably express an anti-GD2 CAR and these CAR NK-92 cells facilitated tumor effective recognition and elimination of $GD2^+$ NB cell lines and primary NB cells (193). Anti-EpCAM CAR engineered NK-92 displayed high and selective cell-killing activity against EpCAM-expressing breast carcinoma cells that were resistant to the natural cytotoxicity of unmodified NK cells (194). Additionally, our group is developing anti-ROR1 CAR engineered expanded primary NK cells through CAR mRNA electroporation technology to target $ROR1^+$ solid tumors with promising *in vitro* anti-tumor effects (195). Anti-mesothelin CAR-NK cells were derived from CAR-expressing iPSCs with the optimized CAR construct

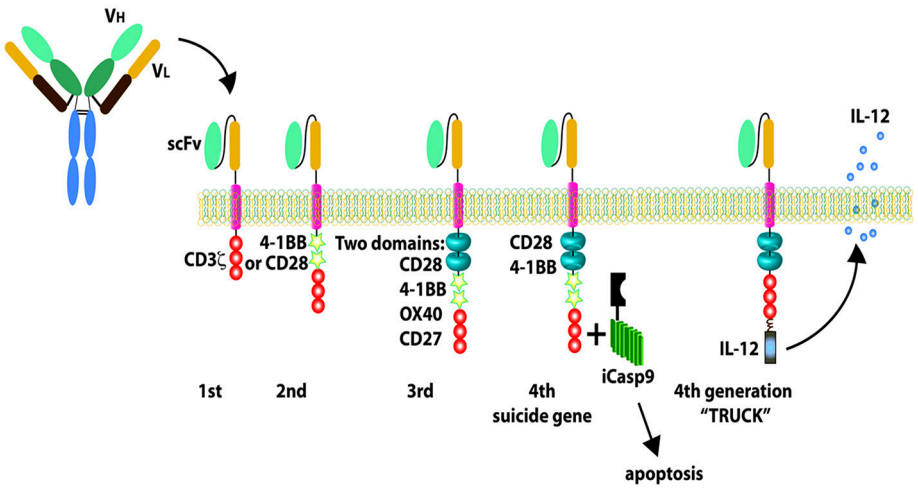

FIGURE 3 | Chimeric antigen receptors (CARs). The first-generation CARs only have CD3 zeta signaling domain; the second-generation CARs include one CD28 or 4-1BB co-stimulatory components combined with CD3 zeta signaling domain; the third-generation CARs include two co-stimulatory domains; the fourth-generation CARs are designed with new elements including a controllable suicide gene like caspase 9 or loaded with IL-12 secretion. Reproduced with permission from: Barth et al. (39).

(197). These CAR-NK cells showed great potent ability to kill mesothelin-expressing tumors both *in vitro* and *in vivo*, demonstrating a potential strategy to produce "off the shelf," targeted allogeneic cell products for refractory malignancies (197). Besides designing a CAR based on the single chain variable fragment (scFv) of a mAb again an antigen on tumor cell surface, CAR can also be formed from a NK activating receptor such as NKG2D followed by transmembrane domain and signal transduction domains. Chang et al. designed a CAR termed NKG2D-DAP10-CD3ζ that was composed of the NK cell activating molecule NKG2D plus 2 key signaling molecules, DAP10 and CD3ζ (196). These NKG2D CAR engineered primary NK cells through retroviral transduction showed significantly enhanced *in vitro* cytotoxicity against a variety of solid tumor cell lines that express NKG2D ligands MICA/B such as the osteosarcoma cell lines U-2 OS, MG-36, HOS, the prostate carcinoma cell lines DU 145 and PC-3, and the rhabdomyosarcoma cell line RH36 (196) and significantly reduced tumor burden in osteosarcoma xenografted NSG mice compared to mock NK cells (196). Similar strategy can be applied to generate other NK activating receptor based CAR like NKp30-CAR to enhance NK cytotoxicity. The advantage of this CAR strategy is that one CAR can be applied for a variety of tumor types in a matter expressing the corresponding ligands. Considering the recent safety concerns such as cytokine release syndrome and neurotoxicity associated with infusion of CAR-modified T cells (187), a suicide gene should be incorporated into the construct as a safety measure for CAR NK therapy but it is debatable because of the short life span of NK cells compared to T cells. Additionally, IL-15 secretion CAR-NK can be generated retroviral transduction by incorporating IL-15 to CAR design (18) to enhance the CAR NK proliferation, persistence and homing in solid tumors.

There are currently 3 registered clinical trials testing the safety and efficacy of CAR-NK cells in patients with solid tumors. One trial is a single-center, single arm, open-label pilot study to evaluate the safety and feasibility of CAR-NK cell treatment in subjects with metastatic solid tumors using autologous or allogeneic NK cells transfected by mRNA electroporation against NKG2D-ligand expressing cancer cells (NCT03415100). Another trial is to evaluate the efficacy and safety of CAR-modified NK Cell lines in MUC1 positive advanced refractory or relapsed solid tumors (NCT02839954). These two trials are being conducted in China. The third trial is being conducted in the USA sponsored by Johann Wolfgang Goethe University Hospital to evaluate the safety and tolerability of NK-92/5.28.z (HER2.taNK) for patients with recurrent HER2-positive glioblastoma (NCT03383978). Pharmacokinetics and pharmacodynamics and potential signs of anti-tumor activity of NK-92/5.28.z cells will also be analyzed.

BISPECIFIC ANTIBODIES TO ENHANCE NK CELL KILLING POTENTIAL

mAbs have revolutionized the development of anticancer therapeutics over past last few decades. However, the efficacy of mAbs has been limited against solid tumors. Advances in protein engineering has made the generation of bispecific molecules possible. Bispecific antibodies are novel molecules where two antigens can be targeted at the same time by combining the specificities of two antibodies. The design of a bispecific antibody constitutes an antitumor scFv targeting a specific malignancy is linked to an anti-CD3/anti-CD16 in order to create an immune connection between cancer cell and the immune effector cells like T cell or NK cell. Recently, there has been a growing interest in development of bispecific

TABLE 3 | Summary of CAR NK in preclinical studies for solid tumors.

Antigen	NK resource	CAR signaling domains	Methods	Diseases	Report year	References
ErbB2/HER2	NK92	CD28 and CD3ζ	Lentiviral transduction	Breast carcinoma	2015	(189)
ErbB2/HER2	NK92	CD28 and CD3ζ	Lentiviral transduction	Glioblastoma	2015	(190)
EGFR	NK92 and primary NK	CD28 and CD3ζ	Lentiviral transduction	Breast cancer brain metastases	2016	(191)
EGFRvIII	KHYG-1	N/A	Lentiviral transduction	Glioblastoma	2018	(192)
GD2	NK92	CD3ζ	Retroviral transduction	Neuroblastoma	2012	(193)
EpCAM	NK92	CD28 and CD3ζ	Lentiviral transduction	Breast carcinoma	2012	(194)
ROR1	*Ex vivo* expanded human NK	4-1BB and CD3ζ	CAR mRNA electroporation	Neuroblastoma, sarcomas	2017	(195)
NKG2D	*Ex vivo* expanded human NK	DAP10 and CD3ζ	Retroviral transduction	Osteosarcoma, prostate carcinoma, rhabdomyosarcoma	2013	(196)
Mesothelin	iPSCs-NK	NKG2D-2B4-CD3ζ, NKG2D-2B4-DAP10-CD3ζ, NKG2D-4-1BB-2B4-CD3ζ	Piggybac transposon integration	Mesothelin+ solid tumors such as ovarian cancer	2018	(197)

antibodies with currently multiple studies evaluating their anti-cancer potential in preclinical and clinical studies. To date, the most success with bispecific antibodies has been seen with T cell specific bispecific molecules like catumaxomab (CD3/EpCAM) against malignant ascites, and blinatumomab (CD3/CD19) and ionotuzumab (CD3/CD22) against B cell lymphoblastic leukemia (198–200). These successes have encouraged the development of diverse bispecific antibodies with varied clinical applications besides cancer, like emicizumab/ACE910 for patients with Hemophilia A. The goal of developing these bispecific engagers is to enhance the therapeutic efficacy, improve targeted delivery to the tumor site, optimize immune cell engagement, and reduce off target effects and relative ease of administering one drug instead of two separate molecules. One major shortcoming of the T cell specific antibodies has been their potential to cause massive cytokine release causing capillary leak, hypotension and respiratory distress in a clinical setting. These shortcomings have made NK cell based bispecific NK cell engagers an attractive alternative. Bispecific NK cell based antibodies can engage the Fc portion of the antibodies through their FcγRIII (CD 16) receptor with the other portion designed to bind a specific epitope on the tumor surface. Several NK based bispecific antibodies are currently in preclinical and clinical development. Early investigations have focused on AFM13, an anti-CD30/CD16A for relapsed or refractory Hodgkin lymphoma. In Phase I trial, AFM13 was shown to be safe, caused activation of NK cells, decreased soluble CD30 in peripheral blood, was found to be active in patients resistant to brentuximab, and achieved disease control in 77% patients at doses ≥1.5 mg/kg (201). Phase II studies with AFM13 are currently ongoing. Multiple preclinical studies are evaluating different bispecific and trispecific NK cell engaging antibodies against solid tumors. A trivalent bispecific antibody targeting ErbB2 and CD16 was shown to be more potent than anti-ErbB2 single-chain variable fragment (scFv)-Fc fusion protein *in vitro* against breast cancer cell lines, and *in vivo* against breast cancer xenograft mouse model (202). Multiple other antibodies targeting the HER2- FcγRIII antigens have been described (203–205). Similarly, a completely humanized bispecific antibody targeting EpCAM and CD16 showed significant increase in ADCC, increased degranulation of NK cells with concomitant increase in IFN-g production against EpCAM positive prostate, breast, colon, and head and neck cancer cell lines (206). Modifications have been made to the dimeric structure of these bispecific antibodies to further improve the efficacy. A tribody targeting human epidermal growth factor 2 where two HER2-specific scFvs were linked to CD16 [$(HER2)_2$xCD16] was found to be superior to trazutumab against HER2-expressing breast, pancreatic, ovarian, and esophageal tumor cells with increased NK cell degranulation and release of granzyme B (207). Insertion of a modified interleukin-15 cross-linker to an EpCAM/CD16 bispecific construct to create a trispecific construct improved NK cell proliferation and survival and showed increased ADCC (208). **Table 4** highlights multiple CD16 targeting bispecific and trispecific antibodies that have undergone preclinical development. The results of these preclinical studies are encouraging and warrant further clinical development of these molecules.

TABLE 4 | Preclinical development of CD16 antigen based bispecific antibodies targeting solid tumors.

Bispecific antibody	Target malignancy	References
Anti-MOV19-AntiCD16	Ovarian cancer	Ferrini et al. (209)
Anti-ovarian carcinoma(IgG2a)-AntiCD16	Ovarian cancer	Ferrini et al. (210)
Anti-NCAM -AntiCD16	Human glioma	Obukhov et al. (211)
Anti-CA19-9- AntiCD16	Colorectal adenocarcinoma	Garcia de Palazzo et al. (212)
Anti-c-erbB-2-Anti CD16	Ovarian cancer	Weiner et al. (213)
Anti-FBP-Anti CD16	Ovarian cancer	Ferrini et al. (214)
Anti-c-erbB-2-Anti FcgammaRIII	c-ERB-2 positive tumors	Weiner et al. (215)
Anti-c-erbB-2-Anti FcgammaRIII	Ovarian adenocarcinoma	Weiner et al. (216)
Anti-HER2/neu- AntiFcgammaRIII	Ovarian adenocarcinoma	McCall et al. (217)
Anti-PDGFR- AntiFcgammaRIII	Lung cancer	Gruel et al. (218)
Trivalent anti-erbB2-anti-CD16	Breast cancer	Xie et al. (219)
Anti-HER2/neu-Anti CD16 minibody	Ovarian cancer	Shahied et al. (220)
Anti-ErbB2-Anti-CD16	Breast cancer	Lu et al. (202)
Anti-EGFR-Anti-CD16	Cholangiocarcinoma	Asano et al. (221)
Anti-EpCAM-Anti-CD16	Prostate, breast, colon, head and neck cancer	Vallera et al. (206)
Anti-HER2-Anti- FcγRIIIA	Breast cancer	Turini et al. (205)
Anti-CD133-Anti-CD16	Colorectal cancer	Schmohl et al. (222)
Anti-CEA and Anti-CD16	Ovarian and colorectal cancer	Dong et al. (223)
Anti-EpCAM-IL-15 crosslinker-Anti-CD16 (TriKE)	Colorectal cancer	Schmohl et al. (208)
Anti-EpCAM-AntiCD133-IL-15 crosslinker-Anti-CD16 (TetraKE)	Colorectal cancer	Schmohl et al. (224)
AntiCD133-IL-15 crosslinker-Anti-CD16 (TriKE)	Colorectal cancer	Schmohl et al. (225)
Anti-Muc1-Anti-CD16	Colorectal, ovarian and lung cancer	Li et al. (226)
Anti-$(HER2)_2$-Anti-CD16 Tribody	Pancreatic, ovarian, esophageal cancer	Oberg et al. (207)
Anti-GPC3-Anti-CD16	Liver carcinoma	Wang et al. (227)

TARGETING NK CELL CHECKPOINTS PD-1, TIGIT, AND IL-1R8

Immune checkpoints are negative regulators of immune cells, especially T cells, to help keep immune responses in check, and maintain self-tolerance during immune responses (228). Malignant cells often express high level of ligands of checkpoint inhibitory receptors, and escape from immune recognition and elimination (228). In recent years, the application of mAbs directed against immune checkpoint receptors or ligands has greatly enhanced the anti-tumor activity of the immune cells, and has resulted in remarkable clinical benefits (229, 230). Similar to T cells, NK cells also express an array of immune checkpoints which include PD-1, cytotoxic T-lymphocyte-associated protein 4, T cell immunoglobulin- and mucin-domain-containing molecule 3, T cell immunoreceptor with Ig and immunoreceptor tyrosine-based inhibition motif (ITIM) domains (TIGIT), CD96, lymphocyte activation gene-3, and IL-1R8 besides the well-known NK inhibitor receptors: KIRs and CD94/NKG2A (231–233). The data of the cytotoxic T-lymphocyte-associated protein 4, lymphocyte activation gene 3 and mucin-domain-containing molecule 3 on NK cells functions are either scarce or controversial. But several lines of evidences strongly demonstrate the inhibitory roles of PD-1, TIGIT, and IL-1R8 on NK cells. PD-1^+ NK cells are confined to CD56dimNKG2A–KIR+CD57+ mature NK population and are functionally exhausted, exhibiting reduced proliferative capability, poor cytolytic activity and impaired cytokine production as compared with the PD-1^- NK cells (234, 235). A recent study demonstrated that the increased PD-1 expression on peripheral and tumor infiltrating NK cells from patients with digestive cancers indicates poorer prognosis (236). And blocking PD-1/PD-L1 signaling markedly enhances cytokines production and degranulation and suppresses apoptosis of NK cells *in vitro* (236). More importantly, a PD-1 blocking antibody was found to significantly suppress the growth of xenografts in nude mice, and this inhibition of tumor growth was completely abrogated by NK depletion, strongly suggesting that PD-1 is an inhibitory regulator of NK cells in digestive cancers (236). PD-1 blockade might be an efficient strategy in NK cell-based tumor immunotherapy. A phase II clinical trial is on-going to assess the effect of pembrozilumab (a humanized anti-PD-1 mAb) on NK cell function and exhaustion in melanoma (NCT03241927). TIGIT competes with the NK activating receptor DNAX Accessory Molecule-1 (CD226) for their common ligands CD112 (PVRL2) and CD155 (PVR) to directly dampen NK cell cytotoxicity (237). *In vitro* TIGIT blockade improves the anti-tumor effect of Trastuzumab (a recombinant humanized anti-HER2 mAb), which partially relies on NK cell-mediated ADCC (238). Recent evidence showed the upregulation of TIGIT on tumor-infiltrating NK cells in mouse models of subcutaneously administered solid tumors and the TIGIT expression on tumor-infiltrating NK cells was associated

with tumor progression and was linked to functional exhaustion of NK cells (233). The blockade of TIGIT via mAbs reversed the exhaustion of anti-tumor NK cells in multiple tumor models, enhanced the infiltration of activated (CD69+) NK cells into tumors and thereafter improved the OS of the host (233). The presence of NK cells was critical for the therapeutic effects of blockade of TIGIT or the PD-1 ligand PD-L1 or combined blockade of both checkpoints (233). These findings demonstrate that the NK cell–associated TIGIT signaling pathway has a role in tumors' evasion of the immune system and that reversing NK cell exhaustion is critical for the therapeutic effects of anti-tumor immunotherapy based on the blockade of TIGIT (239). IL-1R8, also known as toll-interleukin 1 receptor or Single Ig IL-1-related receptor, is a member of interleukin-1 receptor family (IL1Rs) and acts as a negative regulator of IL1Rs and toll-like receptors (TLRs) to suppress ILR- and TLR-mediated cell activation (240). IL-1R8 is widely expressed in several epithelial tissues, in particular by epithelial cells of the kidney, digestive tract, liver, lung, lymphoid organs, and it is also expressed on monocytes, B and T lymphocytes, dendritic cells, and NK cells (240). Recently, Molgora et al. identified IL1R8 as a checkpoint protein in NK cells that regulates antitumor activity of NK cells in solid tumors (232). Utilizing IL-1R8-deficient (Il1r8–/–) mice as a study model, Molgora et al. found that IL1R8- deficient NK cells expressed significantly higher levels of the activating receptors NKG2D, DNAX Accessory Molecule-1 and Ly49H and fas ligand and produced increased levels of IFNγ and granzyme B (232). IL-1R8 partial silencing in human peripheral blood NK cells with small interfering RNA was associated with a significant increase in IFNγ production and upregulation of CD69 expression (232). In a model of sarcoma (MN/MCA1) spontaneous lung metastasis, *Il1r8*–/– mice showed a reduced number of hematogenous metastases, whereas primary tumor growth was unaffected and the protection was completely abolished in NK-cell-depleted *Il1r8*–/– mice (232). Additionally, adoptive transfer of *Il1r8*–/– NK cells significantly and markedly reduced the number and volume of lung and liver metastases in in the mice with MC38 colon carcinoma liver metastasis while *Il1r8*+/+ NK cells had no effect (232). These results suggest IL-1R8 serves as a negative regulator of NK cells and its inactivation unleashes human NK-cell effector function (232).

FOCUSING ON NKG2D AND THE LIGANDS

NKG2D is a C-type, lectin-like, type II transmembrane glycoprotein-activating receptor expressed in humans on NK, natural killer T, activated CD8+ T cells and some CD4+ and γδ+Tcell subsets (241). In humans, NKG2D forms a hexameric structure with the adaptor molecule DNAX-activating protein of 10 kDa (DAP10) to mediate signal transduction and cellular activation upon ligand recognition (242). NKG2D ligands are structural homologs of MHC class I molecules and are upregulated on the surface of many cell types by cellular stress and viral/bacterial infections and frequently during tumorigenesis (243, 244). The currently identified human NKG2D ligands include MICA and MICB and UL16 binding protein (ULBP1–ULBP6) families (245). Several lines of evidence conclusively demonstrated that engagement of NKG2D and NKG2D ligands, such as MIC A/B elicits cytolytic responses overcoming inhibitory signals on NK cells and is sufficient to trigger cytolysis by NK cells expressing NKG2D (246–249) (**Figure 4**). The expression of these ligands on the tumor cell surface are regulated at multiple levels: transcriptional regulation, ribonucleic acid (RNA) splicing, posttranscriptional regulation, posttranslational regulation (245). NKG2D ligands can be cleaved from the tumor cell surface after translation by membrane matrix metalloprotease and be released as soluble ligands (245). The findings from "humanized" transgenic animal studies demonstrated the opposite roles of membrane-bound and soluble forms of NKG2D ligands (250, 251). The membrane-bound ligands binding to NKG2D play an important role in NK cell activation and tumor immune surveillance (247, 252, 253), while the soluble NKG2D ligands suppress tumor immunity by passively blocking NKG2D and inducing receptor internalization to down-regulate NKG2D on the surface of NK cells (254–256). Serum levels of soluble NKG2D ligands significantly correlate with patients prognosis and are used as prognostic markers in some tumor patients (257, 258). Therefore, therapeutic strategies have focused on enhancing NKG2D expression and signaling on NK cells such as expression of NKG2D CAR and applying IL-15 agonist as we discussed in the earlier section; enhancing the level of membrane-bound

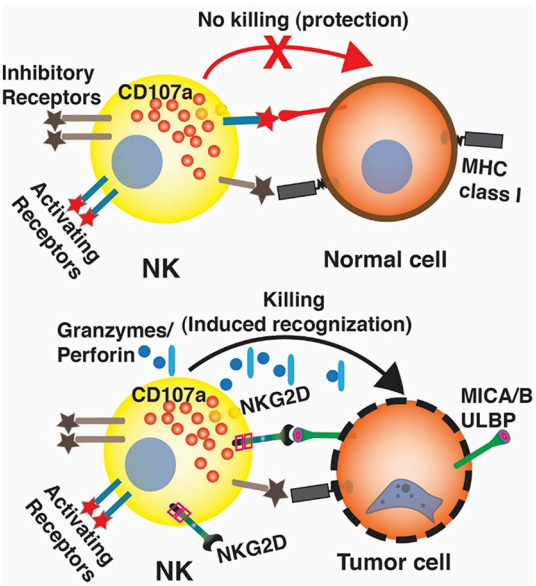

FIGURE 4 | The interaction between NKG2D on NK cells and NKG2D ligands on tumor cells. In normal cells, NKG2D ligands express is very low. The functions of NK cells are balanced by the signals from the inhibitory and activating receptors. In humans, when normal cells are transformed into cancer cells. NKG2D ligands such as MICA/B and ULBP proteins, are often overexpressed. The engagement of NKG2D and NKG2D ligands overcomes inhibitory signals on NK cells, activates NK cells to release cytotoxic molecules such as perforin and granzyme, and trigger apoptosis of tumor cells.

NKG2D ligand on tumor cells; and eliminating soluble NKG2D ligands (**Table 5**). We and others had utilized histone deacetylase inhibitors such as romidespin, entinostat, sodium valproate to enhance NKG2D ligands expression on tumor cell surface to enhance NK based immunotherapy (259, 260, 263, 264). Zhu et al. found that entinostat not only increased the expression of MICA/B on osteosarcoma cells but also simultaneously increased the expression of NKG2D on primary human NK cells to augment the activation pathways for NK cell recognition of cancer cells (259). Their results indicate that entinostat has the potential to enhance concurrent NK-cell therapy for solid tumors such as colon carcinoma and osteosarcoma (259). Proteases, such as ADAM-10, ADAM-17, and the membrane type matrix metalloproteinase 14, have been found to mediate MIC shedding through proteolytic activities (265–267). In an *in vitro* drug screen using a Federal Drug Administration-approved drug library, lomofungin, an antifungal drug, was found to strongly decrease ADAM17 activity in hepatocellular carcinoma cells and resulted in enhanced membrane bound MICA expression and inhibited soluble MICA production (261). Another ADAM17 inhibitor, INCB7839, was used to present HER2 cleavage and to treat patients with HER2-positive breast cancer in combination with trastuzumab and it is also in clinical trials to prevent CD20 cleavage in combination with rituximab for the treatment of diffuse large B-cell NHL (268). It would be interesting to investigate if these inhibitors prevent NKG2D ligands shedding and enhance NK-cell therapy for solid tumors. Applying neutralizing antibodies of soluble NKG2D ligands is another promising strategy to overcome immune suppressive effect of these cleaved ligands. Soluble MIC-specific mAb B10G5 was shown highly effective against primary prostate carcinoma and metastasis in the double transgenic TRAMP/MIC mouse model (262). B10G5 antibody therapy effectively induced regression of primary tumors and eliminated metastasis associated with enriched NK cell infiltration in the prostate tumor parenchyma (262). B10G5 therapy also remarkably restored NK cell pool in the periphery and the ability of NK cell homeostatic to self-renew as evidenced by bromodeoxyuridine uptake and markedly enhanced NK cell function, illustrated by increased production of IFNγ in response to mitogen stimulation and cytolytic ability against NKG2D ligand-positive target cells (262). These data conclude that targeting serum soluble MIC significantly restores NK cell homeostatic maintenance and function in MIC^+ cancer host (262).

ENHANCING NK HOMING AND TUMOR INFILTRATION

Several studies have shown that NK cell homing and infiltration within tumors was associated with improved tumor regression and prognosis (7, 269). The inability of NK cells to migrate to the tumor site limits the clinical outcome of adoptive NK cell infusion in patients with solid tumors (270, 271). Strategies that increase NK homing and infiltration into tumors would be plausible to enhance NK antitumor efficacy and prevent resistance and relapse. The ability of NK cells to home and infiltrate into tumors largely depends on the chemokine receptors they express as well as the chemokines secreted by the tumor cells (272). Wennerberg et al. found that *ex vivo* expansion NK had significantly enhanced CXCR3 expression which resulted in increased migratory capacity toward CXCL10-producing RCC and melanoma tumor cells (273). Following adoptive transfer of these *ex vivo* expanded human NK cells, mice bearing $CXCL10^+$ melanoma tumors had increased intratumoral infiltration of NK cells and a significantly prolonged survival compared with mice bearing $CXCL10^-$ tumors (273). These data demonstrated the importance of CXCL10 in directing the migration and infiltration of CXCR3 human NK cells toward solid tumors (273). Prime the tumor microenvironment to secrete CXCL10 might be a good strategy to attract CXCR3 expression NK and to enhance the efficacy of NK cell-based therapy against solid tumors. Other efforts were made to genetically engineer NK cells with chemokine receptors to improve their migration toward the corresponding ligands on

TABLE 5 | Strategies to enhance NKG2D signaling for solid tumors.

Agent	Function	Diseases	Report year	References
NKG2D CAR NK	Enhance NKG2D expression on NK cells	Osteosarcoma, prostate carcinoma, rhabdomyosarcoma	2013	(196)
HDAC inhibitor Entinostat	Enhance NKG2D expression on NK cells	Colon carcinoma and osteosarcoma	2015	(259)
HDAC inhibitor Entinostat	Enhance NKG2D ligands MICA/B expression on tumor cells	Colon carcinoma and osteosarcoma	2015	(259)
HDAC inhibitor Sodium Valproate	Enhance NKG2D ligands MICA/B expression on tumor cells	Hepatoma cells	2005	(260)
Lomofungin	decrease ADAM17 activity and enhance MICA on tumor cells	Hepatocellular carcinoma	2018	(261)
B10G5	Soluble MIC neutralization monoclonal antibody	Primary prostate carcinoma and metastasis tumors	2015	(262)

tumor cells surface. Various solid tumors, including RCC, secrete ligands for the chemokine receptor CXCR2 to promote angiogenesis, tumor growth and metastasis (274). Kremer et al. genetically engineered expanded human NK cells to express CXCR2 to improve their ability to specifically migrate along a tumor-derived chemokine gradient (271). CXCR2 expressing NK cells obtained increased adhesion properties and resulted in increased killing of target cells (271). Therefore, genetic engineering of *ex vivo* expanded NK cells to express chemokine receptor such as CXCR2 represents a novel strategy to improve anti-tumor effects following adoptive transfer of NK cells. A recent study connected the role of autophagy with CCL5-dependent NK cells infiltration in melanoma. Autophagy is a lysosomal degradation pathway for cells to self-digest their own components such as damaged organelles and misfolded proteins and such a degradation process provides nutrients to maintain cellular functions and allows survival of cancer cells under stress conditions (275, 276). Autophagy involves a Beclin-1 (BECN1)/class III phosphoinositide-3-kinase (PI3K) complex to initiate the formation of phagophore (275, 276). The previous studies from Baginska et al. demonstrated that targeting the autophagy gene *BECN1* prevented the degradation of NK-derived granzyme B, and therefore restored their susceptibility to NK cell-mediated killing and significantly inhibited tumor growth in syngeneic melanoma and breast mouse models (277). A recent study from Mgrditchian et al. found that when the autophagy process was blocked in tumor cells by inhibiting the expression of *BECN1*, the tumor cells produced an increased amount of CCL5 to attract functional NK cells to infiltrate into the melanoma tumor (276). Consequently, this led to a significant reduction in melanoma tumor size (276). These studies highlight the importance of integrating autophagy inhibitors as an innovative strategy in enhancing NK infiltration and killing.

TARGETING THE TUMOR MICROENVIRONMENT AND BLOCKING TRANSFORMING GROWTH FACTOR BETA (TGF-β) PATHWAY

It is well documented that the tumor microenvironment (TME) supports tumor growth, metastasis and suppress immune system (278). A major obstacle of ensuring high cytotoxic activity of NK cells is that these cells are surrounded by immunosuppressive cells and molecules in TME and must overcome the immunosuppressive properties from TME. One of immunosuppressive molecules is TGF-β1 (279). The increased TGF-β level was found in the plasma of advanced cancer patients such as breast cancer, ovarian cancer and neuroblastoma and correlated with worse event-free survival (280–282). Among three isoforms of TGF-β, TGF-β1 is the most abundant and widely studied isoform with 390 amino acids (283). This ligand binds to TGFβ receptor type I which results in its dimerization to TGFβ receptor type II and then phosphorylates SMAD2 and SMAD3 which complex with SMAD4 to modulate transcription of downstream genes (283, 284). TGF-β transmits biological signals to cells also through SMAD independent, alternative signaling pathways such as mitogen activated protein kinases, phosphoinositide 3′ kinase, and TNF receptor-associated factor6-TGF-β-activated kinase 1-p38/c-Jun N-terminal kinase (TRAF6-TAK1-p38/JNK) (283). TGF-β is produced by tumor cells themselves, Tregs, myeloid derived suppressor cells and other stromal cells in TME to downregulates the host immune response via driving the Th1/Th2 balance toward the Th2 immune phenotype, directly inhibiting anti-tumoral Th1-type responses and M1-type macrophages and promoting M2-type macrophages, suppressing cytotoxic CD8+ T-lymphocytes, NK, and dendritic cells functions, and stimulating CD4 + CD25+ T-regulatory cells (Treg) (285). TGF-β inhibits NK-cell proliferation and function in part by Treg cells which are known to produce high levels of TGF-β (286). One of the mechanisms by which TGF-β impairs NK cell function is by down-regulating the expression of NK activating receptors: NKp30 and NKG2D (287, 288). On the tumor side, TGF-β inhibits the transcription of the NKG2D ligands on tumor cells such as down regulation of MICA in the glioma cells, which reduced the recognition and killing by NKG2D expressing NK (289). TGF-β also represses development of NK cells from CD34+ progenitors and resulted in conversion of a minor fraction of $CD56^{bright}CD16^{+}$ cells found in peripheral blood into $CD56^{bright}CD16^{-}$ cells (290). TGF-β inhibits CD16-mediated human NK cell IFN-γ production and ADCC through SMAD3 (291). Further studies demonstrated that blockade of TGF-β signaling in NK cells caused the accumulation of NK cells that produce IFN-γ (292) and neutralization of TGF-β prevented NKG2D downregulation and also restored NK cell anti-tumor reactivity (293). RNA interference of TGF-beta1 and TGF-beta2 prevented the down-regulation of NKG2D on NK cells mediated by glioma cells and strongly enhanced MICA expression in the glioma cells and promotes their recognition and lysis by NK cells (289). These evidences support an immunosuppressive effect of TGF-β on NK cells and also provide a compelling rationale for blunting the inhibitory effect of it on NK cells as an anti-cancer therapy. Some approaches aiming at decreasing circulating TGF-β, blocking ligand-receptor interactions or inhibiting TGF-β signaling pathways to enhance NK based therapies are currently under investigation pre-clinically and clinically including TGF-β neutralizing antibody, TGF-β receptor I kinase inhibitors, SMAD3-Silenced NK Cells, NK cells engineered with a dominant negative receptor II for TGF-β, NK cells engineered to express a chimeric receptor with TGF-β type II receptor extracellular and transmembrane domains and the intracellular domain of NK cell-activating receptor NKG2D (**Table 6**).

Fresolimumab (GC1008) is a high-affinity fully human mAb that neutralizes the active form of all the three isoforms of TGF-β (294). It was designed as an IgG4 isotype to minimize immune effector function. Fresolimumab has been assessed as a potential treatment for RCC and metastatic melanoma. The safety and antitumor activity of repeated doses of fresolimumab administered to patients with advanced malignant melanoma and RCC was evaluated in a Phase I study (NCT00356460) (294). Even the study was not designed

TABLE 6 | Summary of agents utilized to block TGF-β for solid tumors.

Agent	Function	Diseases	Study stage	Clinical trial NCT #	Report year
Fresolimumab (GC1008)	Human monoclonal antibody, neutrolize TGF-β	Advanced malignant melanoma and renal cell carcinoma	Clinical phase I	00356460	2014
Galunisertib (LY2157299)	Inhibitor of TGFβR1	Advanced solid tumors	Clinical phase I	01722825	2015
Galunisertib + Dinutuximab + aNK	Enhance ADCC of expanded NK cells	Neuroblastoma	Preclinical	N/A	2017
Galunisertib + aNK	Enhance cytotoxic function of expanded NK	Glioblastoma	Preclinical	N/A	2018
TGFBR2 knocking-down primary and expanded NK cells	TGF-β signaling pathway deficient	Brain tumor	Preclinical	N/A	2018
SMAD3 knocking-down NK-92 cells	TGF-β signaling pathway deficient	Hepatoma and melanoma	Preclinical	N/A	2018
DNRII CB NK	CB NK cells to express a dominant negative receptor II for TGF-β	Glioblastoma	Preclinical	N/A	2017
NK-92-TN (contains the TGF-β type II receptor extracellular, transmembrane domains and the intracellular domain of NK cell-activating receptor NKG2D (TN chimeric receptor)	Resistant to TGF-β-induced suppressive signaling, but did not downregulate NKG2D	hepatocellular carcinoma	Preclinical	N/A	2017

to evaluate the effect of fresolimumab on NK cells but it showed acceptable safety and displayed encouraging antitumor activity (294). The results warrant further studies of it with NK therapy.

Galunisertib (LY2157299 monohydrate) is a small-molecule inhibitor of TGFβR1 that binds antagonistically to TGFβR1 to prevent the intracellular phosphorylation of SMAD2 and SMAD3 (295). Phase I studies have demonstrated that galunisertib had an acceptable tolerability and safety profile in patients with advanced solid tumors (296). Recently the preclinical studies from Tran et al. demonstrated that galunisertib combined with anti-GD2 antibody Dinutuximab augmented the anti-tumor cytotoxicity of activated NK (aNK) cells which were activated *ex vivo* with K562.mbIL21 artificial antigen presenting cells (297). Galunisertib suppressed SMAD2 phosphorylation and restored the expression of DNAX Accessory Molecule-1, NKp30, NKG2D and TNF-related apoptosis-inducing ligand death ligand expression on aNK cells and also significantly enhanced the release of perforin and granzyme A from aNK cells and the direct cytotoxicity and ADCC of aNK cells against neuroblastoma cells *in vitro* (297). The combination of galunisertib, aNK cells plus dinutuximab reduced tumor growth and increased survival of mice xenografted with two neuroblastoma cell lines or a patient-derived xenograft (297). In another study, galunisertib was shown to preserve the cytotoxic function of *ex vivo* expanded, highly activated NK cells and significantly improved eradication of liver metastases of colon cancer in mice treated with adoptive NK cells compared with mice receiving NK cells or TGF beta inhibition alone (298). Overall these studies demonstrate that the therapeutic efficacy of adoptive NK cell therapy clinically will be markedly enhanced by complementary approaches targeting TGF-beta signaling *in vivo*.

Ex vivo manipulating NK cells by novel strategies such as knocking-down TGF-β receptor 2 (TGFBR2) and SMAD3, expressing a dominant negative receptor II for TGF-β, or engineering with a TGF-β type II receptor based chimeric receptor to block the TGF-β signaling pathway are very attractive for adoptive NK therapy for solid tumors. Kararoudi et al. knocked down *TGFBR2* in human primary and expanded NK cells using the novel DNA-free Cas9 ribonucleoprotein complexes (299). *TGFBR2*-knockdown NK cells showed less sensitive to TGFβ (299). SMAD3 is a downstream factor in TGF-β signaling pathway and plays an essential role in TGF-β-mediated immune suppression, and in regulating transcriptional responses that are favorable to metastasis (300). *SMAD3* knocked-down NK-92 cells showed enhanced cancer killing activities and enhanced IFN-γ production *in vitro* and better anticancer effects than NK-92 empty vector control in non-obese diabetic severe combined immunodeficiency mice bearing human hepatoma (HepG2) or melanoma (A375) *in-vivo* (301). Yvon et al. engineered CB NK cells to express a dominant negative receptor II for TGF-β (DNRII) (302). These CB-derived DNRII-transduced NK cells were expanded to clinically relevant numbers, retained their secretion of interferon-γ, maintained both perforin and NKG2D/DNMA1 expression, and more importantly, retained their killing ability in the presence of TGF-β for glioblastoma cells (302). NK-92 cells were engineered to express a chimeric receptor which contains the TGF-β type II receptor extracellular, transmembrane domains, and the intracellular domain of NK cell-activating receptor NKG2D (TN

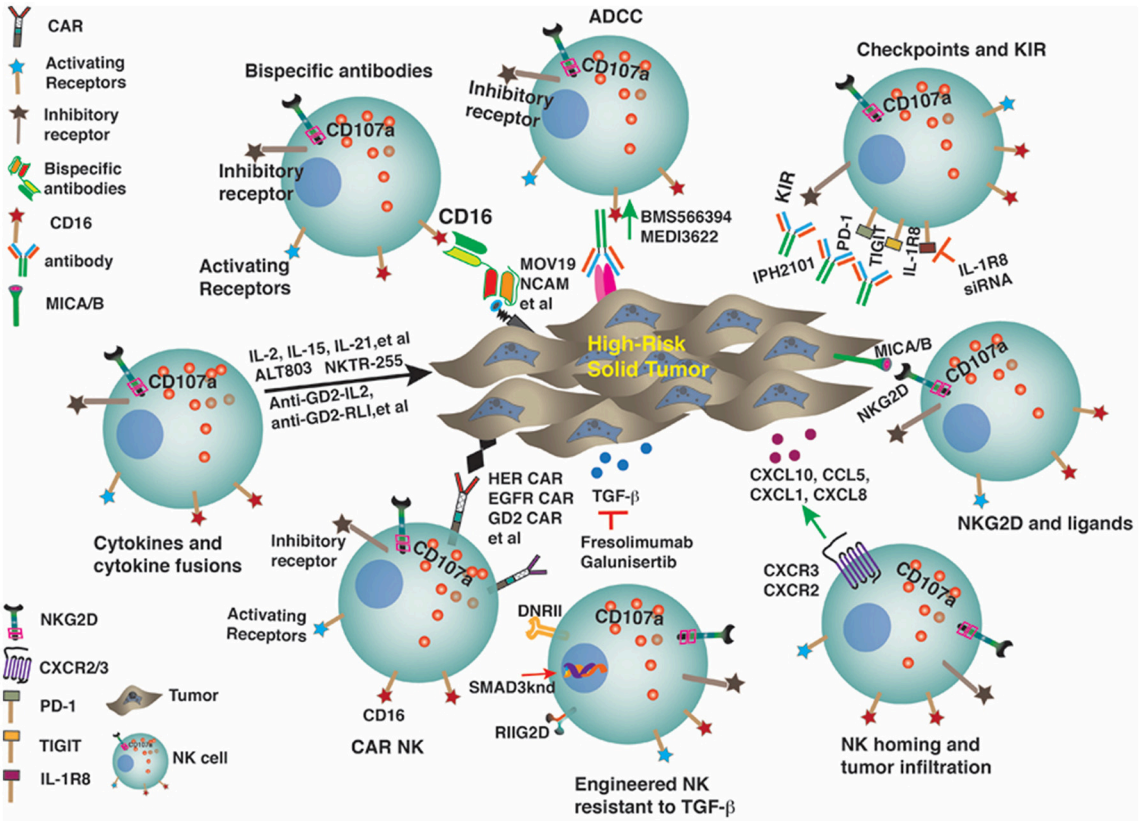

FIGURE 5 | Strategies to overcome NK resistance in solid tumors. To enhance targeting specificity, NK cells have been engineered to express CAR such as anti-HER2 CAR, anti-EGFR CAR, anti-GD2 CAR et al. to target a specific antigen on tumor cell surface. NK cells can be activated by cytokines and cytokine fusion proteins such as IL-2, IL-15, IL-12, IL-18, IL-21, ALT-803 (an IL-15 superagonist), NKTR-255 (a polymer-engineered IL-15 molecule), anti-GD2-IL2, and anti-GD2-RLI fusions et al. Bispecific antibodies are novel molecules where two antigens can be targeted at the same time by combining the specificities of two antibodies. Bispecific antibodies can enhance NK cells targeting and killing. Preventing CD16 shedding and expressing high affinity CD16 on NK cells combined with novel engineered humanized antibodies will enhance NK mediated ADCC. The inhibitory roles of checkpoint proteins PD-1, TIGIT, IL-1R8, and KIR on NK cells are well documented. Blocking PD-1, TIGIT, and KIR with specific antibodies or knocking down IL-1R8 in NK cells unleash human NK-cell effector function. The membrane-bound ligands such as MICA/B binding to NKG2D play an important role in NK cell activation and tumor immune surveillance. Therapeutic strategies have focused on enhancing NKG2D expression and signaling on NK cells and enhancing the level of membrane-bound NKG2D ligand on tumor cells; and eliminating soluble NKG2D ligands. To enhance NK homing and tumor infiltration, NK cells can be enhanced to express chemokine receptors such as CXCR3, CXCR2 to be attracted to tumor cells that secret CXCL10, CXCL1, CXCL8, or CCL5. TGF-β plays an immunosuppressive effect of on NK cells. The approaches to block TGF-β and inhibit TGF-β pathway including TGF-β neutralizing antibody, TGF-β receptor I kinase inhibitors, SMAD3-Silenced (Smad3knd) NK Cells, NK cells engineered with a dominant negative receptor II for TGF-β (DNRII), NK cells engineered to express a chimeric receptor with TGF-β type II receptor extracellular and transmembrane domains and the intracellular domain of NK cell-activating receptor NKG2D (RIIG2D).

chimeric receptor) by Wang et al. (303). These NK-92-TN cells were resistant to TGF-β-induced suppressive signaling, did not downregulate NKG2D (303). These modified NK-92 cells had higher killing capacity and IFN-γ production against carcinoma tumor cells compared with the control cells *in vitro* and in in a hepatocellular carcinoma xenograft tumor model (303). More interestingly, NK-92-TN cells were better chemo-attracted to the tumor cells expressing TGF-β and their cytotoxicity was further enhanced by TGF-β (303). The presence of these modified NK-92-TN cells significantly inhibited the differentiation of human naive CD4+ T cells to regulatory T cells (303). Overall, these engineered NK cells either with *SMAD3* knock-down, expressing a dominant negative receptor II for TGF-β, or with a TGF-β type II receptor based CAR should have functional advantages over unmodified NK cells in the presence of TGF-β-secreting solid tumors and will be important therapeutic approaches for NK resistance in patients with solid tumors.

CONCLUSION AND FUTURE DIRECTIONS

NK cell based applications are a promising alternative for immunotherapy of solid tumors. Improvements in understating of NK cell biology and function are driving the further development of NK cells based novel approaches to effectively target solid tumors. We have described the multiple strategies that have been investigated for improving the cytolytic properties of NK cells (**Figure 5**). In the future, combinations of these approaches need to be optimized to further enhance NK efficacy in targeting solid tumors. There is also a growing need to improve

the current imaging modalities to monitor the accumulation and distribution of NK cells *in vivo* after systemic administration, which could serve as a potential surrogate for monitoring the tumor accumulation and anti-tumor response. Improvements in manufacturing and expansion techniques are desired in order to obtain a true universal "off-the-shelf" NK cell product that is GMP-compatible, lower in cost, has a longer half-life and possesses enhanced antitumor responses.

AUTHOR CONTRIBUTIONS

GN and YC reviewed the literatures, developed the designed for the paper, wrote the manuscript and contributed equally. MC reviewed and approved the final manuscript.

FUNDING

This manuscript was supported by the grant from Department of Defense (W81XWH-17-1-0421, PI: MC), Pediatric Cancer Research Foundation (PI: MC), and New York Medical College (NYMC) School of Medicine (SOM) Translational Science Institute (TSI) Children Health Translational Research Award (PI: YC).

ACKNOWLEDGMENTS

The authors would like to thank Erin Morris, RN and Virginia Davenport, RN for their excellent assistance with the preparation of this manuscript.

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

frontiers in Oncology

REVIEW
published: 12 February 2019
doi: 10.3389/fonc.2019.00056

Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer

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Conventional treatments for pancreatic cancer are largely ineffective, and the prognosis for the vast majority of patients is poor. Clearly, new treatment options are desperately needed. Immunotherapy offers hope for the development of treatments for pancreatic cancer. A central requirement for the efficacy of this approach is the existence of cancer antigen-specific T cells, but these are often not present or difficult to isolate for most pancreatic tumors. Nevertheless, specific T cells can be generated using genetic modification to express chimeric antigen receptors (CAR), which can enable T cell responses against pancreatic tumor cells. CAR T cells can be produced *ex vivo* and expanded *in vitro* for infusion into patients. Remarkable responses have been documented using CAR T cells against several malignancies, including leukemias and lymphomas. Based on these successes, the extension of CAR T cell therapy for pancreatic cancer holds great promise. However, there are a number of challenges that limit the full potential of CAR T cell therapies for pancreatic cancer, including the highly immunosuppressive tumor microenvironment (TME). In this article, we will review the recent progress in using CAR T cells in pancreatic cancer preclinical and clinical settings, discuss hurdles for utilizing the full potential of CAR T cell therapy and propose research strategies and future perspectives. Research into the use of CAR T cell therapy in pancreatic cancer setting is rapidly gaining momentum and understanding strategies to overcome the current challenges in the pancreatic cancer setting will allow the development of effective CAR T cell therapies, either alone or in combination with other treatments to benefit pancreatic cancer patients.

Keywords: chimeric antigen receptor, pancreatic cancer, tumor microenvironment, pancreatic ductal adenocarcinoma, adoptive cell transfer, immunotherapy

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Edited by:
Catherine Sautes-Fridman, INSERM U1138 Centre de Recherche des Cordeliers, France

Reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *16 November 2018*
Accepted: *21 January 2019*
Published: *12 February 2019*

Citation:
Ali AI, Oliver AJ, Samiei T, Chan JD, Kershaw MH and Slaney CY (2019) Genetic Redirection of T Cells for the Treatment of Pancreatic Cancer. Front. Oncol. 9:56. doi: 10.3389/fonc.2019.00056

INTRODUCTION

Pancreatic cancer presents a major challenge in the clinic and is one of the most aggressive tumor types. Pancreatic cancer is the fourth most common cause of cancer death (1, 2) and it is on its way to be the second most common cause of cancer-related deaths by 2030 (3). Patients with pancreatic cancers have a median survival rate of 5 months after diagnosis and the overall 5-years survival rate is <5% (4).

The most common clinical therapeutic approaches against pancreatic cancer include surgical resection, radiotherapy, chemotherapy, and combination of these treatments (5). Surgical resection may lead to longer-term survival, but only a small number of the patients are considered resectable

(6–8) because most patients that present to the clinic are with advanced or metastatic disease (9). In addition, the removal of part or the full pancreas is technically difficult, and even if the operation is successful, the 10-years survival rate is still <10% (10). Radiotherapy is usually not curative on its own and is used to alleviate symptoms. Although in the last two decades, chemotherapy and targeted therapy (such as Erlotinib targeting epidermal growth factor receptor, Sunitinib targeting multiple receptor tyrosine kinases, and Everolimus targeting mTOR kinase) have been used for patients with unresectable locally advanced or metastatic pancreatic cancer (11), these have only generated modest improvements in survival (12, 13). Therefore, the need to develop alternative effective therapies for pancreatic cancer is urgent.

Currently, immunotherapy has been offered as an important cancer treatment for a number of cancer types (14, 15) and recent preclinical and clinical evidence suggest that therapies utilizing the immunity could potentially be effective against this devastating disease (16). However, there has been limited success in the use of checkpoint blockade immunotherapies such as PD1/CTLA4 antibodies or vaccines in the treatment of pancreatic cancer (17).

Adoptive cellular therapies involving an infusion of effector immune cells into patients have generated remarkable responses in some cancers (18) and chimeric antigen receptor (CAR) T cell therapy represents a promising therapeutic modality for some difficult cancers including pancreatic cancers. This review aims to summarize the recent development of cellular therapies and clinical data in CAR T cell trials for pancreatic cancer. As pancreatic ductal adenocarcinoma (PDAC) is the most common malignancy of the pancreas and represents the vast majority of pancreatic cancer deaths, we will emphasize CAR T cell work on PDAC in this article.

CAR T CELLS

CAR T cells present an exciting opportunity for cancer immunotherapy. As a form of adoptive immunotherapy, CAR T cells are generated from patient autologous T cells isolated from peripheral blood. Patient T cells are transduced *ex vivo* to express a CAR specific for a tumor antigen of choice and adoptively transferred into the patient to treat established cancers (19). CARs are composed of an antibody single-chain variable fragment (scFv) conjugated to intracellular signaling domains containing CD3-ζ chain and one or more co-stimulatory domains such as CD28 and CD137 (18, 20–22) (**Figure 1**). The CAR scFv confers the ability to T cells to directly recognize cancer antigens independent of MHC antigen presentation, and CAR specific recognition/binding to tumor antigen drives CAR T cell activation and tumor cell killing (23, 24). The first generation of CARs that was designed to contain CD3ζ or FcRγ signaling domains was limited by the lack of costimulatory signaling. The subsequent second generation of CARs has been designed to incorporate CD28 or CD137 cytoplasmic co-stimulatory domains. The third generation of CARs contains additional signaling domains (CD137, CD28, and/or OX40) (18, 20). The latter generations of CAR T cells are better equipped to overcome the immunosuppressive tumor microenvironment (TME), however, it remains unclear what combination of signaling domains is necessary for maximal anti-tumor response.

The use of CAR T cells for the treatment of B cell malignancies demonstrated significant responses in patients (25, 26). Given the success in clinical trials, the use of CD19-targeted CAR T cell therapies was approved by the FDA in 2017. Approved CAR T cell therapies include tisagenlecleucel (Kymriah) for the treatment of children and adolescents with refractory/relapsed B-cell acute lymphoblastic leukemia (B-ALL), and axicabtagene ciloleucel (Yescarta) for adult relapsed-refractory large B-cell lymphoma patients. However, despite the successes in hematological cancers, clinical trials targeting solid tumors have demonstrated only moderate efficacy. This is largely attributed to the immunosuppressive TME, limited activation and trafficking of CAR T cells to the tumor site, heterogeneous antigen expression/distribution in some solid tumors and availability of validated antibodies that could be utilized in the CAR constructs (27–29).

A range of approaches aimed at enhancing CAR T cell efficacy is currently undergoing investigation. A notable strategy that has demonstrated promising effects *in vivo* is the use of dual-specific T cells. Dual-specific T cells co-express a CAR against a tumor antigen and a TCR against a strong immunogen (30). Through vaccination, dual-specific T cells can engage the cognate immunogen of the chosen TCR presented by antigen presenting cells (APCs) on MHC molecules. A recent study using the "adoptive cell transfer incorporating vaccination" (ACTIV) therapy regimen for dual-specific T cell treatment has demonstrated durable responses in a range of solid tumors *in vivo* (31, 32). Use of the specialized "CARaMEL" dual-specific T cells, expressing a CAR against HER2 and TCR specific for the melanocyte protein gp100 (also known as pMEL), drove dramatic T cell expansion and tumor regression in a number of solid tumor models. Moreover, surviving mice that received ACTIV therapy developed potent immune memory responses against pre-existing tumor cells. These results provide encouraging evidence for the investigation and development of dual-specific T cells for the treatment of difficult cancers including pancreatic cancer.

CAR T CELL THERAPIES IN TREATING PANCREATIC CANCER

In recent years, CAR T cell therapies have been tested in both preclinical and clinical settings for treating pancreatic cancers. However, a focus for the field remains the discovery and validation of pancreatic cancer-specific antigens.

Mesothelin

Mesothelin (MSLN) is a glycoprotein present mainly in mesothelial cells and overexpressed in a variety of human cancers including malignant pleural mesothelioma, ovarian, lung and pancreatic cancers (33). MSLN has been reported to be expressed by >80% of PDACs and its expression correlated with poor

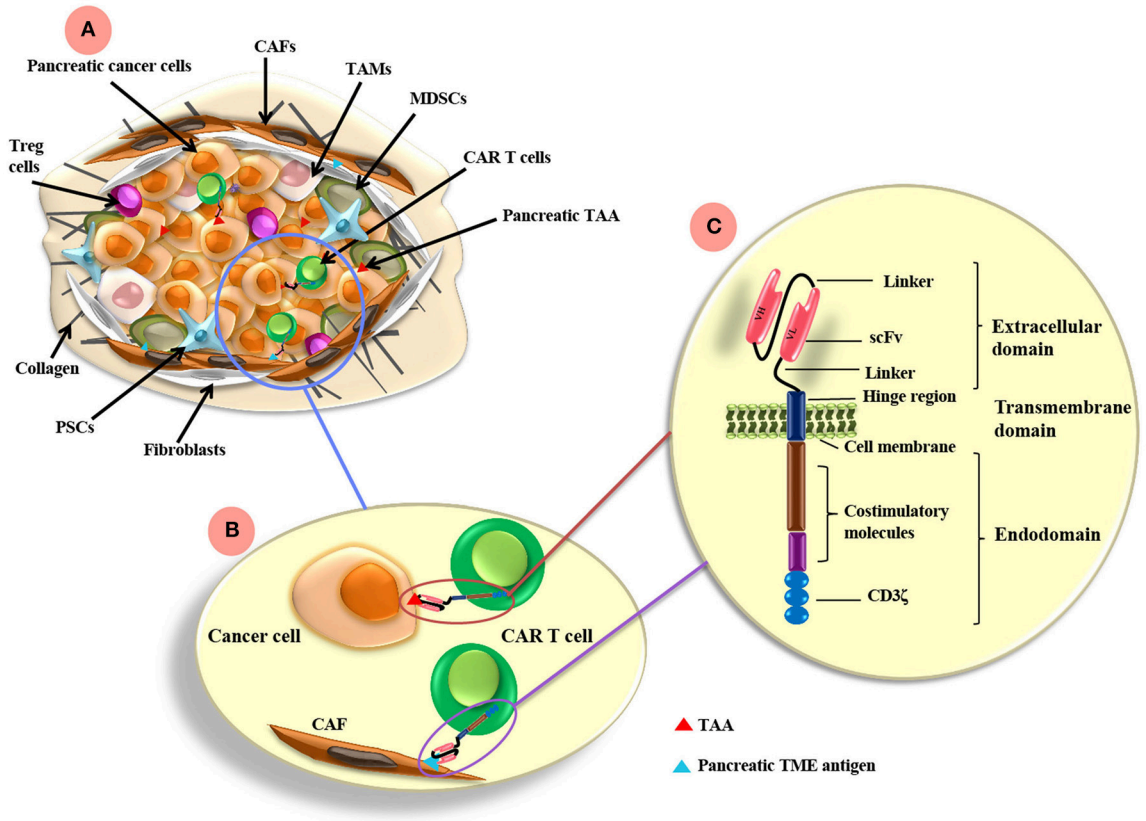

FIGURE 1 | CAR T cell antigen-targeting strategies and pancreatic cancer TME. **(A)** The pancreatic TME consists of tumor cells as well as many immunosuppressive cells, such as CAFs, TAMs, MDSCs, PSCs, and Treg cells. **(B)** CAR T cells can be directed to the TAA expressed on pancreatic cancer cells and/or other antigens targeting the TME components, such as FAP on CAFs. **(C)** CARs are composed of extracellular, transmemebrane and endo-domains. The extracellular domain consists of an antibody variable heavy chain (VH) and a light chain (VL) domain, which are derived from an scFv from an antibody specific for a TAA. A flexible hinge region links the extracellular domain to a transmembrane and endodomain. The endodomain has cytoplasmic signaling regions derived from CD3ζ and costimulatory signaling domains. TAMs, tumor-associated macrophages; CAFs, cancer associated fibroblasts; MDSCs, myeloid-derived suppressor cells; Tregs, regulatory T cells; PSCs, pancreatic stellate cells; FAP, fibroblast activation protein; scFv, single chain variable fragment. TAA, tumor associated antigen; TME, tumor microenvironment.

prognosis (34). Although MSLN is believed to play a role in cell adhesion and positively regulates tumor invasion and growth, its biological function is unclear (34). Because MSLN is expressed only on non-crucial tissues, it is an attractive target for CAR T cell based immunotherapy.

Preclinical studies have demonstrated that CAR T cells against MSLNs could potentially be effective against PDAC. When MSLN-CAR T cells were adoptively transferred intratumorally or intravenously (i.v.) into NSG mice bearing pre-established subcutaneous patient-derived mesothelioma, the tumor burden was greatly reduced in size and some tumors were completely eradicated, demonstrating the potential of targeting this antigen (35). Due to the concerns for potential on-target/off-tumor toxicity, phase I clinical studies used mRNA-based methods to generate CAR T cells that express CARs transiently to limit the duration of toxicity. Given the short-term expression of CARs, multiple injections were required. In a study carried out by Beatty et al (36), a patient with metastatic PDAC was given eight doses of MSLN-CAR T cells by i.v. infusion and two doses via intratumoral injections. The CAR T cells were detected in the extravascular tumor compartments 3 days after the initial i.v. infusion. The patient demonstrated stable disease 3 weeks post MSLN-CAR T cell administration, without overt evidence of toxicity against normal tissues. An anti-tumor effect was observed based on the development of novel humoral immune responses post the cell infusion. In another phase I trial carried out by the same group, six metastatic PDAC patients were treated with mRNA-based MSLN-CAR T cells three times per week for three consecutive weeks. The treatment was well-tolerated and the disease was stabilized in two of the treated patients. Importantly, one patient had total metabolic active volume decreased by 69.2%, although there was no detected effect on the primary tumor (37). Given the promising results, trials of MSLN-CAR T cells engineered by traditional viral transduction methods have also been initiated (**Table 1**).

Prostate Stem Cell Antigen

Prostate stem cell antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell surface protein involved in intracellular signaling, although much of its function

TABLE 1 | Clinical trials involving CAR-based immunotherapy in pancreatic cancer from www.clinicaltrials.gov.

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
MSLN	National Cancer Institute, USA	Pancreatic cancer Cervical cancer Ovarian cancer Mesothelioma Lung cancer	Recruiting/December, 2029	136 estimated	NCT01583686	Intravenous infusion of retroviral transduced MSLN-CAR T cells and low dose IL-2 with cyclophosphamide and fludarabine preconditioning.
	University of Pennsylvania, USA	Pancreatic cancer	Active, not recruiting/September, 2021	18 estimated	NCT03323944	1–3 × 10^7/m^2 (Cohort 1) or 1–3 × 10^8/m^2 (Cohort 2 and 3) lentiviral transduced MSLN-CAR T cells with/without cyclophosphamide preconditioning.
	Shanghai Gene Chem Co., Ltd./Shanghai Cancer Hospital, China	Metastatic pancreatic cancer	Unknown/July, 2018	20 estimated	NCT02959151	Intratumor injection or i.v. at one dose of 1.25~4 × 10^7 CAR^+ lentiviral transduced MSLN-CAR T cells/cm^3 tumor bulk.
	University of Pennsylvania, USA	Metastatic PDAC	Completed/March 2017	16 actual	NCT01897415	1–3 × 10^8/m^2 RNA transfected MSLN-CAR T cells i.v. injected three times weekly for up to 3 weeks. No cytokine release syndrome, neurologic symptoms or dose-limiting toxicities reported. One patient has a response in the live, but no activity in the primary tumor (37).
	University of Pennsylvania, USA	Metastatic PDAC Epithelial ovarian cancer Malignant epithelial pleuralmesothelioma	Completed/November, 2015	19 actual	NCT02159716	1–3 × 10^7 and 1–3 × 10^8/m^2 lentiviral transduced MSLN-CAR T cells i.v. injected with or without cyclophosphamide preconditioning. Safe in patients with recurrent serious ovarian cancer. CAR T cells expanded in the blood of all patients. One patient had clearance of pleural effusion (104).
	Chinese PLA General Hospital, China	Malignant mesothelioma Pancreatic cancer Ovarian tumor Triple negative breast cancer Endometrial cancer Other $MSLN^+$ tumors	Unknown/November 2018	20 estimated	NCT02580747	Retroviral transduced MSLN-CAR T cells.
	China Meitan General Hospital/Marino Biotechnology Co., Ltd. China	$Mesothelin^+$ tumors	Recruiting/August 2019	20 estimated	NCT02930993	i.v. infusion with 5 × 10^4-1 × 10^7/kg MSLN-CAR T cells in a three-day split-dose regime. Cyclophosphamide preconditioning.
	University of Pennsylvania, USA	Pancreatic cancer Mesothelioma	Completed/September 2017	18 actual	NCT01355965	Three infusions of 1 × 10^8-1 × 10^9 mRNA MSLN-CAR T cells every other day for 2-cycle of three infusions. The pancreatic patient was given eight doses i.v. infusions and two intratumor injections. Anti-tumor effect observed and no overt toxicities (36).
	University of Pennsylvania/University of California, USA	Pancreatic cancer	Completed/November, 2017	4 actual	NCT02465983	i.v. infusion of 1–3 × 10^7/m^2 or 1 × 10^8/m^2 combined lentiviral transduced MSLN-CAR T cells and CD19-CAR T cells (to deplete B cells and impede the antibody response against MSLN CAR T cells) with cyclophosphamide preconditioning.

(Continued)

TABLE 1 | Continued

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
	First Affiliated Hospital of Wenzhou Medical University, China	Pancreatic cancer	Active, not recruiting/October, 2020	10 estimated	NCT03497819	Pancreatic arterial or i.v. infusion of lentiviral transduced MSLN-CAR T cells and CD19 CAR T cells with cyclophosphamide preconditioning.
	Shanghai GeneChem Co., Ltd. China	Pancreatic cancer	Unknown/September 2018	30 estimated	NCT02706782	Transcatheter arterial infusion of 1–10 $\times$ 10^6 MSLN-CAR$^+$ T cells/kg. Cyclophosphamide preconditioning.
MSLN, PSCA, CEA, HER2, MUC1, EGFRvIII and other targets	First Affiliated Hospital of Harbin Medical University/Shanghai Unicar-Therapy Bio-medicine Technology Co.,Ltd. China	Pancreatic cancer	Recruiting/June 2019	10 estimated	NCT03267173	A single dose of 10^7 /kg CAR T cells administered i.v.
	Cancer Research UK, UK	Pancreatic cancer Breast cancer Colorectal cancer Gastric cancer Lung cancer Ovarian cancer Unspecified adult solid tumor	Terminated/April 2010	14 actual	NCT01212887	The pancreatic cancer patient received 10^9-5 $\times$ 10^{10} retroviral transduced CEA CAR T cells i.v. and seven doses IL-2 with cyclophosphamide and fludarabine preconditioning. All patients had adverse events with grade $\leq$2 and lack of prolonged CAR T cell persistence led to the premature termination of the trial. No objective clinical response observed although serum CEA level reduced in the pancreatic cancer patient (47).
CEA	Roger Williams Medical Centre/University of Colorado, USA	Liver metastases Pancreatic cancer	Active, not recruiting/August, 2018	5 actual	NCT02850536	Three hepatic artery infusions of CEA-CAR T cells delivered by the Surefire Infusion System at 1-week intervals. Low dose IL-2 for 4 weeks.
	Southwest Hospital, China	Pancreatic cancer Lung cancer Colorectal cancer Gastric cancer Breast cancer	Recruiting/December, 2019	75 estimated	NCT02349724	CEA-CAR T cells were well-tolerated in high doses with some efficacy reported in the colorectal cancer cohort (105).
	Roger Williams Medical Center/Sirtex Medical, USA	Liver metastases	Active, not recruiting/January 2019	8 actual	NCT02416466	Three doses of CEA-CAR T cells and 1 dose of Selective Internal Radiation Therapy (SIRT) with Yttrium-90 at 2-weeks intervals combined with low dose IL-2 for 6 weeks.
PSCA	Bellicum Pharmaceutical, USA	PDAC Gastric adenocarcinoma Prostate adenocarcinoma	Recruiting/December, 2020	138 estimated	NCT02744287	i.v. infusion of retroviral transduced PSCA CAR T cells (BPX-601) with rimiducid (a homodimerizing molecule that enhances BPX-601 activation).
CD70	National Cancer Institute, USA	Pancreatic cancer Renal cell cancer Breast cancer Melanoma Ovarian cancer	Recruiting/January, 2028	113 estimated	NCT02830724	i.v. infusion of retroviral transduced CAR T cells. The CAR consists of the CD70 receptor CD27. Cyclophosphamide and fludarabine preconditioning with high dose IL-2.

(Continued)

TABLE 1 | Continued

Target antigen	Sponsor/collaborator	Cancer type	Status/estimated completion date	Number of patients	Trial ID	Notes
MUC1	PersonGen BioTherapeutics (Suzhou) Co., Ltd./The First People's Hospital of Hefei; Hefei Binhu Hospital, China	Pancreatic carcinoma Hepatocellular carcinoma Non-small cell lung cancer Triple-negative invasive breast carcinoma	Unknown/October, 2018	20 estimated	NCT02587689	
	PersonGen BioTherapeutics (Suzhou) Co., Ltd./The First People's Hospital of Hefei/Hefei Binhu Hospital, China	Pancreatic carcinoma Hepatocellular carcinoma Non-small cell lung cancer Triple negative invasive breast carcinoma Malignant glioma of brain Colorectal carcinoma Gastric carcinoma	Unknown/July, 2018	10 estimated	NCT02839954	Anti-MUC1 CAR-NK cells.
HER2	Southwest Hospital, China	Pancreatic cancer Breast cancer Ovarian cancer Lung cancer Gastric cancer Colorectal cancer Glioma	Recruiting/September, 2019	60 estimated	NCT02713984	
EpCAM	First Affiliated Hospital of Chengdu Medical College, China	Pancreatic cancer Colon cancer Esophageal carcinoma Prostate cancer Gastric cancer Hepatic carcinoma	Recruiting/December, 2020	60 estimated	NCT03013712	1–10 $\times$ 10^6 lentiviral transduced EpCAM-CAR$^+$ T cells/kg. Vascular interventional or endoscopy infusion. Precondition before CAR T cell infusion.
Claudin 18.2	CARsgen Therapeutics, Ltd., China	Pancreatic carcinoma Adenocarcinoma of esophagogastric junction	Not recruiting/December, 2020	48 estimated	NCT03302403	i.v. infusion. Fludarabine and cyclophosphamide preconditioning.
	Changhai Hospital/CARsgen Therapeutics, Ltd., China	PDAC Advanced gastric adenocarcinoma	Recruiting/December 2021	24 estimated	NCT03159819	i.v. infusion of lentiviral transduced CAR T cells with lymphodepletion preconditioning.
CD133	Chinese PLA General Hospital, China	Pancreatic cancer Liver cancer Brain tumor Breast cancer Ovarian tumor Colorectal cancer Acute myeloid and lymphoid leukemias	Unknown/October 2018	20 estimated	NCT02541370	Retroviral transduced CD133-CAR T cells.

remains unclear. PSCA is expressed in the epithelial cells including that of prostate, kidney, skin, stomach, urinary bladder, esophagus and placenta, and also expressed in differentiating cells such as the ones of prostate and gastric epithelial cells. PSCA has also been detected in several cancer types including prostate, urinary bladder and pancreatic cancers (38). Aberrant overexpression of PSCA is detected in nearly 60% of the primary PDACs, while the gene expression is not detected in normal pancreatic duct (39). Therefore, PSCA has been proposed as a specific biomarker for PDAC patients and a promising target for CAR T cell therapies in treating PDAC. An advantage of targeting PSCA is that it is upregulated in pancreatic cancer cells from early stages of malignant transformation (40), including premalignant pancreatic intraepithelial neoplasias. PSCA may therefore serve as a useful target of immunotherapy that could eliminate malignant cells at all stages of PDAC.

Strategies using CARs against PSCA have been tested in preclinical settings. The 1st generation CAR T cells specifically killed $PSCA^+$ pancreatic cancer cell lines without lysing $PSCA^-$ target cell *in vitro* (8). In a more recent study, multiple CAR constructs were compared. Adoptive transfer of these human CAR T cells in this study demonstrated significant antitumor activity in a murine model of human pancreatic cancer. Interestingly, although the third-generation CAR containing CD28 and CD137 costimulatory domains induced greater persistence of CAR T cells *in vivo*, the second generation CAR that does not contain CD137 domain, induced a better antitumor effect, with 40% of mice demonstrating tumor eradication (40). The efficacy between second- and third- generation CARs have been compared by various studies and the discrepancies between these reports indicate that the optimal CAR design needs to be empirically determined for disease and antigen targeted (41, 42). Being encouraged by the preclinical success, a trial using CAR T cells against PSCA has been initiated and is currently recruiting patients (NCT02744287).

Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) is a cell surface glycoprotein belonging to the immunoglobulin (Ig) superfamily and plays a role in cell adhesion. CEA is one of the "oncofetal" antigens, typically produced in the gastrointestinal tissue during fetal development (43, 44). Although CEA is expressed in various healthy epithelia of pulmonary and gastrointestinal tracts, its distribution is often limited to the luminal surface, thus difficult for CAR T cells to access (44). After neoplastic transformation, luminal epithelia cells lose the apical polarity of CEA expression and CEA becomes accessible to immune cells. Some CEA is released to the serum and soluble CEA in the circulation of patients is used as a marker for cancer progression. CEA is highly expressed on the surface of the majority of PDAC cells (45). Together with its restricted expression in normal tissues, CEA is an attractive target for CAR T cell treatment in PDAC. A few versions of anti-CEA CARs have been developed in the past few years, each targeting different CEA epitopes.

CEA-CAR T cells exhibited cytotoxicity against CEA expressing cancer cells *in vitro* and demonstrated anti-tumor effect *in vivo* in a clinically relevant orthotopic CEA^+ murine model. The recipient CEA transgenic mice express CEA in their intestinal and pulmonary tracts. Ten days post intrapancreatic injections of Panc02-CEA^+ cells, the recipient mice received CEA-CAR T cells. Interestingly, the injection of the CAR T cells eradicated tumors without any damage to normal tissues that are CEA^{low} (46).

However, a recent clinical trial using CEA-CAR T cells treating patients with advanced CEA^+ cancers demonstrated acute respiratory toxicity, which resulted in the premature closure of the trial (47). The expression of CEA on lung epithelium was considered to result in the toxicity, which was associated with pre-conditioning. In a recent clinical trial using T cells modified to express an anti-CEA TCR for treating CEA^+ metastatic colorectal cancer, severe autoimmune colitis and pneumonia was observed in all of the three patients and led to the halt of the trial (48). This was likely due to the ability of TCR-redirected T cells to engage CEA presented by MHC.

Mucin 1

Mucins are high-molecular-weight glycoproteins with the presence of a heavily *O*-glycosylated tandem repeat region that is rich in proline, threonine and serine residues. The large gel-forming mucins are an extracellular secretion of goblet cells and their functions include lubrication of the epithelial surfaces and protection from physical and chemical insult. The epithelial membrane-tethered mucins are distinct from the conventional secreted mucins and are transmembrane molecules expressed by most glandular and ductal epithelial cells. It is widely accepted that the transmembrane mucin 1 (MUC1) is overexpressed in multiple epithelial adenocarcinomas, such as that of breast, colon, and pancreatic cancers. Importantly, under normal conditions, MUC1 is heavily glycosylated and expressed on the apical surface of epithelial cells, but in tumor cells MUC1 is aberrantly glycosylated. This modification of the MUC1 antigen reveals epitopes associated with the core protein, which is usually masked by oligosaccharides. Overexpression of aberrantly glycosylated MUC1 is associated with multiple metastatic cancers. In particular, MUC1 is aberrantly expressed in 60% of pancreatic cancers and is correlated with poor prognosis, enhanced metastasis, and chemoresistance (49, 50).

Strategies using CAR T cells targeting aberrantly expressed MUC1 have generated exciting results in preclinical studies. Posey et al. (51) generated a CAR that recognizes the aberrant glycoform Tn (GalNAca1-O-Ser/Thr) antigen on MUC1. These CAR T cells are able to recognize multiple types of tumors *in vitro* and exhibited superior tumor rejection and prolonged survival against disseminated pancreatic cancers in a xenograft model. This elegant study highlighted the potential for protein modifications as a target. As one of the most characteristic features of cancer cells is altered glycosylation, changes in glycosylation may expose a range of different cancer-associated epitopes and can serve as a target for CAR T cells. Given the promising outcomes from preclinical studies, multiple early phase trials have been planned and are active for using MUC1-CAR T cells in treating PDAC (**Table 1**).

CD47

CD47 is a transmembrane protein known to mediate a "do not eat me" signal. Structurally, CD47 contains an extracellular N-terminal hydrophilic Ig superfamily domain and an intracellular hydrophobic domain. Signal regulatory protein alpha (SIRPα) has been identified as the receptor to CD47. The binding of tumor-expressing CD47 to SIRPα on immune cells leads to the activation of the downstream signaling pathway in immune cells and inhibits the immune phagocyte-dependent clearance of tumors. CD47 has been identified in several types of hematological and solid cancers including pancreatic cancer (52, 53). In addition, CD47 is expressed on high levels on cancer stem cells (CSCs), but not on normal cells in the pancreas (52). Therefore, targeting CD47 has been a subject of intense interest in recent years.

CD47-specific CAR T cells were recently developed by ProMab Biotechnologies. These CAR T cells demonstrated high cytotoxicity against a few types of cancer cells including pancreatic cancer cells. Importantly, intratumoral injection of these CAR T cells significantly decreased pancreatic xenograft tumor growth. The same research group also developed humanized CD47-CAR T cells that contain humanized CD47 scFv. These humanized CD47-CAR T cells demonstrated specific killing of $CD47^+$ cancer cells *in vitro* and it will be interesting to assess their efficacy in clinical trials (54).

Tyrosine Kinase Growth Factor Receptors

The tyrosine kinase growth factor receptors are transmembrane proteins that play a key role in medicating intracellular signal transduction cascade for cell proliferation and differentiation. Up-regulation of some of the receptors are mechanisms of cancer development and progression, and the overexpression of a number of these receptors have been identified in many types of cancers (55). Therefore, CARs designed to target tyrosine kinase growth factor receptors have been developed and clinical trials are under way to test the safety and efficacy of many of these targets.

Human epidermal growth factor receptor 2 (HER2, also known as ERBB2) is a transmembrane glycoprotein belonging to the epidermal growth factor receptor (EGFR) family. The binding of HER2 to its ligand induces heterodimerization of the receptors, which mediates the activation of intracellular tyrosine kinase signaling cascades and leads to cell proliferation and differentiation. Overexpression of HER2 induces dimerization of HER2 and initiates signal transduction without ligand binding. Overexpression of HER2 has been reported in multiple cancer types making it an attractive target for CAR T cell treatment (20). However, an early HER2-CAR T cell trial, utilizing a third generation CAR, reported a patient death post the CAR T cell treatment (56). This patient was diagnosed with colon cancer metastatic to the lung and liver. The patient was preconditioned using cyclophosphamide (CY) and flurodarabine. Following intravenous infusion of 10^{10} HER2-CAR cells in 30 min, the patient experienced acute respiratory distress and subsequent death. At autopsy, multiple organs including the lung showed signs of ischemia and injury, and her serum samples post infusion showed significant increased levels of multiple cytokines, including IFN-γ, IL-6, and TNF-α. This report raised safety concerns on targeting HER2, but a more recent study using lower numbers of HER2-CAR T cells, and a second generation CAR in treating 19 patients with $HER2^+$ sarcoma, demonstrated safety (57).

HER2 expression is observed in 20–60% of pancreatic cancer cases and therefore, it is a potential target for CAR T cell treatment (58–61). Recently, a Phase I clinical trial used HER2-CAR T cells to treat two pancreatic cancer patients. Although the trial demonstrated safety, only moderate responses were achieved (62). In contrast, results from preclinical studies have been promising. A recent study used HER2-CAR T cells to treat mice bearing xenografts derived from stage IV PDAC patients and achieved complete remission in both local and disseminated disease settings. In addition, in this study, the authors used an antibody-based switchable CAR system. These switchable CAR T cells bind to a specific peptide that is genetically engrafted onto a tumor-binding Fab molecule. The switch acts as a bridge between the tumor cells and CAR T cells and has a short half-life and thus, limits potential immunogenicity. The comparison between the switchable and conventional CAR T cells demonstrated that the anti-tumor efficacy of these switchable CAR T system was not compromised (63). The discrepancy in efficacy results from clinical and preclinical studies may be due to the different levels of HER2 expression in patients and xenografts, and the immunosuppressive TME in the patients.

Other putative growth factor receptors that could be targeted against pancreatic cancer using CARs include insulin-like growth factor receptor-1 (IGF1R), EGFR, vascular endothelial growth factor receptor (VEGFR), fibroblast growth factor receptor (FGFR) and platelet-derived growth factors (PDGFRs) that are expressed at elevated levels in pancreatic cancers and contribute the cancer's malignant phenotype (55). IGF1R is expressed in a variety of cancers and blocking IGF1R expression enhances apoptosis and suppresses metastasis in pancreatic cancer cells (64). IGF1R-CAR cells have demonstrated efficacy in murine models of sarcoma xenograft (65) and could be potentially useful in the treatment of PDAC. EGFR is a surface glycoprotein that belongs to the EGFR family of tyrosine kinase receptors. EGFR is aberrantly activated in a number of epithelial tumors and its overexpression has been detected in up to 90% of pancreatic tumors (66, 67). The value of EGFR-CAR T cells in treating solid cancers has been demonstrated in both preclinical (68) and clinical settings (69) in a few different cancer types but its potential in treating PDAC is yet to be tested.

CD24

CD24 is a mucin-like protein. It was originally discovered as the ligand for P-selectin and involved in signal transduction mediated by the members of the protein tyrosine kinase family. CD24 expression is observed in over 70% of PDAC tumors and in putative PDAC cancer stem cells (CSC). CSC can also express CD44 and epithelial specific antigen (ESA) or CD133. Given the prevalence of early-disseminated metastases, CSCs are believed to play a key role in the pancreatic cancer development

and progression. Among these PDAC CSC protein markers, CD24 has the lowest expression in normal tissue and thus is proposed as a suitable target antigen for immunotherapy (70, 71). A study carried out by Maliar et al. examined the therapeutic efficacy of both HER2-CAR and CD24-CAR in treating PDACs in murine models. In mice bearing subcutaneous human PDAC cell line Capan-1 (positive for both HER2 and CD24), intratumor injection of the CAR T cells targeting either HER2 or CD24 greatly inhibited tumor growth, in some cases, eradicated tumors. In mice bearing orthotopic Capan-1 tumors, i.v. injections of these two different CAR T cells also demonstrated anti-tumor effects to both primary and metastatic tumors at the liver and lymph nodes. Interestingly, the cells that were dissociated from patient pancreatic cancers for xenograft in this study, displayed a heterogeneous expression pattern of antigens, including both HER2 and CD24. The adoptive transfer of HER2-CAR or CD24-CAR to mice bearing these xenografts significantly arrested the tumor growth, but to a different degree, dependent on the CAR-targeted antigen specificity. The results from this study highlight the antigen heterogeneity nature of human pancreatic tumors (72). It remains to be seen if the administration of CAR T cells targeting both antigens will enhance efficacy. This study highlights potential therapeutic limitations using CARs targeting a single tumor antigen in treating complex cancers that are heterogeneous in antigen expression patterns and distributions.

Fibroblast Activation Protein

Fibroblast activation protein (FAP) is a type II integral membrane serine protease. Healthy adult tissues have no detectable FAP expression. However, under certain biological circumstances, such as remodeling, wound healing, and embryogenesis, FAP expression has been observed. FAP is also present in a large proportion of tumor stromal fibroblasts in the majority of epithelial carcinomas including pancreatic cancers (73) and its expression correlates with poor prognosis in pancreatic cancer patients (74). The carcinoma-associated fibroblasts (CAFs) are a central player in tumorigenesis and metastasis and the key characteristics of CAFs is the expression of FAP (75). Due to its high expression in CAFs, FAP has been tested as a CAR target.

Tran et al. investigated the use of FAP-CAR T cells targeting tumor stromal fibroblasts in a number of mouse tumor models and human pancreatic cancer xenografts. Despite *in vitro* activity observed, the injection of the FAP-CAR T cells only elicited limited *in vivo* anti-tumor effect. Unexpected side effects such as cachexia and lethal bone toxicities were observed. The off-target effect was due to the expression of FAP on murine bone marrow stromal cells (BMSCs). In this study, human BMSCs were also identified in expressing FAP and could be recognized by FAP-CAR T cells (76). The finding that FAP is expressed by BMSCs raised safety concerns for therapies targeting FAP.

Interestingly, in separate studies carried out by Wang et al. (77) and Kakarla et al. (78) using FAP-CAR T cells, no toxicity was observed. The reason may be due to the different scFvs used. The scFv used by Wang et al. targets a different FAP epitope and only eliminate FAP^{hi} cells, while sparing FAP^{low} cells including BMSCs.

CLINICAL TRIALS USING CAR T CELLS AGAINST PANCREATIC CANCER

A substantial number of clinical trials involving the use of CAR technology have recently been undertaken on pancreatic cancer patients in an attempt to investigate the potential safety, effectiveness and feasibility of the approach. A variety of tumor associated antigens (TAA) expressed on pancreatic cancer cells have been targeted in various clinical trials to redirect CAR T cells against pancreatic cancer including MSLN, CEA, PSCA, MUC1, and HER2 (**Table 1**).

A range of CAR formats are used in these clinical studies, although chiefly second-generation formats, with either CD28 or CD137 cytoplasmic domains. Transduction methods also vary, including electroporation with CAR-encoding RNA, but viral vectors, either retroviral or lentiviral, are the main method of CAR gene delivery (**Table 1**). Typically, dose escalation is used in these trials, which aim to determine safety, with doses ranging from 1×10^7 to 3×10^8 per m^2. Higher doses of RNA transfected T cells are used, since they present less of a risk due to their limited duration of CAR expression. The majority of studies involve a single dose of CAR T cells; some trials use multiple doses (**Table 1**).

There is substantial variation in the use of preconditioning, although some level of lymphodepletion is proposed for most studies. The combination of CY and fludarabine is often preferred to induce a deep level of lymphodepletion to enhance engraftment of transferred CAR T cells. While most of the studies detailed in **Table 1** involve the transfer of CAR T cells alone, some propose to use additional drugs to enhance CAR T cell activity. Thus, IL-2 is proposed for some studies to provide T cells with a supporting growth factor.

To enhance the effectiveness and the safety of CAR T cell therapy, some clinical trials use reagents that activate CAR T cells. For example, in trial NCT02744287 targeting PSCA, a dimerizer agent, Rimiducid (AP1903) is used. Rimiducid is administered with PSCA-specific CAR T cells that contain an inducible MyD88/CD40 (iMC) costimulatory domain. In another trial (NCT02416466), radiation is delivered using Yttrium-90 microspheres to maximize the tumoricidal effects of CAR T cells and minimize the effects on healthy liver parenchyma in patients with liver metastases.

The majority of clinical trials of CAR T cells in pancreatic cancer are in early stages of recruitment, but some have been completed and initial reports are available. CAR T cells are generally well-tolerated in pancreatic cancer patients, although some toxicity was reported when targeting CEA (47). Despite the early nature of most trials, there are some reports of CAR T cell efficacy. The CARsgen trial reported their early results at the 2018 CAR-TCR Summit in Boston that using an anti-Claudin-18.2 CAR treating pancreatic and gastric cancer patients resulted in some objective responses without overt toxicities (79, 80). However, there are no descriptions of significant responses in most trials to date, some unique challenges presented by pancreatic cancer may have to be overcome to maximize responses.

TUMOR MICROENVIRONMENT AS A UNIQUE CHALLENGE FOR CAR T CELLS IN PANCREATIC CANCER

A major obstacle for immunotherapies, in particular CAR T cell therapies, in solid tumors is the immunosuppressive tumor microenvironment (TME) (75). The TME impacts on the efficacy of CAR T cells both by limiting their infiltration and suppressing their function within the tumor (29, 81).

A unique feature of the pancreatic TME is the desmoplastic stromal reaction, which promotes tumor growth and provides a physical barrier for therapeutic drugs and T cell infiltration. In fact, PDAC is one of the most stroma-rich cancers and in some cases, the stromal components precede pancreatic cancer cells. In normal pancreas, the pancreatic stellate cells (PSCs) are a rare population and function to store retinoids in a form of lipid droplets in the cytosol. During pancreatic cancer progression, the pancreatic stellate cells (PSCs) become activated by tumor-secreted cytokines, lose retinoid droplets and transform into a myofibroblast phenotype. The activated PSCs secrete extracellular matrix (ECM) proteins and deposit collagens to form a dense fibrotic cancer stroma (82). In addition, activated PSCs secrete cytokines (such as IL-6 and IL-11) and chemokines (such as CXCL12, CCL5, CCL2, and CCL17) that recruit immunosuppressive leukocyte subsets (82–85). Indeed, as discussed above, CAR T cells that target the highly expressed CAF protein, FAP, are considered in pancreatic cancer due to the significant role these cells play in tumorigenesis (77, 86, 87) and in addition, FAP is positive in PDAC-derived ECMs but negative in normal PSCs (88). The particularly harsh TME is a major barrier to CAR T cell efficacy in pancreatic cancer and thus additional modulators will be required for durable responses (**Figure 1**).

Approaches that enable CAR T cells to sustain and function in the TMEs have been investigated in a large number of preclinical and clinical studies in a range of malignancies, and the results have revealed various potential strategies against pancreatic cancers. For example, depleting immunosuppressive cell subsets such as Tregs, MDSCs and TAMs has demonstrated enhanced efficacy of CAR T cell therapies (29). These suppressive immune cell types are enriched in pancreatic cancer and are associated with increased tumor growth and poorer prognosis (89–91). Various reagents that have shown to eliminate these suppressive immune cells or modulate their functions have been tested in murine models in recent years, such as IL-2 toxin and anti-CD25 (for Treg depletion), CSF-1R inhibitor and CCR2 toxin (for MDSCs/TAMs) (92–95). A few anti-TAM drugs such as antibodies against CCL2, CSF1R that inhibit the recruitment and survival of TAMs and MDSCs are currently under clinical investigation (96). Strategies of using CARs against these suppressive cells are emerging. For examples, inserting CSF1R-CARs into NK and T cells for killing TAMs have demonstrated promising outcomes *in vitro* (97). In our laboratory, using ACTIV therapy that involved injecting dual-specific CAR T cells and a vaccine, TAMs decreased significantly post the treatment, coinciding with tumor regression (32). In humans, the engraftment of T cells is enhanced with myeloablative preconditioning regimes and thus specific depletion methods could enhance CAR T cell efficacy in pancreatic cancer patients (98, 99).

Another strategy for enhancing CAR T cell efficacy in solid tumors is blocking the inhibitory signals received by the T cells from immunosuppressive populations in the TME. Checkpoint inhibitors, against molecules such as PD-1, CTLA4, TIM-3, and LAG-3 have shown promise as single agents in a number of cancer types (100) but unfortunately, none of these treatment as a single therapy has generated significant clinical benefit in pancreatic cancers. CAR T cells can express high amounts of these checkpoint molecules, which can lead to apoptosis and hypo-function. Results from preclinical studies clearly demonstrated that CAR T cell treatments could benefit from the addition of checkpoint inhibitors (101, 102). Some early clinical data also support the use of a combination of the checkpoint inhibitors with CAR T cell therapy in treating difficult cancers. A study carried out by Chong et al. reported that in a diffuse large B cell lymphoma patient refractory to CART19, after PD-1 blockade, the patient had an expansion of the CAR T cells and clinically significant antitumor response (103).

In addition, a number of strategies have been tested to enhance CAR cells to sustain in a suppressive cytokine milieu. Methods such as introducing the CD137 signaling domain within the CAR intracellular domain to increase mitochondrial biogenesis (104), expressing regulatory subunit I anchoring disruptor (RIAD) peptide to CAR T cells to disrupt protein kinase A (PKA) activation (105), and constitutively expressing CD40L by CAR T cells (106) have all demonstrated potential in enhancing CAR T cell treatment efficacy in solid cancers. A recent elegant study combined MSLN-CAR T cells with an oncolytic adenovirus expressing TNF-α and IL-2 to treat human PDAC xenograft models and a sygeneic mouse tumor model. This strategy significantly enhanced CAR T cell anti-tumor efficacy. The anti-tumor effect was linked to increased tumor-infiltrating lymphocytes and altered TME including altered polarization of macrophages and maturation of dendritic cells (107).

Given the limited options for efficacious pancreatic cancer treatment and the success of CAR T cells in hematological malignancies, in which TME differs in its degree of immunosuppression, overcoming this obstacle in pancreatic cancer will be an important consideration for future research.

CONCLUSIONS AND FUTURE PERSPECTIVES

Pancreatic cancer is the most lethal cancer and new therapies are urgently needed. CAR T cell therapy represents a revolutionary treatment for cancers and has generated remarkable responses in hematological malignancies. The extension of CAR T cell therapy into pancreatic cancer recently started and this field is moving forward rapidly. Due to its unique immunosuppressive TME and antigen complexity and heterogeneity (108), pancreatic cancer presents one of the most difficult cancers for immunotherapies. Understanding the pancreatic cancer TME and how this TME affects CAR T cell efficacy is key in designing effective

CAR T cell treatments. In addition, besides expanding the CAR-antigen landscape, targeting multiple antigens simultaneously (109) and using strategies targeting neoantigens (16) could provide significant opportunities for treating pancreatic cancer.

AUTHOR CONTRIBUTIONS

AA, MK, and CS: conception and design; AA, AO, TS, JC, MK, and CS: write, review, and revision of the manuscript; MK and CS: supervision.

FUNDING

This work was supported by grants from the Peter MacCallum Cancer Center Foundation, the National Health and Medical Research Council (NHMRC) of Australia (1103352 and 1132373), the National Breast Cancer Foundation (NBCF) of Australia (IIRS-18-064) and Susan G. Komen Breast Cancer Foundation (16376637). CS was supported by a Fellowship from the NBCF. AA was supported by a University of Melbourne International Research scholarship, AO by an Australian Postgraduate Award.

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

frontiers in Immunology

REVIEW
published: 20 February 2019
doi: 10.3389/fimmu.2019.00263

Fueling Cancer Immunotherapy With Common Gamma Chain Cytokines

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Adoptive T cell transfer therapy (ACT) using tumor infiltrating lymphocytes or lymphocytes redirected with antigen receptors (CAR or TCR) has revolutionized the field of cancer immunotherapy. Although CAR T cell therapy mediates robust responses in patients with hematological malignancies, this approach has been less effective for treating patients with solid tumors. Additionally, toxicities post T cell infusion highlight the need for safer ACT protocols. Current protocols traditionally expand T lymphocytes isolated from patient tumors or from peripheral blood to large magnitudes in the presence of high dose IL-2 prior to infusion. Unfortunately, this expansion protocol differentiates T cells to a full effector or terminal phenotype *in vitro*, consequently reducing their long-term survival and antitumor effectiveness *in vivo*. Post-infusion, T cells face further obstacles limiting their persistence and function within the suppressive tumor microenvironment. Therapeutic manipulation of T cells with common γ chain cytokines, which are critical growth factors for T cells, may be the key to bypass such immunological hurdles. Herein, we discuss the primary functions of the common γ chain cytokines impacting T cell survival and memory and then elaborate on how these distinct cytokines have been used to augment T cell-based cancer immunotherapy.

Keywords: chimeric antigen receptor, T cell, adoptive cell transfer, gamma chain cytokines, TRUCKs

OPEN ACCESS

Edited by:
Prashant Trikha, Nationwide Children's Hospital, United States

Reviewed by:
Nejat K. Egilmez, University of Louisville, United States
Doug Palmer, National Cancer Institute (NCI), United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *03 December 2018*
Accepted: *30 January 2019*
Published: *20 February 2019*

Citation:
Dwyer CJ, Knochelmann HM, Smith AS, Wyatt MM, Rangel Rivera GO, Arhontoulis DC, Bartee E, Li Z, Rubinstein MP and Paulos CM (2019) Fueling Cancer Immunotherapy With Common Gamma Chain Cytokines. Front. Immunol. 10:263. doi: 10.3389/fimmu.2019.00263

INTRODUCTION

The field of cancer immunotherapy, encompassing vaccines, checkpoint modulators, and adoptive T cell transfer therapy (ACT), has improved treatment outcomes in patients by harnessing the immune system to target their malignancy, sometimes resulting in cures (1). ACT uses either tumor-infiltrating lymphocytes (TILs) already equipped with tumor-specificity or peripheral blood lymphocytes genetically redirected with tumor-specific T cell receptors (TCRs) or chimeric antigen receptors (CARs) (2). Two different groups in the 1980's first revealed that T cells could be successfully redirected with an antigen receptor. Kuwana and team engineered a CAR that combined the immunoglobulin variable regions with a TCR constant region and they reported specificity against phosphorylcholine-specific bacteria (3). Gross et al. then used a similar construct but made the transformants specific for TNP-expressing cancer cell lines. They demonstrated that these CAR T cells could secrete IL-2 and lyse tumor cells in an antigen-specific manner (4). In some instances, engineering cells with a CAR instead of a TCR can be advantageous. This advantage stems from the fact that CARs, similar to antibodies, are able to recognize free unmodified antigen

while TCRs require antigen modification and presentation by the major histocompatibility complex (MHC), which is often down-regulated on tumor cells (5). However, unlike TCRs, CAR antigen specificity is restricted to cell surface antigens.

Following the two initial studies, CAR designs have been further modified to enhance their antitumor properties and persistence. First-generation CARs use a single chain variable fragment (scFv) for antigen recognition and an intracellular signaling domain, CD3ζ or FcεRIγ (6). In recent years, the incorporation of one or more co-stimulatory domains (i.e., second and third generation CARs) was instrumental to the success of CAR T cell efficacy for patients in clinical trials. As reviewed by Knochelmann et al., donor lymphocytes have been further modified in many ways by (1) incorporating targets to multiple antigens, (2) converting suppressive signals such as TGF-β or IL-4 into activating signals, (3) overexpression of chemokine receptors to enhance migration, and (4) secreting cytokines or soluble factors to modulate donor TIL or CAR T cells and endogenous immune cell function to induce a proinflammatory or "hot" tumor microenvironment (7, 8).

The most notable recent successes with CAR T cell therapy have resulted from the use of second generation CD19-CAR T cells for B cell derived malignancies that incorporate CD28 or 4-1BB costimulatory domains. Administration of CD19-CAR T cells leads to near complete eradication of $CD19^+$ malignant and B cell lineage cells in patients with advanced lymphomas (9–14) and multiple forms of chemo-refractory or advanced leukemias (15–23). In many of these studies, CAR T cell therapy induced long term remissions in patients who had been heavily pre-treated with various ineffective therapies. Due to their unprecedented success in multiple patients in clinical trials, the second generation CD19-CAR containing 4-1BB-CD3ζ (Tisagenlecleucel) was FDA approved for patients with B cell acute lymphoblastic leukemia in 2017 and diffuse large B cell lymphoma in 2018 while the second generation CD19-CAR containing CD28-CD3ζ (Axicabtagene ciloleucel) was approved for diffuse large B cell lymphoma in 2017 (7). Indeed, these therapies have revolutionized treatment for many patients around the world suffering from advanced hematological malignancies.

Though CAR T cell therapy has demonstrated incredible success with certain hematologic cancers, challenges still remain today in using this therapy to successfully treat patients with solid tumors. There also remains challenges in managing treatment-associated toxicity. Toxicities associated with CAR T cell therapy can be numerous including (1) cytokine release syndrome (CRS) which is characterized by a fever induced by high serum levels of IL-6 and IFNγ (2) respiratory distress and (3) neurological symptoms (23–26). All of these toxic side effects can be lethal in individuals if left untreated (23–26). To manage these adverse events, patients are treated with drugs to block CRS such as IL-6 inhibition with tocilizumab (anti-IL-6R) or corticosteroids (23, 24). For the treatment of solid tumors, CAR T cell therapies have poor efficacy due to tumor mediated suppression by (1) inhibitory receptor engagement, (2) soluble factors, (3) recruitment of suppressive immune cells, (4) nutrient deprivation and (5) loss of tumor antigen (5, 27–30). As solid tumor-specific antigens are difficult to identify, patients can experience toxic side effects due to on-target off-tumor reactivity leading to autoimmune-like symptoms (26, 31–37). Consequently, investigators have more recently designed CAR T cell constructs containing an inducible suicide gene to rapidly eliminate CAR T cells from the patient with a pharmacological reagent. The hope is that this approach will theoretically reverse or reduce the onset of these adverse events (38–42).

Novel ways to improve the potency of CAR T cells in the tumor are desperately needed for patients that fail conventional chemotherapies or other forms of cancer immunotherapy. T cell function, survival, and proliferation are strongly influenced by cytokine signaling. Notably, the members of the common γ chain (γc) cytokine family play pivotal roles in fueling T cells to thrive, lyse tumors and drive long-lived memory to tumor relapse or metastasis. While IL-2 has been widely used to expand T cells *ex vivo* in preparation for infusion into patients, preclinical work reveals that other members of the γc cytokine family should be considered for clinical use. Consequently, this review will detail the basic biology of various γc cytokines, including IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 and discuss how each cytokine has have been used in cellular therapy. Lastly, we will discuss a subset of fourth generation CARs known as TRUCKs (T cell redirected for universal cytokine-mediated killing) in cancer immunotherapy and discuss our vantage of how to best augment their antitumor potency using γc cytokines *in vitro* and *in vivo* to safely improve treatment outcomes in patients with advanced blood or solid tumors.

OVERVIEW: COMMON γ CHAIN CYTOKINE SIGNALING AND FUNCTION IN T LYMPHOCYTE BIOLOGY

Common γ chain cytokines exert numerous functions on T lymphocyte survival, function and proliferation. As illustrated in **Figure 1**, the γc family consists of six members—IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21—which all have unique receptors. Upon receptor ligation, γc cytokines through JAK1 and JAK3 activate various developmental pathways including STAT1, STAT3, STAT5, MAPK, and PI3K/AKT pathways (43–55). The one exception is IL-4, which in addition to STAT5, MAPK and PI3K/AKT pathways, activates STAT6 signaling (56–62). Below, we will further discuss receptor composition and the biological functions exerted by each of these six γc cytokines.

IL-2

IL-2 is primarily produced by activated T cells upon TCR and costimulatory signaling (43). As displayed in **Figure 1**, the IL-2 receptor (IL-2R) is a trimeric receptor that consists of IL-2Rα, IL-2Rβ and the γc where signaling is ultimately mediated through IL-2Rβ and the γc (43, 44). High affinity IL-2Rs (αβγ) are expressed on activated T cells and constitutively expressed on T regulatory cells (Tregs) while the intermediate affinity IL-2R (βγ) is expressed on natural killer (NK) cells and memory $CD8^+$ T cells (43). IL-2 has non-redundant functions in both Treg and effector T cell biology. For Tregs, IL-2 is

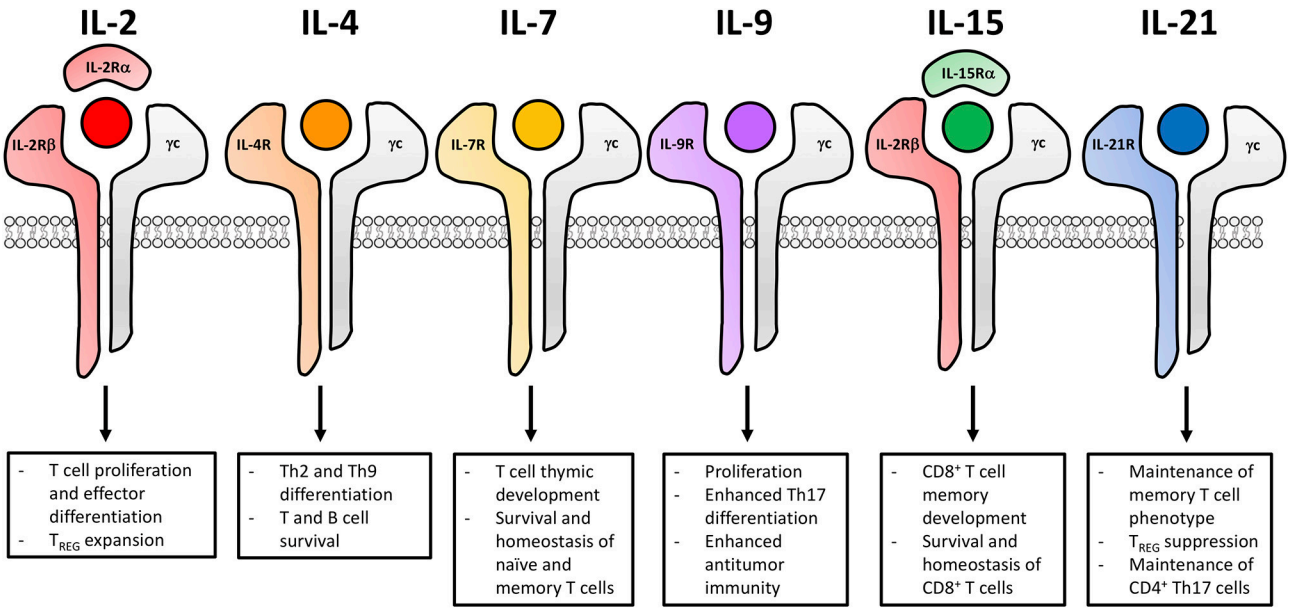

FIGURE 1 | Common γ chain cytokine signaling impacts the functional fate of T cells for adoptive cell transfer. The six members of the γc cytokine family (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21) and the composition of their unique cytokine receptors. Signaling cascades from these receptors lead to distinct biological outcomes impacting differentiation, effector function and memory development of T cells.

essential for thymic development, peripheral homeostasis, and suppressive function (63–74). In IL-2-, IL-2Rα-, and IL-2Rβ-deficient models, mice succumb to lethal autoimmunity within 8–12 weeks due to impaired thymic development of Tregs (63–66). Conversely, effector T cells readily develop in IL-2-, IL-2Rα-, and IL-2Rβ-deficient models. However, IL-2 is essential for the optimal proliferation and differentiation of effector T cells and this cytokine influences their contraction through activation induced cell death (AICD) (75). Additionally, IL-2 plays distinct roles in the function of various $CD4^+$ T helper (Th) subsets. The differentiation of Th1, Th2, Th9, and iTreg subsets is promoted by increased expression of IL-2 while Th17 and Tfh differentiation is suppressed by IL-2 (76–81). IL-2R signaling intensity influences the development, survival and recall response of T cell memory (82–85). Low IL-2R signaling favors the development of central memory T cells (Tcm) whereas high IL-2R signaling favors the development of effector memory T cells (Tem) and terminally-differentiated effector cells (86). IL-2 also influences effector development through the upregulation of IFNγ, perforin, granzyme B and Blimp-1, which drive terminal effector differentiation and suppresses expression of makers associated with memory (such as Bcl-6, CD127, and CD62L) (86–89).

IL-4

The cytokine IL-4 has long been appreciated to impact humoral immunity. IL-4 is primarily produced by $CD4^+$ T cells (specifically Th2 and Tfh cells), basophils, eosinophils, mast cells and NKT cells (90–98). Along with the γc receptor, IL-4 binds to IL-4Rα (**Figure 1**). Upon IL-4 receptor (IL-4R) signaling, cascades promote the up-regulation of IL-4Rα, which induces a positive feedback loop (62, 99, 100). IL-4 is required for the differentiation of naïve $CD4^+$ T cells to a Th2 phenotype. This cytokine also induces and immunoglobulin class switching in B cells, promotes the survival of T and B cells and drives long-term development of $CD8^+$ T cell memory (101). Humoral immunity is dependent on IL-4, as IL-4-, or IL-4R-deficient mouse models have impaired antibody production, high susceptibility to parasitic infection and diminished Th2 differentiation (101). IL-4 is thought to be controversial for cancer therapy because multiple forms of cancer express the IL-4R. Increased IL-4R expression has been observed in renal cell carcinoma, melanoma, breast, glioblastoma, lung, prostate, bladder and head neck cancers (102–107). Angiogenesis of human breast tumor cells has been shown to be inhibited by the addition of IL-4, preventing metastatic growth and proliferation (108, 109). However, since both adipose tissue and cancer cells secrete IL-4 to promote a suppressive tumor microenvironment, blocking IL-4R signaling was found to decrease the viability of breast tumor cells (110). Finally, recent data has emerged that, along with TGF-β, IL-4 can support the generation of a new subset called Th9 cells. These cells secrete IL-9 and have been reported to augment immunity to tumors in ACT models (111, 112). Indeed, future investigations are required to better understand the role of IL-4 in regulating Th2 and Th9 cells in adoptive immunotherapy for cancer.

IL-7

In contrast to IL-2, cytokine IL-7 is not produced by hematopoietic cells but rather is secreted by stromal cells (113–115). Its receptor consists of the γc and a unique IL-7Rα (**Figure 1**) (113). The fundamental role of IL-7 has been demonstrated in both humans and in mice with

deficiency in either IL-7 or the IL-7 receptor (IL-7R) resulting in impaired thymic development of mature lymphocytes resembling severe combined immune deficiency (116–118). Moreover, IL-7 supports the survival and homeostasis of naïve and memory T cells (119–124). Upon activation and IL-7R signaling, the IL-7R is down-regulated on naïve T cells. Interestingly, IL-7R is re-expressed on Tcm and Tem cells (125–128). It is important that cells express IL-7R as IL-7 signaling promotes the homeostasis and survival of naïve and memory T cells via the up-regulation of Bcl-2 and the suppression of pro-apoptotic mediators (49, 129–131). Unlike IL-2, IL-7 does not induce Treg proliferation, as IL-7Rα is expressed at low levels on this suppressive lymphocyte population (132, 133). Due to the deleterious role of Tregs in cancer immunotherapy, many investigators are now exploring the role of IL-7 in potentiating checkpoint modulators or T cell therapies, as discussed in greater detail later in this review.

IL-9

IL-9 was initially described as a T cell growth factor. However, IL-9 is more recently appreciated for its role in the proliferation and differentiation of mast cells as well as its involvement in B cell maturation (134–137). IL-9 is primarily produced by various $CD4^+$ T cell subsets (naïve, Th2, Th9, Th17, and Tregs) but can also be made by mast cells, NKT cells and type 2 innate lymphoid cells (ILC2) (112, 138–145). As shown in **Figure 1**, IL-9 signals through the γc and IL-9Rα which is expressed on activated T cells, mast cells and macrophages (52, 146). In IL-9- and IL-9 receptor (IL-9R)-deficient mice, there was no effect on T cell differentiation or development, but these mice had diminished mast cell proliferation (147). Additional investigations revealed that experimental autoimmune encephalomyelitis was markedly reduced in IL-9R-deficient mice compared to wild-type cohorts, as $CD4^+$ T cells and macrophages from these mice secreted less IL-17 and IL-6, respectively (148). Importantly, IL-9 also plays roles in regulating transplant tolerance, promoting anti-parasitic immunity, exacerbating allergy and autoimmunity (149). The role of IL-9 in tumor immunity has been controversial, both promoting antitumor immunity and enhancing transformation and tumor growth. It has been reported that IL-9 overexpression promotes cell proliferation, metastasis and survival of pancreatic cancer and lymphomas (150–152). However, the adoptive transfer of antitumor Th9 or Tc9 cells regress melanoma in mice through IL-9-dependent mechanisms and are highly cytolytic, hyperproliferative, and persistent post transfer into animals (153–157).

IL-15

As depicted in **Figure 1**, IL-2 and IL-15 share common receptor subunits, IL-2Rβ and the γc, only differing between the unique α subunits (53). IL-15Rα is expressed on activated monocytes and dendritic cells and because of IL-15Rα's high affinity for IL-15, IL-15 can be trans-presented to IL-2Rβ and the γc on NK and $CD8^+$ T cells, unlike IL-2 which is primarily cis-presented (53, 158). Also, in contrast to IL-2, IL-15 is primarily produced by innate immune cells (including dendritic cells, macrophages and monocytes) (159–162). IL-15 and IL-15R signaling are important for the development and homeostasis of NK cells and $CD8^+$ T cells though the up-regulation of anti-apoptotic markers Mcl-1 and Bcl-2 while inhibiting AICD (163–174). This discovery became clear in studies using IL-15- and IL-15R-deficient mouse models, which have impaired NK cell and $CD8^+$ T memory cell development and compromised lymph node homeostasis (164, 175). IL-15, unlike IL-2, preferentially expands $CD8^+$ T cell memory and NK cells in the presence of Treg cells while promoting resistance to Treg suppression (176, 177).

IL-21

IL-21 has been reported to improve antitumor T cell immunity but has also been identified as a potent mediator of autoimmunity (178). IL-21 is primarily produced by activated $CD4^+$ T cells, particularly Th17 and Tfh but can also be produced by NKT cells (179–181). As shown in **Figure 1**, the IL-21 receptor (IL-21R) is comprised of the γc and IL-21Rα (182, 183). Receptor expression is low on resting T cells but is upregulated upon TCR activation or IL-21 stimulation (183–185). Both adaptive and innate immune cells are influenced by IL-21 as T, B, NKs, macrophages and DCs all express the IL-21R (179, 181, 183, 184, 186). IL-21 promotes the proliferation, survival and differentiation of Th17 and Tfh subsets while enhancing the function of cytotoxic $CD8^+$ T cells (187–197). Additionally, IL-21 blunts Treg expansion by suppressing Foxp3 expression and favors the enrichment of antigen-stimulated $CD8^+$ T cells (198). Th17 and Th2 immune responses are impaired while Tregs are increased in IL-21- and IL-21R-deficient mice (190, 191, 199, 200).

Collectively, γc cytokines play a major role influencing the development, differentiation, and survival of innate and adaptive immune cells. For cancer treatment, γc cytokines have been used systemically as monotherapies to harness endogenous immune responses, or in combination with ACT to improve antitumor efficacy. The presence of γc cytokines at various points in the T cell development including priming, *ex vivo* expansion, or post adoptive transfer can influence the function of tumor-specific T cells. As both IL-4 and IL-9 have not been thoroughly explored for ACT and have controversial roles in both promoting tumorigenesis and mediating antitumor immunity, we will focus the rest of our discussion on the clinical uses of IL-2, IL-7, IL-15, and IL-21 for immunotherapy, and their potential to improve patient responses to T-cell based therapies.

CLINICAL USES OF IL-2, IL-7, IL-15, AND IL-21 IN CANCER IMMUNOTHERAPY

Interleukin-2: T Cell Proliferation at the Cost of Treg Expansion

Currently, IL-2 is the only γc cytokine to be FDA-approved to treat patients with cancer. In anti-cancer therapies, this cytokine is commonly administered to patients to augment the engraftment and function of adoptively transferred T cells. For treatment of several autoimmune disorders such as type 1 diabetes, HCV-induced vasculitis and graft vs. host disease (GVHD), IL-2 is administered at low doses and has been beneficial for patients because it targets the constitutive

expression of the high affinity IL-2R leading to selective proliferation of Tregs (201–204). Conversely, effector T cells do not readily express the high affinity IL-2R. High dose IL-2 is administered to cancer patients to support the proliferation and function of cytotoxic T lymphocytes (CTLs) (205, 206). In fact, since the 1980s high dose IL-2 has been used to treat patients with renal cell carcinoma and metastatic melanoma (207–210). Standard treatment protocols involve the administration of 720,000 IU IL-2/kg every 8 h for up to 14 consecutive doses. Using high-dose IL-2 for patients with renal cell carcinoma, 14% of patients (255 patients total) had an objective response, while 12 patients experienced a complete response (209). Similar efficacy was observed with high-dose IL-2 treatment for metastatic melanoma, where 16% of patients (270 patients total) had an objective response with 17 patients having a complete response and 26 patients experiencing a partial response (210). High dose IL-2 treatment was FDA-approved for renal cell carcinoma in 1992 and for metastatic melanoma in 1998 (211, 212). However, due to toxicities associated with this therapy such as hypotension, capillary leak syndrome, cardiac toxicity, and renal failure, many cancer centers stopped using this therapy to treat patients (213–215). Today, IL-2 is mainly used to expand TILs or CARs *ex vivo* for ACT and is administered to the patient to support donor cell expansion post-transfer.

As IL-2 promotes the differentiation of naive $CD8^+$ T cells to full effectors and generates Tregs in the ACT products (**Figure 2**), immunologists have focused on preferentially targeting IL-2 to effector T cells. One promising way to target IL-2 to effectors has been by complexing this cytokine with anti-IL-2 antibodies. This IL-2 complex uniquely presents IL-2 to the intermediate but not high affinity IL-2Rs thereby reducing Treg expansion (216–219). The importance of targeting IL-2 to transferred T cells has also shown promise in the field of cancer immunotherapy. For example, Rubinstein and colleagues discovered that IL-2Rα on transferred T cells sustained signaling by promoting recycling of endocytosed IL-2 back to the cell surface (220). This recycling mechanism raised the possibility of engineering TILs or CARs to express IL-2Rα to improve IL-2-based therapies (220). Furthermore, other groups have recently discovered novel ways to specifically target transferred T cells with IL-2. In fact, Sockolosky et al. engineered a synthetic IL-2 and IL-2R (distinct from native IL-2 and the IL-2R) and expressed them on transferred T cells. The synthetic IL-2R did not interact with native IL-2, could mediate IL-2R signaling, thereby leading to the selective proliferation of CTLs and regression of melanoma in mice (221).

TIL therapies require expansion of ample numbers of lymphocytes from the suppressive tumor microenvironment. *Ex vivo*, patient tumor samples are treated with high dose IL-2 to preferentially expand TIL. These TIL are then rapidly expanded in the presence of anti-CD3, IL-2 (6000IU/mL) and irradiated feeder cells for several weeks in order to propagate them to the billions (222). After expansion, TIL are infused into the patient who has been preconditioned with a non-myeloablative preparative regimen (2, 212, 223). Upon transfer, IL-2 is administered to patients to promote the expansion of donor TILs *in vivo* because these cells have increased IL-2Rα as expression is positively regulated by TCR and IL-2R signaling (43). In a preclinical model using Epstein Barr Virus positive tumors, EBV-specific CTLs were engineered to express IL-2 or IL-15. Transgenic expression of IL-2 or IL-15 increased T cell expansion *in vitro* and *in vivo* ultimately enhancing their *in vivo* efficacy (224). Treating melanoma patients with *ex vivo* expanded TIL and high dose IL-2 (720,000 IU/kg) led to complete remission in 20 of 93 patients and some patients experienced long-term remission (225). Transducing melanoma TIL to continually secrete IL-2 bypassed the need for exogenous administration of IL-2 to the patient. These modified cells survived in the patient but surprisingly did not improve clinical outcomes compared to TIL administered with exogenous IL-2 (226, 227).

Similar to TIL therapies, IL-2 promotes the proliferation of CAR T cells. Yet this cytokine also drives their differentiation into terminal effector phenotypes. In pancreatic ductal adenocarcinoma xenograft models, treatment of mesothelin-specific CAR T cells with a TNFα and IL-2-secreating adenovirus increased their activation, proliferation and antitumor response in mice (228). IL-2 also increases resistance of CD28-CD3ζ CARs *in vitro* to TGFβ-mediated suppression compared to 4-1BB-CD3ζ CARs. CD28 costimulation activates Lck, which promotes IL-2 production and if Lck is nonfunctional, CAR T cells have impaired antitumor activity (229). It has also been reported that CAR T cells expanded with IL-2 (100 IU/mL) for 3 days, compared to 10 days, generated lymphocytes with an increased proportion of "younger" memory-like cells (230, 231). With longer culture time and increased differentiation, CARs mediated slightly reduced anti-leukemia immunity in mice (230). Ablation of IL-2Rα on CAR T cells did not improve their function but did decrease their expansion capabilities *in vitro* (230). While IL-2 promotes the differentiation of naïve T cells to an effector phenotype, IL-2Rβ signaling has been clearly shown to improve the function of CAR T cells. In a recent study conducted by Kagoya et al. CAR T cells were engineered to express a truncated IL-2Rβ domain (232). This truncated domain increased STAT3 and STAT5 signaling and improved their expansion *in vivo*. When these cells were transferred into mice bearing leukemia or melanoma, they had improved survival and regressed hematological and solid tumors more effectively compared to their traditional CAR cohorts (232). To circumvent the negative attributes of IL-2, investigators have also been turning their focus to other γc cytokines including IL-7, IL-15, or IL-21 which may prove to be better candidates to improve methodology for ACT therapy.

Interleukin-7: Naïve and Memory Cell Proliferation Without Treg Expansion

Similar to IL-2, IL-7 promotes the proliferation of naïve and memory T cells. Thus, IL-7 is a promising cytokine for cancer immunotherapy. The benefit of IL-7 for ACT was first shown in preclinical models treating CTL's *in vitro* with either IL-7 or IL-2. When transferred into mice, IL-7-treated CTLs controlled metastatic disease to the same extent as those treated with IL-2 (233). In clinical trials using recombinant human IL-7 (rhIL-7) as a monotherapy, IL-7 was shown to be well tolerated

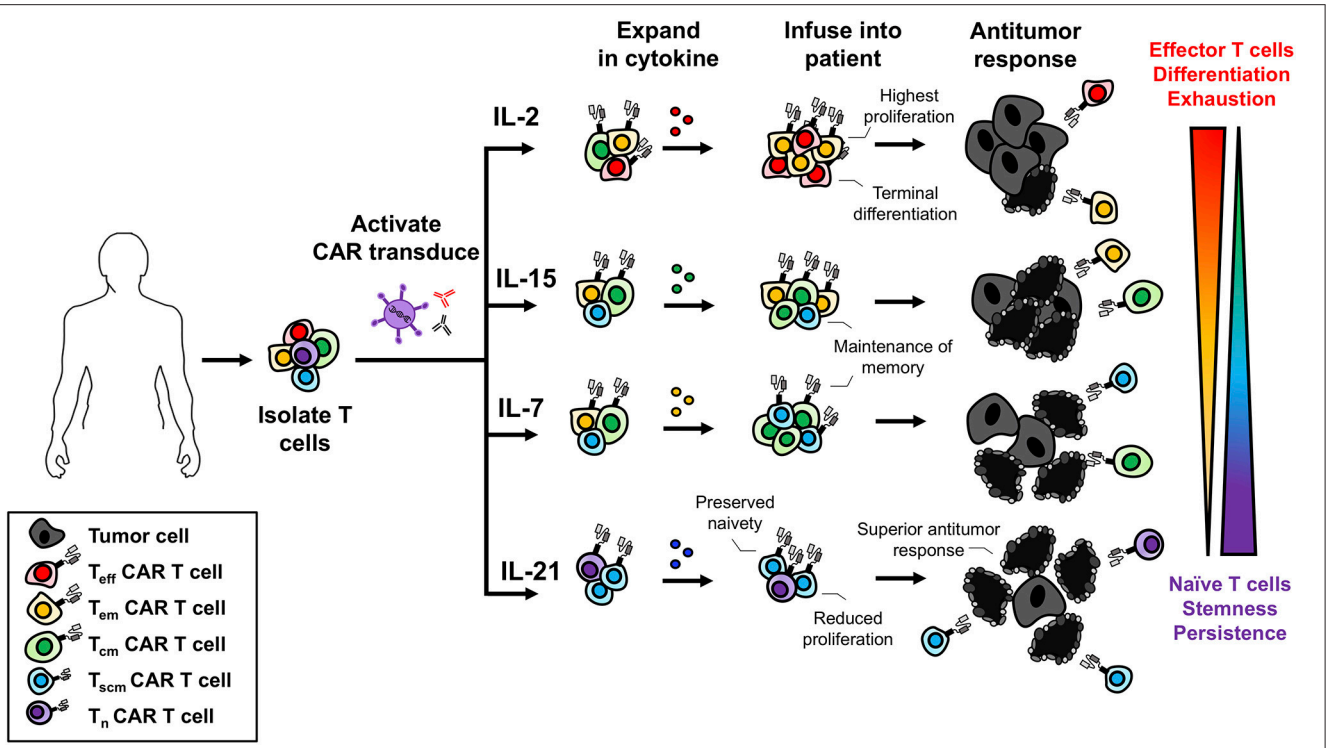

FIGURE 2 | Use of γc cytokines for *ex vivo* T cell expansion generates T cells with variable memory phenotypes. γc cytokines promote different biological programs that influence the differentiation of T cells. While IL-2 promotes robust proliferation it also promotes terminal effector differentiation. IL-7 and IL-15 maintain the homeostasis and survival of memory T cells and treatment *ex vivo* promotes a Tscm/Tcm phenotype. IL-21 slows T cell expansion but prevents differentiation and maintains a naïve-like T cell phenotype. With respect to antitumor immunity, less differentiated cell products are more therapeutic leading to the understanding that IL-2 is not the best option for *ex vivo* expansion.

by patients with advanced malignancies (234, 235). Rosenberg and colleagues treated a cohort of 12 patients (11 metastatic melanoma and 1 metastatic sarcoma) with 8 doses of IL-7 and found dose-dependent increases in $CD4^+$ and $CD8^+$ T cells with a decrease in Tregs (234). Following this work, Sportes and colleagues conducted an IL-7 dose-escalation study on 16 patients with non-hematologic non-lymphoid cancers and found similar results with increased $CD8^+$ T cells and decreased Tregs (235). TCR-repertoire analysis of T cells indicated a more diverse repertoire, signifying IL-7's role in promoting a broader immune response and the selective expansion of naïve T cells (235). Culturing naïve T cells from healthy donors with IL-7, expands T stem cell memory (Tscm) cells to a greater extent than IL-2 treatment (236). These Tscm were defined as $CD62L^+$ $CCR7^+$ $CD45RA^+$ $CD45RO^+$ $IL\text{-}7R\alpha^+$ $CD95^+$ and were shown to have increased expansion as well as a high capacity for self-renewal (236). IL-7 preferentially expanded naïve T cells to a Tscm phenotype compared to Tcm and Tem, likely because naïve cells express more IL-7R, as portrayed in **Figure 2**. As Tscm have been reported by several groups to mediate potent memory responses to tumors, it has become increasingly clear that IL-7 has promise in the clinical setting (237–240).

IL-7 has been used in CAR T cell therapy, often in combination with other cytokines, during the *in vitro* expansion phase. For example, CAR T cells expanded in the presence of IL-7, IL-4 and IL-21 were found to express less inhibitory receptors compared to IL-2-expanded cells. These cells also had an increased Tscm/Tcm phenotype and co-expressed CD27 and CD28 (241). In the presence of IL-7 and IL-15, CAR T cells possess a naïve/Tscm phenotype with improved proliferation upon antigenic-rechallenge compared to IL-2-treated CARs (242, 243). IL-7/IL-15 expanded CAR T cells have increased *in vivo* persistence, leading to improved antitumor immunity (244). MUC-1-specific CAR T cells have been engineered with a switch receptor containing an IL-4 ectodomain and an IL-7 endodomain to counter the IL-4-rich tumor microenvironment (245). By converting an IL-4 signal into an IL-7 signal, these T cells expanded robustly and mediated potent antitumor immunity in mice bearing breast tumors (245). Anti-CD20 CAR T cells engineered to express CCL19 and IL-7 migrate and expand to a greater extent than conventional CARs and led to complete remission of mastocytoma and Lewis lung carcinoma in mice (246). Additionally, IL-7 was critical to this response, as anti-IL-7Rα administration diminished the therapeutic benefit of these cells (246). Shum and colleagues engineered a GD-2-specific CAR T cell with constitutive IL-7R signaling, CD34 ectodomain and an IL-7Rα endodomain, leading to constitutive STAT5 activation (247). These CARs were able to undergo multiple rounds of expansion and mediate a robust response against glioblastoma and metastatic neuroblastoma tumors (247). These

CAR constructs highlight the beneficial role of IL-7 and IL-7R signaling in improving the antitumor functions of T cells for ACT.

Interleukin-15: CD8$^+$ Memory T Cell Expansion With Some NK Cell Assistance

IL-15R signaling is promising for T cell-based cancer immunotherapies. This pathway selectively induces the expansion and function of CD8$^+$ memory and NK cells (163–168). For ACT, IL-15 has been used to enhance the activity of TIL and CAR T cells *ex vivo*. Moreover, IL-15 has also been complexed with IL-15Rα and this novel agent has been used as an immunotherapy in cancer patients *in vivo* (248). When cultured *in vitro*, T cells expanded with IL-15, rather than IL-2, are predominately a Tcm phenotype with very few Tem (249, 250). Conversely, IL-2-expanded cells are mostly effectors (**Figure 2**) (249). In turn, IL-15 generates a cellular product that mediates improved antitumor immunity, as IL-15-propogated Tcm have an improved engraftment potential and migratory capacity compared to IL-2-expanded cells (250, 251). Administration of recombinant IL-15 can promote immunity through the expansion of endogenous CD8$^+$ T cells and NK cells (252, 253). In combination with checkpoint inhibition, IL-15 improved CD8$^+$ T cell function marked by increased IFNγ production and mice treated with the combination had improved control of metastatic disease (253). Additionally, IL-15 is able to reverse tumor-tolerant CD8$^+$ T cells, when IL-2 and IL-7 were unable to, restoring antigen responsiveness and leading to tumor clearance (254–256). In the first clinical trial using recombinant human IL-15 (rhIL-15) to treat 18 patients with metastatic cancer (11 metastatic melanoma and 7 renal cell carcinoma), rhIL-15 was administered intravenously in a dose escalating study for 12 consecutive days (257). From the 18 patients treated, there was only stable or progressive disease. The dosing regimen led to elevated serum levels of IL-6 and IFNγ along with grade 3 toxicities such as hypotension, lymphopenia and elevated aspartate and alanine aminotransferases at higher doses. However, minutes after administration of rhIL-15, NK, γδ, and CD8$^+$ T cells effluxed from the blood and proliferated robustly for many days after administration (257). This study implicates the promising role of IL-15 to selectively target the homeostasis and expansion of NK and CD8$^+$ T cells.

Complexing IL-15 with IL-15Rα drastically increases the half-life of this cytokine, maximizing its activity while preferentially presenting IL-15 to cells expressing IL-2Rβ and γc (258, 259). For example, ALT-803, an IL-15/IL-15Rα sushi domain complex, mediated improved therapeutic benefit over native IL-15 (260, 261). Administration of ALT-803 in mice led to selective expansion of NK and CD8$^+$ T cells with no expansion of Tregs, increased production of IFNγ, TNFα, and IL-10, and reduced metastasis of breast carcinoma, colon carcinomas, and myeloma in mice (260, 261). Therapeutic benefit was mediated by CD8$^+$ T cells as their depletion diminished antitumor immunity (260–262). In a phase 1b clinical trial conducted by Wrangle et al. ALT-803 was administered with nivolumab (anti-PD-1) to 21 patients with metastatic non-small cell lung carcinoma (263). ALT-803 could be safely administered to these patients in combination nivolumab. In fact, there were no dose limiting toxicities experienced by patients on this trial (263). Moreover, this therapy dramatically increased the proliferation of NK and CD8$^+$ T cells in the blood (263). Although this study was not designed to assess efficacy, the authors reported evidence of the re-induction of antitumor responses in patients who failed to respond to nivolumab therapy alone (263). This study emphasizes the promise of using IL-15/IL-15Rα complex in cancer therapy and also implies that ALT-803 may improve the antitumor activity of TIL or CAR therapies in patients without increased toxic side effects.

In a clinical trial treating 22 patients with advanced stage lymphoma, patients with positive tumor responses and complete remissions had increased IL-15 serum levels (12). Investigators have engineered IL-15-producing CARs to enhance T cell memory development and incorporate NK cell responses for tumor clearance *in vivo*. Anti-leukemia CAR T cells that express IL-15 have increased expansion, viability, and improved antitumor immunity compared to conventional CAR T cells in lymphoma xenograph models (264). In glioma xenograph models, IL-13Rα2-specific CAR T cells that secrete IL-15 showed increased proliferation, sustained cytokine production and improved survival (265). In this model, tumor relapse was observed due to the expansion of tumor cells that had lost expression of the target antigen. However, in some instances retroviral transduction of IL-15 can transform human primary T cells leading to prolonged cell survival, increased telomerase activity and resistance to apoptosis (266). Membrane-bound IL-15 on CAR T cells mediated similar results, as demonstrated by their increased persistence and immunity against leukemia (238). Thus, IL-15 bolsters NK and CD8$^+$ T cell expansion and function, which leads to improved immunity, implicating IL-15 as a beneficial cytokine for ACT (237). In the future, it will be paramount to understand the best way to deliver IL-15 therapy in combination with ACT and CAR T cell therapy.

Interleukin-21: Preventing T Cell Differentiation to Increase Antitumor Immunity

In a phase 1 clinical trial using recombinant human IL-21 (rhIL-21) in a dose-escalation study with 43 patients (24 melanoma and 19 renal cell carcinoma patients), rhIL-21 was administered consecutively for 5 days for two full cycles. rhIL-21 was safe for patients and mediated antitumor immunity in some individuals, as demonstrated by 1 complete response and 4 partial responses (267). To follow this trial, Davis et al. conducted a phase IIa clinical trial treating 24 patients with metastatic melanoma with 30 μg/kg doses of IL-21 (268). Treatment with IL-21 led to 1 complete response and 1 partial response in this study. Additionally, IL-21 lead to the selective activation of NK and CD8$^+$ T cells marked by increased expression of CD25, IFNγ, perforin and granzyme B (268). In a phase II trial with 40 metastatic melanoma patients, most of which had metastasis to the lungs, liver or lymph nodes, were treated with either 30 μg/kg or 50 μg/kg of IL-21 (269). Nine patients experienced partial

responses where 16 patients had stable disease. There were 6 patients who experienced some dose-limiting toxicities amongst the treatment groups (267). Collectively, these trials indicate the benefit of IL-21 as a monotherapy and warrant the investigation combining IL-21 with other agents for cancer therapy.

IL-21 augments ACT therapy by preserving T cells in a less differentiated state *ex vivo* (88, 237, 250, 270, 271) (see **Figure 2**). While IL-2 drives robust proliferation and differentiation of $CD8^+$ T cells, IL-21 enriches $CD8^+$ T cells with a "younger" phenotype that express less IL-2Rα, CD44, and Eomes but have reduced expansion compared to those expanded with IL-2 (88). However, when IL-21-stimulated $CD8^+$ T cells were transferred into mice, they mediated superior anti-melanoma immunity compared to T cells treated *in vitro* with IL-2 or IL-15 (88). Additional investigation revealed that IL-21 supported the propagation of lymphocytes that expressed CD62L and secreted IL-2, consistent with Tscm phenotype. Moreover, these cells expressed Tcf1 and Lef7, which are transcription factors critical for the self-renewal of stem cells (88, 272, 273). The benefits of IL-21 have been demonstrated on TILs isolated from ovarian or non-small cell lung carcinoma patients. While IL-2 greatly bolsters TIL expansion, IL-21 is unable to expand TIL alone (274). Importantly, IL-21 does not support the expansion of Treg cells in contrast to IL-2 (274). For human $CD8^+$ T cells isolated from the peripheral blood of healthy donors, IL-21 promotes Tscm development *in vitro* leading to improved immunity upon adoptive transfer into mice with melanoma compared to IL-2-stimulated $CD8^+$ T cells (275). In addition to using IL-21 for treatment of $CD8^+$ T cells *ex vivo*, IL-21 is a potent agent for the expansion of NK cells. Using membrane-bound IL-21 on artificial antigen-presenting cells, NK cells can be expanded to large numbers to elicit graft vs. leukemia responses without inducing GVHD (276, 277). These expanded NK cells had increased cytotoxicity and cytokine production without exhaustion. When combining membrane-bound IL-21 expansion with IL-18, IL-15, and IL-12, NK cells had increased expression of IFNγ and TNFα. These results indicate IL-21 as a potent agent for improving efficacy of T and NK cells for ACT (278).

IL-2 and IL-21 regulate opposite immune programs (88, 279). However, IL-21 is able to synergize with IL-7 and IL-15. For example, IL-15 and IL-21 synergistically promote the expansion $CD8^+$ T cells with a Tscm phenotype and have increased persistence when infused into the host (196). Also, cells stimulated with IL-15 and IL-21 mediated enhanced immunity in mice with melanoma compared to T cells expanded in the presence of either IL-15 or IL-21 alone (196). Together these cytokines increase the effector molecules and cytokines produced by T cells *in vitro* (280, 281). Likewise, combining IL-7 and IL-21 promotes the expansion of cells with a Tscm phenotype with high CD28 and CD27 expression (241). The synergy between these two cytokines may be due to IL-21 augmenting IL-7-induced expansion of T cells and by preventing the down regulation of IL-7Rα, all of which lead to increased immunity *in vivo* (282). IL-21/IL-7-treated cells have increased proliferation and production of inflammatory cytokines, directing improved lysis of tumor cells (282). These data support the use of cytokines in combination in next generation clinical trials for patients.

As IL-21 prevents T cell differentiation and preserves their naïve-like phenotype, investigators have used this cytokine to generate "younger" CAR T cells. Interestingly, culturing CD19 CD28-CD3ζ CARs with IL-21 led to CAR^+ T cell expansion and increased expression of IFNγ and granzyme B (283). Compared to IL-2-treated CARs, IL-21-treated CARs had increased expression of CD45RA, CD62L, CCR7, and CD28. When transferred into mice with leukemia, IL-21-treated CARs had improved tumor control compared to those treated with IL-2 *ex vivo* (283). Moreover, membrane-bound IL-21 on CAR T cells recapitulated the effects of soluble IL-21 in culture (283). To improve the activity of CAR T cells, Sabatino et al. isolated naïve $CD8^+$ T cells ($CD62L^+$ $CD45RA^+$ $CCR7^+$) from healthy donors and transduced them with CD19 CD28-CD3ζ CAR constructs. During expansion, cells were cultured in IL-7, IL-21 and TWS119 (glycogen synthase kinase 3β inhibitor), which enriched for Tscm (284). CD19-CAR Tscm had no changes in their transcriptome compared to non-transfected Tscm and were polyfunctional (284). When transferred into mice with leukemia, CD19-CAR Tscm cells were maintained with intraperitoneal injections of IL-15 and displayed improved survival over conventional CD19-CAR T cells (284). This study demonstrates that *in vitro* cooperation of IL-7, IL-21, and TWS119 and the *in vivo* functions of IL-15, lead to improved T cell functionally improving therapeutic outcome *in vivo*.

TRUCKs: Putting Cytokines to Work in the Tumor Microenvironment

Engineering CAR T cells with inducible or constitutive cytokine secretion reinforces transferred T cell function in the host and manipulates the endogenous immune response within the tumor. T cells redirected for universal cytokine-mediated killing, termed TRUCKs, are such CAR T cells equipped with the expression of IL-2, IL-7, IL-15, or IL-21 (285, 286). In a study conducted by Markley and Sadelain, human CD19-CAR T cells were engineered to constitutively express either IL-2, IL-7, IL-15, or IL-21 (285). Using lymphoma model, constitutive expression of the γc cytokines improved antitumor immunity and animal survival. Even though IL-2- and IL-15-expressing TRUCKs led to the upregulation of effector molecules such as granzyme A, TNFα and IFNγ, TRUCKs that produced IL-7 or IL-21 were most efficacious (285). IL-21-expressing TRUCKs mediated the best overall tumor immunity in mice, demonstrated by their capacity to increase survival. These TRUCKs were found to co-express CD27 and CD28 and were able to persist long-term in the animals (285). IL-7-expressing TRUCKs mediated improved antitumor immunity, while upregulating Bcl-2 expression and promoting improved cell expansion *in vitro* compared to IL-2-expressing TRUCKs. This preclinical study suggests that IL-7 or IL-21 TRUCKs could be efficacious in patients.

Other research has revealed that cytokines not in the γc cytokine family, such as IL-12 and IL-18, could be efficacious in TRUCK constructs. For example, in ovarian carcinoma xenograft models, $MUC\text{-}16^{ecto}$-specific second-generation CAR T cells were engineered to secrete IL-12 which led to

improved expansion of TRUCKs and a 27-fold increase in IFNγ production compared to non-IL-12-secreting constructs (287). MUC-16^{ecto}-specific IL-12-secreting TRUCKs had enhanced immunity compared to non-IL-12-secreting CARs in mice with ovarian cancer, leading to near complete survival (287). IL-18-secreting TRUCKs have similar benefits to IL-12-secreting TRUCKs with enhanced immunity and increased proliferation in both mice and humans (288). However, IL-18 secretion had preferential effects on CD4$^+$ TRUCKs and was able to promote significant T cell expansion without costimulatory signaling. IL-18-secretion expanded both CAR$^+$ and CAR$^-$ T cells in an antigen-independent manner which could be beneficial in cases of epitope spreading but detrimental for autoimmune manifestations (288). As both IL-12 and IL-18 promote immunity and expansion of TRUCKs, these cytokines could be potential candidates to improve therapy for solid tumors. However, as IL-12 and IL-18 upregulate the expression of several inflammatory cytokines, such as IFNγ, and have a historic reputation of toxic side effects, administration could further exacerbate CRS already associated with CAR T cell therapy (289–292). Because of these toxic side effects both preclinically and in patients, the γc cytokines, IL-7, Il-15 and IL-21 might prove to be better options for TRUCK therapies. These cytokines have well documented roles in improving cell products for ACT and as shown by Markley and Sadelain, can improve antitumor immunity of TRUCKs. Additionally, IL-7, IL-15, and IL-21 reinforce the essential T cell functions of proliferation, effector function and memory warranting further investigation. Overall, it is clear that further work must be done to investigate whether the γc cytokine-secreting TRUCKs would be beneficial to overcome the suppressive tumor microenvironment. Findings from future work will be instrumental to apply this therapy to patients with solid tumors, as these constructs have been preclinically shown to be efficacious for blood cancers.

Conclusion: Ideal Use for γc Cytokines in TIL and CAR T Cell Therapy

In summary, we have discussed how the various γc cytokines play fundamental roles in shaping T lymphocyte biology. We have also highlighted important preclinical work that reveals their potential for immunotherapy via several modalities: (1) infusion as monotherapies or in combination with adoptive T cell transfer therapy, (2) *ex vivo* expansion of TILs and CAR T cells to generate "younger" more agile cell products, and (3) *in vivo* constitutive or inducible production by genetically engineered T cells (TRUCKs) to bolster not only the transferred cells but to enhance immune cells in the oppressive tumor microenvironment. While exploration of TRUCKs has been largely preclinical to date, promising results indicate high potential for successful future clinical translation. Though IL-2 is the only currently FDA-approved γc cytokine, it is possible that this cytokine alone may not be ideal for future trials. As depicted in **Figure 3**, we envision the ideal application of the γc cytokines for T cell therapy to involve a combinatorial approach. Based on preclinical work, perhaps the ideal way to

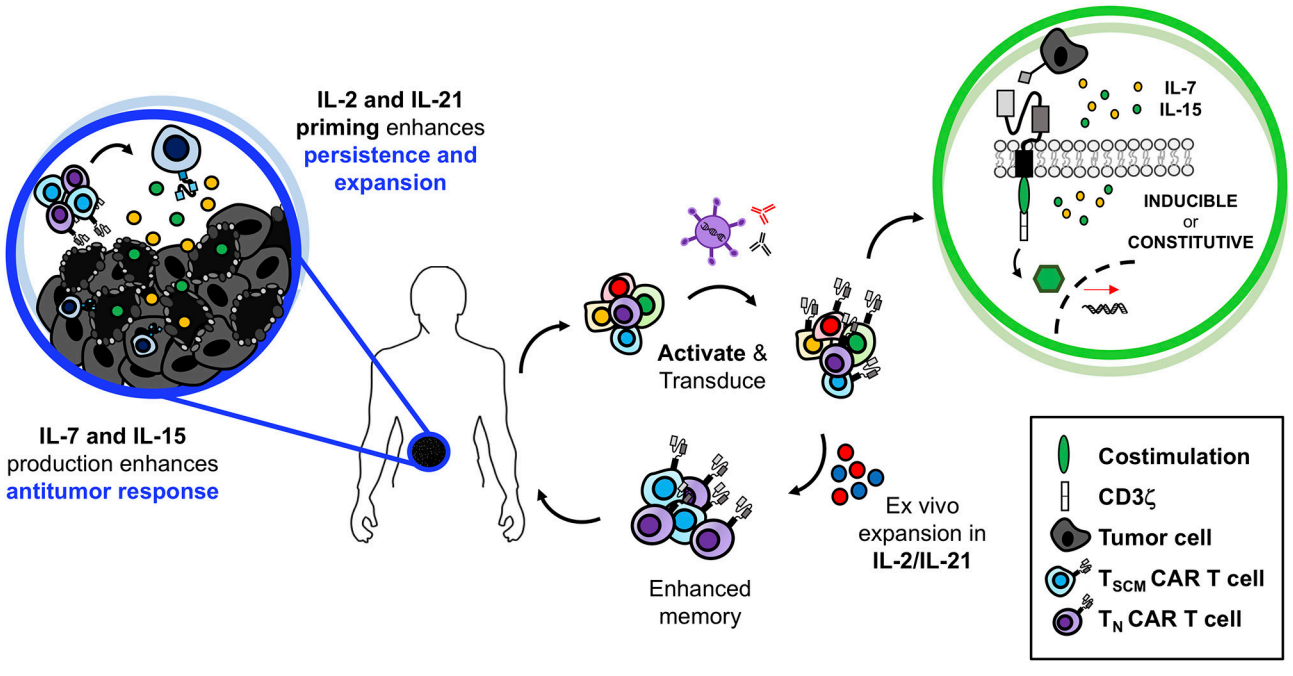

FIGURE 3 | Superior antitumor immunity of IL-2/IL-21-primed CAR T cells producing IL-7 and IL-15 at the tumor site. Generating TRUCKs *ex vivo* in the presence of IL-2 and IL-21 would prevent terminal differentiation, promote enhanced antitumor immunity with robust T cell proliferation. While at the tumor site, secretion of IL-7 and IL-15 would maintain TRUCK proliferation and memory function allowing for robust and persistent antitumor immunity against solid tumors.

expand T cells *ex vivo* may require the presence of both IL-21 and IL-2. As published by Hinrichs and team in murine T cells, we propose that IL-21 will effectively prevent the terminal differentiation of T cells while preserving a "younger" phenotype whereas IL-2 will support their expansion to large enough numbers to effectively treat patients (88). Upon administration, we suspect that these IL-21/IL-2-expanded TILs or TRUCKs would be best maintained by engineering them to secrete IL-7 and IL-15, which we hypothesize will further promote their persistence and memory recall responses to prevent tumor relapse in patients. The concept portrayed in **Figure 3** is just one of many possible ways to combine γc cytokines to bolster T cell-based therapies. We certainly realize that it is also possible that other cytokine combinations will be important in generating T cells with long-lived responses to aggressive tumors. Future studies are also necessary to turn off inhibitory signals (such as TGFβ and IL-10) that dampen T cell responsiveness (293, 294). Regardless, it has become increasingly clear that the γc cytokine family represents a group of cytokines that support the fundamental attributes T cells and understanding how to exploit these cytokines for therapeutic use is critical for next generation cancer clinical trials involving vaccines, checkpoint inhibitors and ACT therapy.

AUTHOR CONTRIBUTIONS

CD and CP conceptualized, wrote and edited the manuscript. CD and HK conceptualized and created the figures. HK, AS, MW, GR, DA, EB, ZL, and MR critically reviewed and provided feedback for this manuscript.

FUNDING

This work was supported by the NIH Training grant T32 AI132164-01 to CD, NIH Training grant T32 GM08716 to HK, R01 CA175061 and R01 CA208514 grants to CP, KL2 South Carolina Clinical and Translational Research grant UL1 TR000062 to CP, ACS-IRG grant 016623-004 to CP and MUSC Start-up funds to CP. Supported in part by pilot research funding, Hollings Cancer Center's Cancer Center Support Grant P30CA138313 at the Medical University of South Carolina.

ACKNOWLEDGMENTS

We would like to thank the researchers and clinicians for their dedication to improving cancer immunotherapy practices for patients.

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

frontiers in Oncology

MINI REVIEW
published: 12 March 2019
doi: 10.3389/fonc.2019.00146

Multi Targeted CAR-T Cell Therapies for B-Cell Malignancies

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Chimeric antigen receptor (CAR) modified T cell therapy has revolutionized the treatment of relapsed and refractory hematological malignancies. Through targeting of the CD19 antigen on B cells durable remissions have been achieved in patients with B cell non-Hodgkin lymphoma and acute lymphoblastic lymphoma. Despite impressive responses, multiple escape mechanisms to evade CAR-T cell therapy have been identified, among which the most common is loss of the target antigen. In this review we will highlight outcomes to date with CD19 CAR-T cell therapy, describe the current limitations of single targeted CAR-T therapies, review identified tumor escape mechanisms, and lastly discuss novel strategies to overcome resistance via multi-targeted CAR-T cells.

Keywords: CAR-T, antigen escape, B-cell NHL, B-cell ALL, immunotherapy

OPEN ACCESS

Edited by:
Conrad Russell Cruz, Children's National Health System, United States

Reviewed by:
Yang Xu, University of North Carolina at Chapel Hill, United States
Alessandro Poggi, Ospedale Policlinico San Martino, Italy

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *15 November 2018*
Accepted: *20 February 2019*
Published: *12 March 2019*

Citation:
Shah NN, Maatman T, Hari P and Johnson B (2019) Multi Targeted CAR-T Cell Therapies for B-Cell Malignancies. Front. Oncol. 9:146. doi: 10.3389/fonc.2019.00146

INTRODUCTION

Adoptive cell transfer utilizing autologous T cells genetically engineered *ex vivo* to target tumor antigens has revolutionized the treatment of relapsed, refractory hematological malignancies. T cells can be engineered to express a new T cell receptor (TCR) or a chimeric antigen receptor (CAR) to target tumor-associated antigens. CAR-modified T-cells are composed of a single-chain variable fragment (scFv) that binds tumor antigens and is fused to a spacer and transmembrane domain with intracellular costimulatory signaling domains, most commonly CD28 or 4-1BB with CD3ζ (1, 2). While multiple tumor antigens are under active clinical investigation, CAR-T cell therapy against the CD19 receptor on B cells is most clinically advanced. CD19 is a 95kDa glycoprotein present on the B cell surface from early development until differentiation into plasma cells. Its normal function involves regulation of signal transduction through the B cell receptor. CD19 was an ideal first target as its expression is restricted to B lineage cells and it is not found on pluripotent blood stem cells or on most other normal tissues (3). These anti-CD19 CAR-T (CAR-19-T) cells have demonstrated significant efficacy in the treatments of patients with relapsed, refractory B cell lymphoid malignancies (4–7). Their potential was first highlighted in a series of case reports that demonstrated the potential of CD19 targeting in patients with non-Hodgkin lymphoma (NHL) (8, 9). Since these initial few reports, the field of CAR-T cell therapy has exploded and now data is available from several large multi-center studies reporting clinical outcomes from Phase II trials (4, 6, 10). Although these studies demonstrated unprecedented efficacy, it also became apparent that not all patients respond to CAR-19-T cells, and even for those who initially respond, durability of response remains a limitation. Amongst the earliest identified resistance mechanisms was the downregulation of target antigen CD19 from tumor cell surface (11, 12).

To date three Phase II studies have reported on efficacy data in B cell NHL and B cell acute lymphoblastic lymphoma (ALL). First, in NHL, Neelapu et al. reported their results of ZUMA-1, a Phase II study of CD28 CD3ζ CAR-19-T cells for relapsed, refractory large B cell lymphoma. Among 108 patients treated and followed for a minimum of 1 year, 42% of patients remained in

response at the time of publication. In a subset of patients who relapsed and had available data, CD19-negative relapse was observed as the likely mechanism of failure (6). The JULIET study evaluated the efficacy of a 41BB CD3ζ CAR-19-T cell as part of an international, phase 2 clinical trial. Among 93 treated patients, the 3 month CR rate was 32% (13). This identical construct was concurrently explored in a similar international phase II study for pediatric and young adult patients with relapsed, refractory B cell ALL. Following treatment, the 3 month overall response rate was 81% with 59% of these patients remaining alive and relapse-free at 12 months. Among relapsed patients, the majority (15/22) presented with CD19-negative disease, demonstrating a major limitation of currently FDA-approved CAR-T therapies. For patients with CD19-negative relapse, options are limited with few approved therapies (14), and prognosis is generally poor although there is great promise with a number of clinical trials underway targeting alternative B-cell antigens such as CD22 (15). In this review we will focus on the role of target antigen loss as a mechanism of CAR-T failure and strategies for overcoming this current limitation through novel CAR constructs.

ANTIGEN LOSS AS A MAJOR LIMITATION OF CAR-T CELL THERAPIES FOR B CELL MALIGNANCIES

While initial response rates in patients treated with CAR-T cells for B cell malignancies have been impressive when compared to historical outcomes for patients with relapsed, refractory disease, many patients fail to respond, and others relapse after initially responding. Of the known escape mechanisms, the best defined etiology of disease relapse has been due to target antigen loss, and recent clinical data indicated that 7–33% of responders in CAR-19-T cell trials for B-ALL have relapsed due to loss of cell-surface CD19 (12, 16), which supports the immunoediting hypothesis proposed by Schreiber and colleagues in 2002 (17). CD19 loss after CAR-T therapy was recognized early on when one of two B-ALL patients relapsed 2 months after treatment with CAR-T cells following an initial complete response (11). Deep sequencing identified that the malignant CD19-negative clone was actually present in peripheral blood and marrow at day 23, a time when the patient was initially felt to not have residual disease (11).

With the recognition that antigen loss is a major barrier to CAR-T therapies, research has uncovered that there are multiple mechanisms responsible for the antigen loss (**Figure 1**). Following CAR-19-T cell treatment, Sotillo et al. identified both acquired mutations and alternatively spliced CD19 alleles in the malignant B cells of pediatric patients with relapsed disease (19). This resulted in either no cell surface CD19 expression or surface of expression of CD19 variants that no longer contained the epitope recognized by the CAR-T cells. A study by Fischer et al. suggested that CD19 isoforms lacking the CAR-T binding epitope are present in some patients prior to treatment, predisposing these individuals to treatment failures (20). These observations have been questioned in a more recent study where antigen loss in a cohort of 12 B-ALL patients was found to be due to a variety of loss of heterozygosity mutations, and alternative splicing only occurred with rare frequency (21). Bagashev et al. identified retention of mutated, misfolded CD19 proteins in the endoplasmic reticulum, suggesting another possible mechanism responsible for antigen loss (22).

Another mechanism involved in antigen loss after CAR-T cell therapy is cell lineage switch. One of the first observations regarding lineage switch was reported in 2015 by Evans and colleagues, where a CLL patient with Richter transformation relapsed after CAR-19-T cell treatment with a plasmablastic lymphoma which is inherently CD19 negative (23). This finding has been followed up by a report showing that 2 of 7 patients with mixed lineage leukemia (MLL)-rearranged B-ALL relapsing with CD19-negative AML following treatment with CD19 CAR-T cells (24) and a recent case report where a pediatric patient with TCF3-ZNF384 fusion-positive B-ALL had a myeloid switch after therapy (25). In an intriguing recent report, Ruella et al. described a novel mechanism of CD19 evasion. This patient with CD19-negative relapse was identified to have a single CD19-positive leukemic cell transformed during the CAR-T manufacturing process (18). The investigators showed that CD19 CAR on the leukemia surface bound in cis to CD19, thereby masking it from being recognized by the CAR (18). Although this is likely an extremely rare event, it represents a not previously described mechanism of resistance and highlights the importance of having rigorous manufacturing standards in place when engineering T cells for adoptive immunotherapies.

Partial antigen loss due to antigen down-regulation, in contrast to complete loss of antigen, has also been implicated as a mechanism for resistance to CAR-T cell therapy (15, 16, 26–28). Using a CD20 CAR, Murata and colleagues were the first to document that a threshold level of around 200 antigen molecules per target cell were required to induce lytic function, while approximately 10-fold higher numbers of molecules were needed to stimulate cytokine production (26). Another study documented that a CD30 CAR could selectively target lymphoma cells while "ignoring" CD30+ hematopoietic progenitor cells (HPCs) due to differential levels of antigen expression (27). The low levels of CD30 on HPCs were insufficient to trigger significant cytolysis, unlike the high levels that were present on the lymphoma cells. Mackall et al. showed that not only is CAR-T cell function regulated by target antigen density on malignant cells, but also by CAR density on the engineered T cells (28). More recently, this same laboratory documented that relapses in patients treated with a CD22 CAR directly correlated with diminished levels of CD22 on the B-ALL cells (15). The investigators went further to show in animal studies that differential levels of CD22 on leukemia cells could have a dramatic impact on anti-cancer efficacy. These results have future implications not only for the use of CAR-T therapy in hematologic malignancies, but also as the use of CAR-T cells for solid tumors moves forward.

While the body of evidence for antigen loss in B cell leukemias after CAR-T therapy is indisputable, the role for antigen loss in similarly treated lymphoma patients has been more challenging since immunohistochemistry has typically been used to assess antigen levels rather than flow

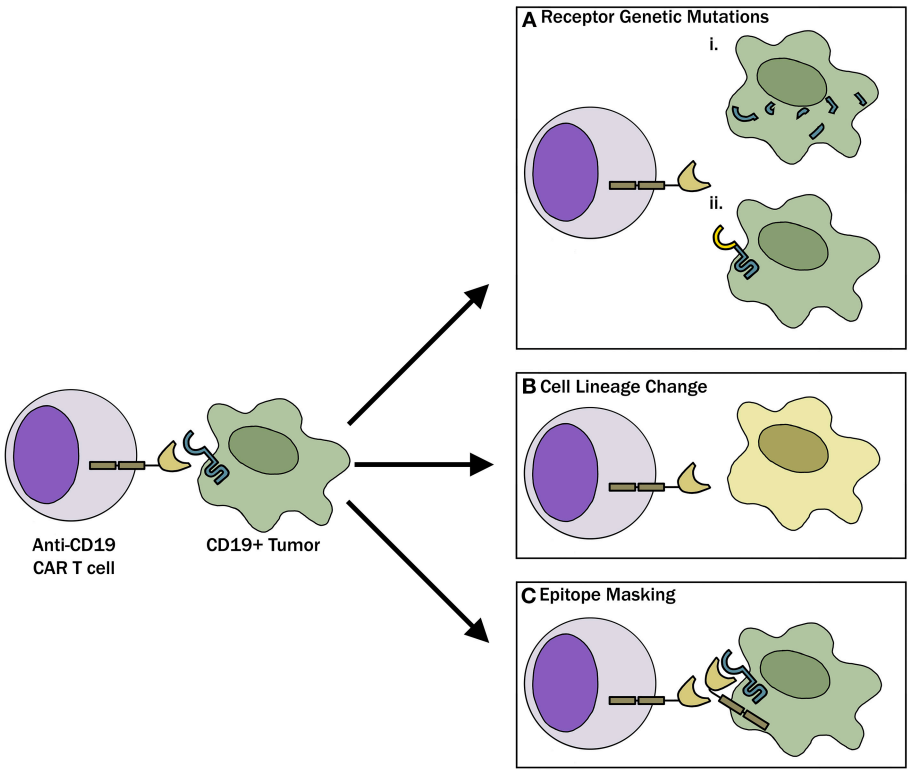

FIGURE 1 | Mechanisms of CAR-T evasion. **(A)** Tumor cells, through genetic mutations, can either (i) completely lose CD19 receptor expression or (ii) modify the CD19 receptor such that CAR-T cells can no longer recognize and bind the target. **(B)** Tumor cells can undergo phenotypic switch to a different lineage that is inherently CD19 negative to evade CAR-T cells. **(C)** As described in the case report by Ruella et al. (18) lentiviral modification of a single leukemic cell allowed for epitope masking and evasion of CAR-T cell therapy.

cytometry. Suggesting the role of antigen loss in lymphoma is the report by Shalabi et al. that documented sequential loss of CD19 and CD22 antigens in a patient with DLBCL following CAR T cell therapies that targeted these proteins (29). It is clear that more sophisticated ways of assessing antigen loss after CAR T cell treatment of lymphoma patients will be required to determine just how frequently this occurs.

TARGETING MULTIPLE MOLECULES TO OVERCOME THE LIMITATION OF ANTIGEN LOSS IN CAR-T CELL THERAPIES

One obvious way to combat the problem of antigen loss following CAR-T cell therapy is by targeting more than one antigen receptor. This can be accomplished by 1 of 4 different approaches: (a) Generate 2 or more cell populations expressing different CARs and infuse them together or sequentially (coadministration); (b) Use a bicistronic vector that encodes 2 different CARs on the same cell; (c) Simultaneously engineer T cells with 2 different CAR constructs (cotransduction), which will generate three CAR-T subsets consisting of dual and single CAR-expressing cells; or (d) Encode 2 CARs on the same chimeric protein using a single vector (i.e., bi-specific or tandem CARs) (**Figure 2**). These different approaches are highlighted in a recent review article by Majzner and Mackall (16).

One of the first pre-clinical studies that advocated for the use of more than one CAR to prevent emergence of antigen escape was in glioblastoma (30). In this study, T cells were either separately engineered to express HER2- or IL-13Rα2-specific CAR and mixed, or sequentially transduced to co-express the two constructs (approaches a & c above). Both approaches helped prevent antigen escape and provided better anti-tumor efficacy (30). Pre-clinical data in support of using dual-targeting in B cell malignancies emerged soon thereafter. In one of the first publications documenting successful use of a tandem CAR pre-clinically, Zah et al. developed a CD19-CD20 CAR and showed that the dual construct could prevent the spontaneous development of CD19-negative tumor cell variants in immune deficient mice (31). Later in 2016, Gill and colleagues tested 3 of the 4 approaches noted above (approaches a, b & c) where they simultaneously targeted CD19 and CD123 (IL-3 receptor α chain) (32). Using a xenograft mouse model, the investigators demonstrated that mixtures of CD19 and CD123 CAR-T cells or cells engineered to express both receptors on the same T cell, through co-transduction with separate lentiviral vectors or a bicistronic vector, could prevent antigen escape.

Preclinical results with another CD19–CD20 tandem CAR (approach d) were published by Schneider et al. (33). Constructs

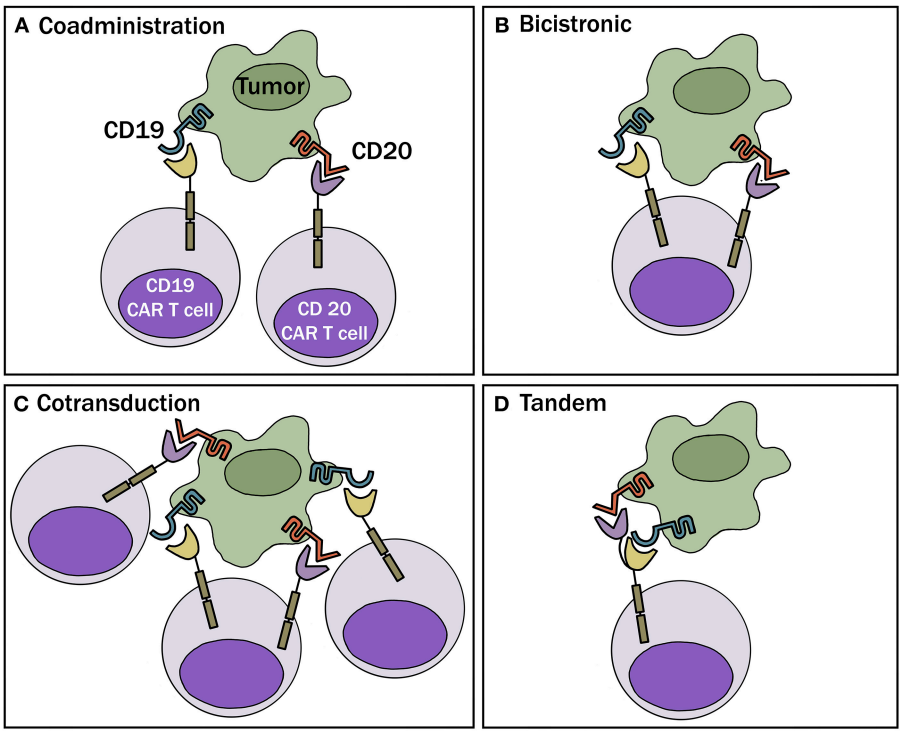

FIGURE 2 | Multi-targeted CAR-T approaches. **(A)** Coadministration—involves production of two separate CAR-T cell products infused together or sequentially. **(B)** Bicistronic vector—allows expression of 2 different CARs on the same cell. **(C)** Cotransduction—encode 2 CAR constructs via transduction with multiple vectors. With this process, one will also obtain cells that express each CAR alone. **(D)** Tandem—encode 2 CARs on same chimeric protein using a single vector.

were generated where CD19 or CD20 was expressed as the distal receptor on the CAR protein (designated as CAR 1920 or CAR 2019, respectively) and compared to single antigen CARs. Both CAR 1920 and CAR 2019 tandem constructs were superior to CD19 single-CAR in a murine xenograft leukemia model. CD19 expression on Raji leukemia cells (express both CD19 & CD20) was strongly diminished by coincubation with CD19 single CAR-T cells but maintained at higher levels by coincubation with CAR 1920 or CAR 2019 T cells. Interestingly, when CAR-T cells were stimulated with antigen-positive leukemia cells, expression of the CD19/CD20 tandem constructs resulted in less cytokine production than CD20 CAR alone, suggesting some attenuation of signaling when the CD19 and CD20 receptors were co-expressed in tandem on the same CAR. Finally, in a high-burden mouse leukemia model, CAR 2019 T cells provided improved anti-leukemia efficacy over that induced by single CD19 or CD20 CAR-T cells or mixtures of CD19 and CD20 single-expressing CAR T cells (33). There did not appear to be a clear advantage of expressing the CD20 receptor distal or proximal to the CD19 receptor, but the CAR 2019 did show better binding of CD20 peptide and improved killing against some cancer cell lines *in vitro* (33). These preclinical findings were translated into a Phase 1, first-in-human bispecific CAR-T cell trial with an anti-CD19/anti-CD20 tandem receptor (NCT03019055). Early results from this dose-escalation study demonstrated an ongoing complete response (CR) or partial response (PR) in 3/6 heavily pre-treated and relapsed B cell NHL patients treated with CAR-20.19-T cells. Interestingly among the three patients who progressed or relapsed, all retained either CD19 or CD20 positivity on subsequent biopsy suggesting other mechanisms rather than antigen loss as the etiology of therapy failure (34).

Similar to the development of a CD20.CD19 CAR-T cell, Fry and colleagues developed a bispecific CD19-22 CAR (15). The CD19-22 CAR was able to efficiently kill CD19+ and CD22+ human leukemia target cells *in vitro*, secrete IFN-γ in response to the target cells, and eradicate the leukemia in immune deficient mice. A Phase 1 clinical trial is currently underway testing this construct in patients with relapsed, refractory Diffuse Large B-cell Lymphoma and B-cell ALL. Early results from this dose escalation trial has demonstrated 2 patients with a CR among seven treated patients (35).

As a result of the encouraging preclinical data, several tandem CARs and combined or sequential administration of single CARs are being tested in the clinic (**Table 1**). **Table 1** also includes an ongoing clinical trial that uses an "armored" CAR, which encodes a CD19 receptor, CD3 and CD28 signaling motifs, the costimulatory ligand 4-1BBL, as well as a suicide gene safety system if the cells mediate severe acute toxicities. Although this vector does not target more than one antigen receptor, the idea is that the armored CAR-T cells might be able to prevent antigen escape by providing a more vigorous initial response that would eliminate the malignant cells before antigen escape develops.

TABLE 1 | Actively recruiting ClinicalTrials.gov registered studies using tandem CARs or administration of multiple single CARs.

CAR	NCT number	B cell malignancy	Site
Sequential CD19, CD20	NCT03207178	Non-specified	Shanghai, China
Multiple mixtures (CD19 + CD22, CD38, CD20, CD123, CD70, or CD30)	NCT03125577	Non-specified	Guangzhou, Shenzhen & Kunming, China
"Armored" CD19	NCT03085173	CLL	New York, NY, USA
CD19–CD20 dual	NCT03398967	Leukemia, Lymphoma	Beijing, China
	NCT03019055	Lymphoma, CLL	Milwaukee, WI, USA
CD19–CD22 dual	NCT03614858	Leukemia	Suzhou, China
	NCT03593109	Lymphoma	Xi'an, China
	NCT03468153	Lymphoma	Shanghai, China
	NCT03448393	Leukemia, Lymphoma	Bethesda, MD, USA
	NCT03398967	Leukemia, Lymphoma	Beijing, China
	NCT03330691	Leukemia, Lymphoma	Seattle, WA, USA
	NCT03289455	Leukemia	London & Manchester, UK
	NCT03287817	Lymphoma	London, Manchester & Newcastle, UK
	NCT03241940	Leukemia	Palo Alto, CA, USA
	NCT03233854	Lymphoma	Palo Alto, CA, USA

OTHER MULTI-TARGETING APPROACHES FOR HEMATOLOGIC MALIGNANCIES INVOLVING CARs

One interesting approach that evolved from work done by Vie and colleagues (36) is the engineering of T cells to express CD16 (FcγRIII) CARs so that they are capable of mediating antibody-dependent cellular cytotoxicity (ADCC). The first of these CARs contained CD16 linked to intracytoplasmic domains of FcγRIII (36). More recently, CD16 CARs have been created by adding CD3ζ and CD28 or 4-1BB signaling domains (37–39). Basically, one can administer engineered CD16 CAR-T cells along with one or more of the several tumor antigen-specific monoclonal antibodies that are known to facilitate ADCC [reviewed in Caratelli et al. (40)]. This is an attractive approach because it could allow for the targeting of multiple antigens simultaneously, as long as each of the monoclonal antibodies facilitates ADCC. Two clinical trials using this approach to treat patients with B cell malignancies in conjunction with anti-CD20 (rituximab) are currently recruiting patients (NCT02776813, NCT02315118).

Other armored CARs in development include an IL-18-secreting CD19 or MUC16 CAR, which appears to modulate the tumor microenvironment of both hematologic malignancies and solid tumors and helps enhance endogenous anti-tumor T cell responses (41). CARs with the same specificities have also been modified to co-express a PD-1 blocking moiety or to secrete IL-12 (42). Interestingly, local PD-1 blockade at tumor sites could increase anti-tumor activity of the CAR T cells while avoiding the toxicities associated with systemic PD-1 blockade (42). The IL-12-secreting MUC16 CAR was able to modify the tumor microenvironment by deleting tumor-associated macrophages and enhancing CAR T cell proliferation and cytotoxicity (43).

Finally, it is notable that the development of trivalent CARs has now been reported (44, 45). It will be interesting to see if these, and other current and future multi-targeting CAR approaches, are able to obviate the problem of antigen loss. Only time will tell.

LIMITATIONS OF MULTI TARGETED CAR-T APPROACHES

While potential advantages of multi-targeted CAR-T approaches over the current standard of care have been discussed, there are several unanswered questions regarding safety, efficacy, and feasibility of these products. First, multi-targeted CAR-Ts do not address other proposed resistance mechanisms outside of target antigen loss. Recently, Fraietta et al. reported on the determinants of efficacy and resistance of CD19 CAR-T cells in CLL (46). They demonstrated that the intrinsic transcriptome profile of the CAR-T cells determined efficacy with CAR-T cells enriched in memory-related genes and IL-6/STAT3 signaling seen in responding patients, while upregulation of genes involved in T-cell differentiation and exhaustion were found in non-responding patients (46). Other proposed mechanisms include inhibition of CAR-T cells due to engagement of PD-L1 on tumor cells (42). In both scenarios, it is unlikely that multi-targeting would be able to overcome these resistance mechanisms. Second, there is limited understanding on the safety profile and *in vivo* activity of multi-targeted CAR-T cells in patients. It is possible that multi-targeting, through availability of increased target antigen, may lead to a more robust form of CRS, making their administration prohibitive. It is also unclear if the cytotoxic activity that is seen *in vivo* is due to preferential engagement of one target over the other, or in the setting where more than

one CAR-T cell product is co-administered, whether there will be equal engraftment and distribution of the modified cells. Lastly, a significant concern of multi-targeting is the cost associated with production. Several approaches to multi-targeted CAR-T cells requires >1 viral transduction or >1 manufacturing run, which when commercialized, can significantly increase the cost of therapies that are already exceedingly expensive.

CONCLUSIONS

CD19 CAR-T cell treatments have transformed the management of B cell hematological malignancies. Despite the remarkable outcomes in relapsed, refractory patients, soon after its development the presence of resistance mechanisms was identified, and CD19-negative relapse was the dominant pathology described. Loss of CD19 has occurred through a variety of mechanisms including genetic modification, leading to partial or complete down regulation of the CD19 receptor, or truncation of the protein preventing binding by CD19 CAR-T cells (16). Other mechanisms include lineage switching and the development of a phenotype that is intrinsically CD19-negative (23, 24). Finally, it was most recently reported that through viral transfection of a CAR in a single leukemic cell, the patient developed a CD19 resistant leukemic clone that resulted in patient death (18). Regardless of the mechanism, it is apparent that single targeting of CD19 leads to selective pressure and development of tumor cell clones that can evade CD19 CAR-T therapy. It is possible that multi-targeted CAR-T cell therapy may overcome this resistance mechanism and improve clinical outcomes. Many trials are now in development or actively accruing patients to determine if targeting multiple antigens can prevent treatment failure due to CD19 loss and improve response rates and durability of response. Pending results of these studies, FDA approved CAR-19-T cell products will remain the mainstay treatment option for relapsed, refractory B cell NHL and ALL.

AUTHOR CONTRIBUTIONS

NS contributed to development, writing, and final review of the article. TM contributed to illustrations and final review of the article. PH contributed to development and final review of the article. BJ contributed to development, writing, and final review of the article.

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Conflict of Interest Statement: NS, PH, and BJ have research funding from Lentigen Technology. NS has participated in advisory boards for Juno and Kite pharmaceuticals.

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

frontiers in Immunology

MINI REVIEW
published: 19 March 2019
doi: 10.3389/fimmu.2019.00456

Building Potent Chimeric Antigen Receptor T Cells With CRISPR Genome Editing

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Chimeric antigen receptor (CAR) T cells have shown great promise in the treatment of hematological and solid malignancies. However, despite the success of this field, there remain some major challenges, including accelerated T cell exhaustion, potential toxicities, and insertional oncogenesis. To overcome these limitations, recent advances in CRISPR technology have enabled targetable interventions of endogenous genes in human CAR T cells. These CRISPR genome editing approaches have unleashed the therapeutic potential of CAR T cell therapy. Here, we summarize the potential benefits, safety concerns, and difficulties in the generation of gene-edited CAR T cells using CRISPR technology.

Keywords: chimeric antigen receptor, CRISPR, gene editing, immunotherapy, cancer, CAR T

OPEN ACCESS

Edited by:
Conrad Russell Cruz, Children's National Health System, United States

Reviewed by:
Donald R. Shaffer, Jounce Therapeutics, Inc., United States
Gengwen Tian, Driscoll Children's Hospital, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *16 October 2018*
Accepted: *20 February 2019*
Published: *19 March 2019*

Citation:
Liu J, Zhou G, Zhang L and Zhao Q (2019) Building Potent Chimeric Antigen Receptor T Cells With CRISPR Genome Editing. Front. Immunol. 10:456. doi: 10.3389/fimmu.2019.00456

INTRODUCTION TO CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY

Major histocompatibility complex (MHC) molecules play key roles in the surveillance of aberrant proteins of tumor cells. T cell receptors (TCRs) on the surface of T lymphocytes recognize antigenic peptide fragments derived from these aberrant proteins in complex with MHCs (1, 2). The expression of MHC/peptide complexes constitutively occurs on all nucleated cells. Tumor-specific MHC/peptide complexes are considered ideal targets for T cell-based immunotherapies. Diverse strategies have been developed to induce T cell immunity against these tumor epitopes, including cancer vaccination (3), adoptive T cell transfer (4), and TCR engineering (5). In cancer patients, however, tumor cells can effectively escape adoptive immunity via regulatory mechanisms, such as downregulation of MHCs or mutation. Because the presence of relatively fewer MHC molecules on the tumor cell surface limits naive TCR recognition, T cells fail to respond and trigger cascades of immune activation (6).

Recently, the most promising development has been the use of chimeric antigen receptor (CAR) T cell immunotherapy (7). CAR T cell immunotherapy has emerged as a leading curative strategy in the treatment of relapsed hematological malignancies. CAR T cell therapy is based on the immune effect of T cell activation and the principle of transformation through the genetic engineering of T cells. A typical CAR construct comprises a binding domain (single chain antibody fragment, scFv), a transmembrane domain and intracellular signaling domains capable of activating T cells (**Figure 1**). CARs allow the T cells to be activated independently of MHC. Donor-derived

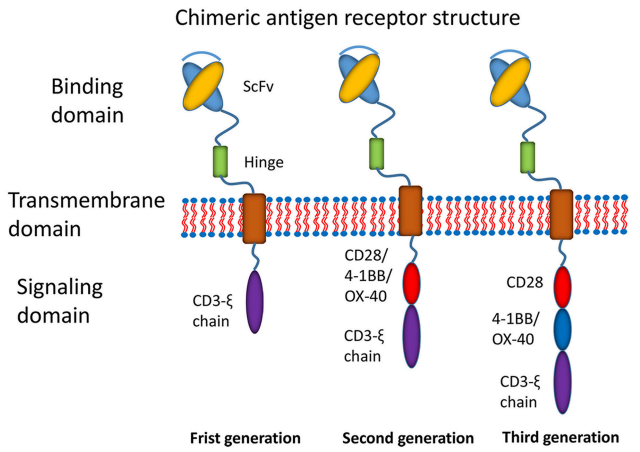

FIGURE 1 | Main structures of chimeric antigen receptors. Three generations of CAR structures. In the first generation of CARs, the binding domain (single chain antibody fragment, scFv) is linked to the signaling domain (CD3ζ) via the transmembrane domain. In the second generation of CARs, the costimulatory molecule (CM1, such as CD28 4-1BB or OX-40) is introduced with the signaling domain (CD3ζ). In the third generation of CARs, the additional costimulatory molecule (CM2) is included.

T cells are modified to express multivalent CARs on the cell surface that are responsible for recognizing the tumor-associated antigen (TAA) of tumor cells. Thus, T cells are activated via intracellular signal transduction. CAR designs differ not only in their signaling domains but also in their functional properties. The CAR structures have progressed since the first generation was described in 1989 (8). The first generation of CARs was designed as an scFv linked to the CD3ζ intracellular signaling domain of the TCR through a hinge and a transmembrane domain. Although the CD3ζ signaling domain can trigger activation of T cells, this pattern most likely results in T cell anergy, attenuating T cell activation. Therefore, the first generation of CARs exhibited limited responses in clinical trials (7). To address this limitation, a costimulatory molecule, such as CD28, OX40, or 4-1BB, was incorporated into the intracellular domain for the second generation of CARs. The additional costimulatory domain in the second generation of CARs strikingly improved T-cell proliferation and persistence. To optimize T-cell efficacy, the third generation of CARs has been developed by introducing two costimulatory domains into the CAR structure. Although dual costimulatory domains can enhance the activation and proliferation of T cells, the abundance of cytokines remains to be considered.

The CAR T cell approach has provided great advances in the treatment of hematological malignancies. Anti-CD19 CAR T cells have significantly advanced the therapy of human hematological malignancies and were shown to achieve a 90% complete response rate in acute lymphoblastic leukemia (ALL) (9). Tisagenlecleucel, the first anti-CD19 CAR T cell therapy, was approved by the US Food and Drug Administration (US FDA) for the treatment of children and adults with advanced leukemia in 2017 (10, 11). As 2017 ended, there were hundreds of ongoing CAR T cell trials for the treatment of hematologic and solid tumor malignancies (12).

POSSIBLE SIDE EFFECTS OF CHIMERIC ANTIGEN RECEPTOR T CELL THERAPY

Although most patients infused with CAR T cells show mild or moderate side effects, potentially severe side effects are still challenging. The prominent toxicities include cytokine release syndrome (CRS), insertional oncogenesis, and neurologic toxicity (13, 14).

Cytokine Release Syndrome

CRS is an unintended side effect due to overactivation of the host immune system. Severe CRS was observed in some patients who received infusion of CAR T cells (15). An abundance of cytokines is released by either the infused CAR T cells or other polarized immune cells. Several clinical studies indicated that 19–43% of patients exhibited CRS when they were treated with anti-CD19 CAR T cells for relapsed/refractory ALL (13, 16). Clinical features of CRS include high fever, muscle pain, malaise, unstable hypotension, fatigue, ang capillary leakage (17). A wide variety of cytokines can be elevated in the serum of patients. Dramatic elevations of inflammatory cytokines, such as INF-γ, IL-2, IL-6, and IL-10, are observed in CRS (18). Occasionally, neurologic toxicity can be associated with anti-CD19 CAR T cell therapy, probably due to the elevated levels of cytokines (16). The use of the anti-IL-6 receptor antibody tocilizumab was demonstrated to exert curative effects for serious cases of CRS in all patients with a high proliferation of CAR T cells (19).

Insertional Oncogenesis

Continuous CAR expression in T cells relies primarily on the delivery of the CAR gene by integrated gamma retroviral (RV) or lentiviral (LV) vectors. The advantages of both systems are high gene-transfer efficiency and stable expression of the CARs. Although both RV and LV vectors have been shown to be safe in intensive biosafety testing, this safety issue remains a concern. LV- or RV-mediated random and uncontrollable integration in the genome are unpredictable (20). Uncontrollable insertions of CAR genes lead to potential oncogenesis, variegated transgene expression, and transcriptional silencing (21). This possibility poses an oncogenic risk for RV/LV-engineered T cells (22). Although RV-driven oncogenesis has not yet been reported in CAR T cell therapy, this phenomenon was observed in clinical trials of hematopoietic stem cell transplantation (23). Additionally, random integration into the genome causes substantial variations in CAR expression levels in a batch of CAR T cells because of the different copy numbers per cell.

Graft-vs.-Host Disease

With the gradual initiation of clinical trials, autologous CAR T cells have shown some disadvantages. In infants or adults who are receiving chemotherapy or radiotherapy, it is difficult to harvest sufficient lymphocytes for CAR T cell manufacture. Thus, the quality of CAR T cells for each patient is uncontrollable and unpredictable. The use of allogeneic CAR T cells has become

a solution for these problems. Allogeneic CAR T cells can be expanded *ex vivo* on a large scale and can be reserved to treat multiple patients (24). The concerned with allogeneic infusion is graft-vs.-host disease (GVHD) between the donor cells and recipients. The repertoire of TCRs and MHCs expressed on allogeneic CAR T cells may potentially induce GVHD in recipients who receive donor CAR T cells (25). A study showed that allogeneic anti-CD19-CAR T cells had clinical benefits for relapsed hematologic malignancies (26). No obvious GVHD was observed in these recipients.

GENERATION OF POTENT CAR T CELLS WITH CRISPR TECHNOLOGY

Efforts to enhance the efficacy of CAR T cell therapy have been undertaken, including the selection of extracellular receptors (27), optimization of intracellular costimulatory molecules (28), combination with cytokines(29), and improvement of "on-target/off-tumor" toxicity (30). Effective gene-editing technologies have emerged as tools for cell engineering (31). The properties of three gene-editing tools, including CRISPR, zinc-finger nucleases (ZFNs), and transcription activator-like effector nucleases (TALENs), are summarized in **Table 1**. The use of CRISPR in genome editing is highly efficient and enables a simple and efficient way to multiplex the processing of T cells (32, 33). Both ZFNs and TALENs have also been adopted to modify T cells for clinical applications (34, 35). However, the recognition of the targetable DNA sequences with ZFNs and TALENs in T cells remains complicated and tedious, resulting in a low gene-editing efficiency. The simultaneous multiplexed genetic manipulations of these techniques are challenging (36). CRISPR/Cas9 systems have been used for the knock-out and knock-in of sequences in mammalian genome editing (**Figure 2**). In principle, a deletion or insertion at a target gene is introduced by a small RNA (sgRNA)-guided Cas9 nuclease that induces a double-stranded DNA break, which is subsequently repaired by non-homologous end joining (NHEJ) (37). Nucleotide insertions or deletions result in non-sense mutations and loss of gene function. In comparison to NHEJ, a relatively large gene sequence can be delivered to a precise locus in the genome through homology directed repair (HDR) after double-stranded DNA is cleaved by sgRNAs (38–40). The HDR process enables precisely targeted nucleotide replacements at the defined site of interest. Currently, several strategies based on CRISPR are being applied to develop next-generation CAR T cells by multiplexed genome editing (41–43). Such approaches include the knockout of endogenous genes (such as TCRs, MHCs, or self-antigens) to build allogeneic universal CAR T cells (41, 44, 45), the disruption of inhibitory receptors (such as CTLA-4, PD-1, or LAG-3) (44, 46, 47), and the integration of the CAR cassette into the endogenous TCR α constant locus (TRAC) (48, 49) or the C-C chemokine receptor type 5 (CCR5) locus (32) (**Table 2**).

TABLE 1 | Comparison of ZFN, TALEN, and CRISPR.

Property	ZFN	TALEN	CRISPR
Anchor site	18–36 nt	30–36 nt	23 nt
Off-target	Low	low	High
Complication	High	High	Low
Efficiency	Relatively low	Relatively low	High
Multiplex	Low	Low	High
Methylation sensitivity	High	High	Low
Mechanism of action	Zinc finger nuclease for DNA recognition and cleavage	transcription activator-like effector nuclease recognition and DNA cleavage	Guide RNA for DNA recognition and Cas9 endonuclease for cleavage

Universal CAR T Cells

Although autologous CAR T cells against B cell malignancies have shown promising results, some clinical studies demonstrated that for some patients, autologous T cells could not be manufactured due to poor lymphocyte counts or low T cell quality and quantity (50). Especially for some patients in infancy, sufficient peripheral blood mononuclear cells (PBMC) cannot be harvested to support T cell manufacture *ex vivo*. These limitations can be circumvented by utilizing allogeneic T cells. Endogenous TCRs that allogeneic T cells express can recognize the alloantigen of the recipient, resulting in major graft-vs.-host disease (GVHD). Before these allogeneic T cells can be widely used clinically, the issue of GVHD must be resolved (45). Universal allogeneic CAR-T cells are ideal because their manufacture and quality may be more easily controlled and GVHD may be avoided. Several groups have generated allogeneic universal anti-CD19 CAR T cells by deleting multiple genes, such as TRAC, β2M, and MHC, using CRISPR methods (41, 42). Meanwhile, ongoing clinical trials have shown that a suicide gene in the CAR construct can also be used to avoid GVHD after allogeneic CAR T cell injection (25). These results suggest that CAR T cells that utilize multiplexed gene editing generate CAR T cells that are as potent as non-gene-edited T cells.

Until now, most successful CAR T cell therapies have been applied to B cell malignances. For T cell malignances, patients would receive allogeneic T cells rather than autologous CAR T cells. Genomic editing of some antigens, which recognize those "non-self" molecules and are attacked by the host immune system, can broaden the application of CAR T cells. DiPersio et al. reported that fratricide-resistant "off-the-shelf" universal T cells generated with CRISPR gene editing were used for treatment of T-cell malignancies (44). CD7 is a molecule commonly expressed in T lymphocytes. To avoid self-elimination, the CD7 target antigen against malignancies, which is recognized by anti-CD7-CARs, is deleted on CAR T cells (51, 52).

Resistance to PD-1 Inhibition

It is widely accepted that the existence of immune checkpoints (such as PD-1, CTLA-4, and LAG-3) can attenuate the activation of CAR T cells and accelerate T cell exhaustion. PD-1 is a primary inhibitory molecule in T cell transduction (53, 54). The

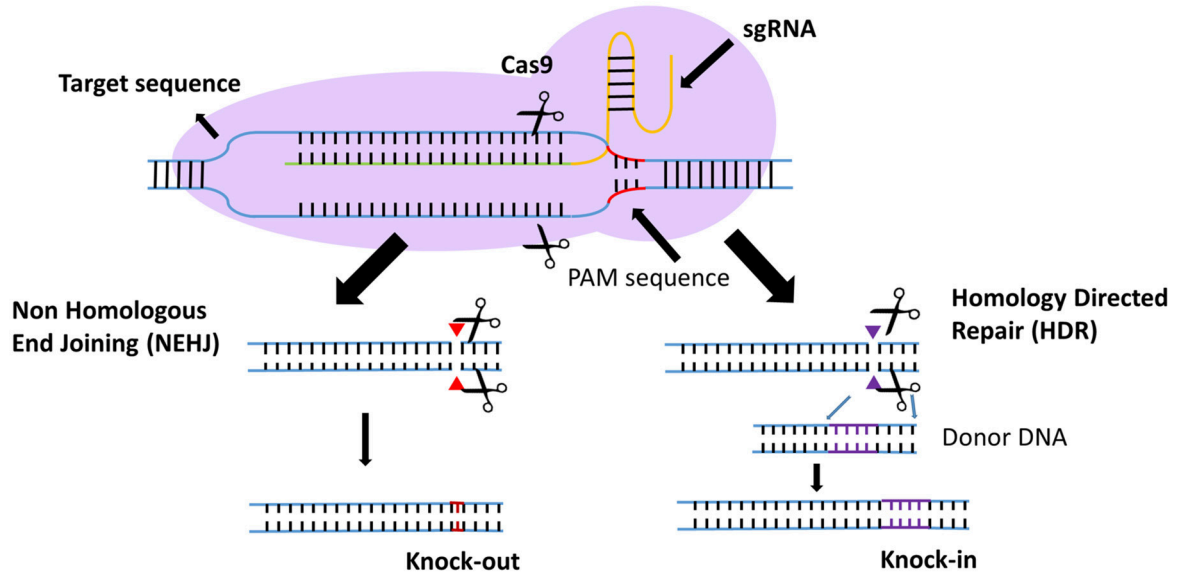

FIGURE 2 | Introduction to the CRISPR gene-editing system. Guided by sgRNAs, the CRISPR-Cas9 nuclease can target short DNA sequences. The PAM specifically creates a sgRNA–target DNA heteroduplex and generates double-strand breaks. Then, the DNA double-strand breaks are repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR). In the NHEJ pathway, indels lead to nucleotide deletions or insertions. In the HDR pathway, accessory factors can facilitate genome recombination through the two homology arms, resulting in the knock-in of a gene of interest.

PD-1/PD-L1 pathway plays an important role in the regulation of T cell activation and differentiation (55). High expression of PD-1 accelerates T cell tolerance and exhaustion (56–59). Increasing evidence indicates that blocking the PD-1/PD-L1 axis could partially restore the function of exhausted T cells (54, 60). A recent clinical study demonstrated that treatment with anti-CD19 CAR T cells in combination with an anti-PD-1 antibody was effective in patients with relapsed chronic lymphocytic leukemia (CLL) (61). This anti-PD-1 antibody treatment revives the antitumor response of anti-CD19 CAR T cells in patients who fail to respond to CAR T cell treatment (62). In other cases, unanticipated autoimmune responses are associated with anti-PD-1 checkpoint inhibitors (63). Therefore, ablation of PD-1 with gene editing by CRISPR/Cas9 is an alternative to enhance the antitumor response of CAR T cells in anti-CD19 CAR T cell therapy (41, 42). Ren et al. suggested that depletion of PD-1 genes in anti-prostate stem cell antigen (PSCA) CAR T cells with a Cas9/RNP method significantly enhanced T cell immunity *in vivo* (42). A significant antitumor response was observed after PD-1 was disrupted by genome editing. Controversially, a study indicated that T-cells without PD-1 were susceptible to exhaustion and lacked long-term durability (64). In regard to other checkpoint targets, no obvious improvement was confirmed when LAG-3 genes were deleted in CAR-T cells using CRISPR/Cas9 (47). Nevertheless, these studies still support the promise of checkpoint inhibition in CAR T cell therapy.

Targeted Integration of CARs

Recently, effective homologous recombination was shown to promote the site-specific integration of large transgenes in the T cell genome (65). In this method, after the DNA of the target gene is cleaved using Cas9 RNPs, a gene of interest is subsequently delivered to the cleavage site using adeno-associated viruses (AAVs). Site-specific transgene integration is achieved by HDR. An anti-CD19 CAR gene has been successfully integrated into the TRAC locus using the combined action of Cas9/RNP and AAV donor vectors (49). Targeting the CAR gene to the TRAC locus not only results in uniform CAR expression but also delays effector T-cell differentiation and exhaustion. Moreover, the insertion of a CAR transgene into a defined location avoids the risk of insertional oncogenesis and places CAR expression under the control of endogenous regulatory elements.

SAFETY CONCERNS OF CRISPR GENE-EDITED CAR-T CELL THERAPY

To date, although many limitations of conventional CAR T cells have been addressed with CRISPR gene editing, safety issues must be addressed before these gene-edited cells start to move into clinic. Multiple elements, such as off-target effects, Cas9 activity, target site selection, and sgRNA design, and delivery methods, can determine the efficiency and safety of the CRISPR/Cas9 system.

The first concern of CRISPR gene editing is off-target effects (66). These off-target effects might be beneficial to bacteria and archaea (67). However, several recent studies have reported unintentional CRISPR/Cas9-induced large genomic

TABLE 2 | Summary of the CAR-T cells modified with gene editing.

CAR	Gene-editing method	Targeted gene	Gene editing efficiency (%)	Malignancy	Reference
KNOCK-OUT					
CD19 scFv/4-1BB/CD3ζ	Cas9 RNP electroporation	TRAC	85	B cell acute lymphoblastic leukemia	(41, 42)
		β2M	100		
		PD-1	64.7		
CD19 scFv/4-1BB/CD3ζ	Cas9 RNP electroporation	TRAC	81.7	B cell acute lymphoblastic leukemia	(45)
		TRBC	49.3		
		β2M	79.9		
CD7 scFv/CD28/4-1BB//CD3ζ	Cas9 RNP electroporation	CD7	89.14	T cell acute lymphoblastic leukemia	(44, 52)
EBV-LMP2A CTL	Cas9 plasmid electroporation	PD-1	47.4	Epstein-Barr virus-associated gastric cancer	(46)
CD19 scFv/4-1BB/CD3ζ	Cas9 RNP electroporation	LAG-3	45–70	B cell acute lymphoblastic leukemia	(47)
KNOCK-IN					
CD19 scFv/4-1BB/CD3ζ	Cas9 RNP electroporation and transfection with AAV6 encoding CAR	TRAC exon 1	50	B cell lymphoma	(48)
CD19 scFv/CD28/CD3ζ	Cas9 RNP electroporation and transfection with AAV encoding CAR	TRAC exon1	40	Adult B acute lymphoblastic leukemia	(49)

deletions or gene inversions in various species, including mouse, *C. elegans*, and rabbit (68–70). For human therapies, clinical safety is particularly important. Several recent studies have reported off-target effects of CRISPR in T-cells. Off-target effects introduce random mutations, thus impacting tumor-suppressor genes or activating oncogenes. Off-target effects were also observed when the *TRAC* or *TRBC* locus of CAR-T cells was inserted with CRISPR/Cas9 electroporation (42). A controversial study indicated that CRISPR gene editing could cause hundreds of unintended mutations in the genome when whole-genome sequencing was performed on a CRISPR–Cas9-edited mouse (68). Notably, another study showed that CRISPR/Cas9 genome editing resulted in a p53-mediated DNA damage response in human retinal pigment epithelial cells (71). p53 activation may lead to chromosomal rearrangements and other tumorigenic mutations in cells. Although the outcome of CRISPR-induced p53 activation is unconfirmed, it seems to decrease the gene editing efficiency. Therefore, the off-target issues must be considered in the future development of CRISPR/Cas9-edited CAR T cells. Off-target assays during CRISPR target selection may be performed to manage the safety risk of clinical CAR T trials.

Another safety concern is that unpredicted translocations may occur between double-strand breaks when multiple genes are edited (72). Although such events are rare in T cells, transformation analysis should still be performed to ensure the safety of gene-edited CAR-T therapy. In addition to the safety risk of translocations, altered functions of gene-edited CAR-T cells most likely would cause adverse effects in patients. For example, CRISPR gene disruption in CAR T cells can cause unintended innate immune responses (73).

PERSPECTIVES OF CRISPR GENE-EDITED CAR-T CELL THERAPY

In recent, many antitumor approaches have been developed, including target small molecules (74, 75), antibody drugs (76–84), immune cell therapy (85). Among them, CAR T cell therapy aims to treat cancer through the use of the patient's immune system. This type of therapy has many advantages, such as low toxicity and a long duration (86). However, CAR T cell therapy appears to be effective only in a limited portion of patients with hematological malignancies. CRISPR is a cutting-edge technique that can be used to generate CAR T cells with enhanced potency and safety. Although the clinical use of allogeneic donor CAR cells has been recently reported, their use is highly dependent upon either rigorous patient selection or T cell selection (25). Potential GVHD still limits the wide application of allogeneic CAR cells. Taking advantage of CRISPR, the risk of GVHD may be minimized through the deletion of endogenous TCR and MHC molecules. The additional disruption of PD-1 is believed to optimize the antitumor activities of CAR-T cells through the regulation of T-cell functions (32). The safety of gene-edited CAR T cells is the primarily concern because of notorious off-target effects. To minimize the safety risk of off-target effects, careful selection of the target site combined with prior off-target assays will be required during target site selection of CAR T cells. Although skeptics question whether CRISPR gene-edited T cell therapy is safe and ready for the clinical stage, the first

CRISPR gene-editing trial using autologous T cells was initiated to treat patients with melanoma, synovial sarcoma, and multiple myeloma in 2016 (87). These potent T cells have shown merits in preclinical studies. The long-term safety profile of gene-edited CAR-T cells should be further examined in the clinic.

AUTHOR CONTRIBUTIONS

JL, GZ, and LZ wrote part of the manuscript; QZ wrote the manuscript.

FUNDING

This work was supported by the Science and Technology Development Fund of Macau (FDCT/131/2016/A3, FDCT/0015/2018/A1), the Guangzhou Science and Technology Program (201807010004), and Start-up Research Grand (SRG2016-00082-FHS) and the intramural research program of Faculty of Health Sciences, University of Macau, and National Natural Science Foundation of China (31440041).

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

frontiers in Oncology

REVIEW
published: 10 April 2019
doi: 10.3389/fonc.2019.00196

Beyond CAR T Cells: Other Cell-Based Immunotherapeutic Strategies Against Cancer

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Background: Chimeric antigen receptor (CAR)-modified T cells have successfully harnessed T cell immunity against malignancies, but they are by no means the only cell therapies in development for cancer.

Main Text Summary: Systemic immunity is thought to play a key role in combatting neoplastic disease; in this vein, genetic modifications meant to explore other components of T cell immunity are being evaluated. In addition, other immune cells—from both the innate and adaptive compartments—are in various stages of clinical application. In this review, we focus on these non-CAR T cell immunotherapeutic approaches for malignancy. The first section describes engineering T cells to express non-CAR constructs, and the second section describes other gene-modified cells used to target malignancy.

Conclusions: CAR T cell therapies have demonstrated the clinical benefits of harnessing our body's own defenses to combat tumor cells. Similar research is being conducted on lesser known modifications and gene-modified immune cells, which we highlight in this review.

Keywords: cell therapy, gene modified cells, immunotherapy, gamma delta T cells, NK cells, NKT cells, dendritic cells

OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *02 January 2019*
Accepted: *07 March 2019*
Published: *10 April 2019*

Citation:
Patel S, Burga RA, Powell AB, Chorvinsky EA, Hoq N, McCormack SE, Van Pelt SN, Hanley PJ and Cruz CRY (2019) Beyond CAR T Cells: Other Cell-Based Immunotherapeutic Strategies Against Cancer. Front. Oncol. 9:196. doi: 10.3389/fonc.2019.00196

INTRODUCTION

Chimeric antigen receptors and engineered T cell receptors (based on previously identified high affinity T cell receptors) function by redirecting T cells to a predefined tumor-specific (or tumor-associated) target. Most of these modifications use retroviral or lentiviral vectors to integrate the construct, and most of the receptors feature a costimulatory signal—enhancing T cell activation following recognition of the target antigen. These modified T cells have collectively shown promising success rates, particularly against hematologic malignancies (1), with growing excitement for these novel treatments (2). Pioneering work at the NIH resulted in promising therapies for melanoma (3) and synovial sarcoma (4). Some of these therapies have been approved as licensed drugs.

CAR T cells targeting commonly overexpressed leukemia and lymphoma markers such as CD19 have shown promise in the prevention and treatment of malignancies such as Acute Lymphoblastic Leukemia (ALL), Chronic Lymphocytic Leukemia (CLL), Non-Hodgkin's lymphoma (NHL), Diffuse Large B cell lymphoma (DLBCL), and other B cell malignancies (5–8). These CD19-CAR

Phase I and II trials have demonstrated safety and efficacy, with substantial partial and complete response rates (PR and CR, respectively). There are however, important concerns about toxicity—as resulting from on target off tumor effects, cytokine release syndromes, and neurotoxicity (9). Current CAR clinical trials are expanding to target other tumor-associated markers including GD2 (10), BCMA (11), CD20, CD30, CD33, CD7, HER2 (human epidermal growth factor receptor 2), and mesothelin (12–17). CAR T cells have been highlighted as Advance of the Year, by the American Society of Clinical Oncology in 2018 (18). A similar technology involves using high affinity T cell receptors (TCRs) and introducing these into cells (19). In the hopes of extending this success, other immune cell-based therapies are in current development.

The first group, non-CAR/non-TCR gene modified cell therapies for cancer, incorporates methods to overcome the barriers presented by cancer and the tumor microenvironment, as well as strategies for enhancing potency of T cell therapies. The second group focuses on immunotherapies generated from less frequently studied cell types including gamma-delta T cells, invariant natural killer T (iNKT) cells, natural killer (NK), and dendritic cells.

This review explores these lesser known cancer cell immunotherapy strategies, highlighting advances that have been made in recent preclinical and clinical efforts, and presents platforms for which they could demonstrate efficacy and may be critical for treating different cancer subtypes.

NON-CAR/TCR MODIFICATION OF T CELLS

The ease by which T cells can be genetically modified has led to other gene modifications that aim to further enhance activity of T cells [a strategy that some groups have labeled as "armored" CARs (20), initially dubbed as "TRUCKS" (21)], including modifications to introduce dominant negative receptors, chemokine receptors, cytokines, cytokine receptors, and checkpoint inhibitors **Figure 1**.

Dominant Negative Receptors

Translation of successful T cell therapies to solid tumors has been hampered by the immunosuppressive tumor microenvironment. Cancers secrete immunosuppressive cytokines which impair immune cell proliferation and function, and recruit regulatory T cells. These cytokines include TGFβ which inhibits the function of host immune cells (even those that successfully infiltrate the tumor), and induces epithelial-to-mesenchymal transition leading to cancer metastasis. Upregulation of TGFβ in the tumor microenvironment has been described in many aggressive malignancies including those of the brain, gastrointestinal tract, bone, breast, lung, and pancreas (22). TGFβ downregulates the secretion of critical Th1 cytokines, such as IFNγ, and impairs T cell and natural killer (NK) cell cytolytic activity and proliferation (23, 24).

A mutated form of the TGFβ receptor has previously been shown to exert a dominant-negative effect by abrogating the negative signaling cascade in cells that express this protein (25). This dominant negative receptor of the type II subunit (TGFβRII DNR) encompass the extracellular and transmembrane region of the endogenous cytokine receptor but exclude intracellular signaling domains, preventing downstream signaling when bound to ligand. Expression of this DNR has led to decrease in downstream signaling following TGFβ ligation—for example SMAD phosphorylation in the presence of TGFβ is abrogated by this receptor (26). T cells genetically engineered to express a TGFβRII dominant negative receptor (DNR) are resistant to the antiproliferative and anti-cytolytic effects of this cytokine (27). Genetically modified tumor antigen-associated T cells (in this case directed against Epstein-Barr virus antigens) expressing DNR show enhanced persistence and activity, resulting in superior antitumor activity (28). In this study, TGFβRII DNR restored proliferation of EBV-specific T cells in the presence of TGFβ, restored cytotoxicity against EBV-expressing lymphoblastoid cell lines, and demonstrated greater antitumor activity and migration *in vivo* (28). Other studies have also demonstrated the benefits of this DNR on the activity of T cells (see **Table 1**) (27, 29, 30, 32–34).

A dose escalation study (using TGFβRII DNR antigen-specific T cells directed against EBV) of patients with EBV-positive lymphoma showed that these T cells were resistant to the inhibitory cytokine, with increased signals from peripheral blood, corresponding to increased frequencies of T cells. Persistence extended to more than 4 years, and four of seven evaluable patients had clinical responses (28). Other clinical trials incorporating TGFβRII DNR expressing cells have targeted a number of cancers including nasopharyngeal carcinoma (using antigen-specific T cells directed against EBV), metastatic melanoma (using tumor infiltrating lymphocytes TILs), EBV-positive Hodgkin disease and non-Hodgkin lymphoma using antigen-specific T cells directed against EBV), and HER2+ breast cancer (using chimeric antigen receptors directed against HER2) (see **Table 2**).

It is important to note that there may potentially be unintended consequences of conferring resistance to a regulatory cytokine: disruption of normal T cell homeostasis may result from expression of TGFβRII DNR. A study by Lucas et al. show that expression of the dominant negative receptor resulted in massive expansion of CD8 T cells in lymphoid organs (36). So far, no dysfunction has been observed in patients (28).

Cytokine Receptors

Besides TGFβ, other negative/regulatory cytokines in the tumor environment limit T cell persistence and activity—these include

Abbreviations: ALL, Acute lymphocytic leukemia; AML, Acute myeloid leukemia; BCR, B cell Receptor; BMT, Bone Marrow Transplant; CAR, Chimeric Antigen Receptors; CLL, Chronic lymphocytic leukemia; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DC, Dendritic Cell; DLBCL, Diffuse Large B Cell Lymphoma; DNR, Double Negative Receptor; ESC, Embryonic Stem Cell; FcR, Fc Receptor; HLA, Human Leukocyte Antigen; HSCT, Hematopoietic Stem Cell Transplant; iPSC, Induced Pluripotent Stem Cell; MHC, Major Histocompatability Complex; mRCC, metastatic renal cell carcinoma; NHL, Non-Hodgkin's Lymphoma; NK, Natural Killer Cell; NKT, Natural Killer T Cell; TALEN, Transcription Activator-Like Effector Nuclease; TCR, T cell Receptor; ZFN, Zinc Finger Nuclease.

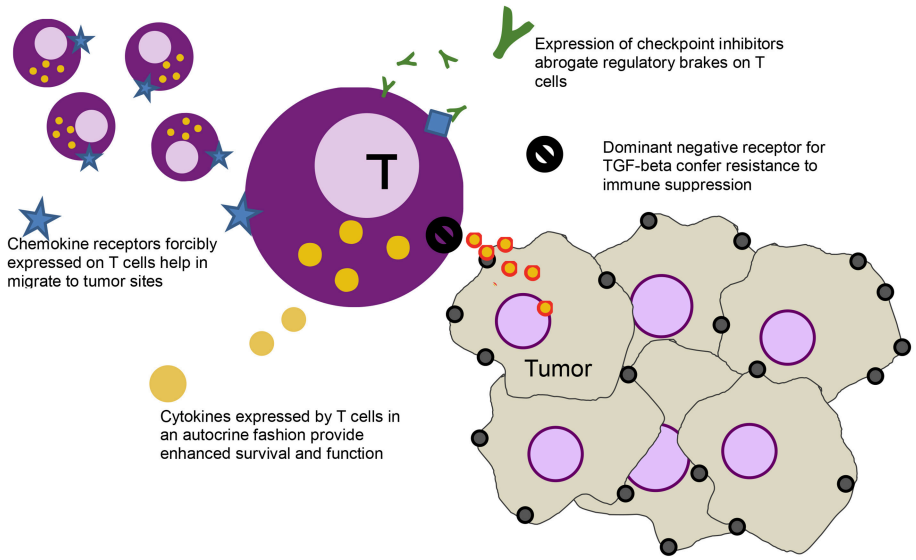

FIGURE 1 | Schematic of a T cell (purple) and various modification - clockwise from left: forcibly expressed chemokine receptors (blue star) that help the cell migrate down the relevant chemokine gradient secreted by the tumor, secreted checkpoint inhibitors (green Ys) that bind to the checkpoint receptors on T cells (blue diamonds), abrogating their inhibitory function, a dominant negative receptor (black no sign) that helps block immune suppressive effects of cytokines like TGF-beta, and cytokines (yellow) that help stimulate the T cells in an autocrine function.

TABLE 1 | Examples of preclinical research evaluating DNR-expressing T cells for the treatment of malignancies.

Disease	Effector cell	Observed effects	References
Prostate cancer	PSMA CAR T cells	Specific lysis of tumor, insensitivity to TGF-beta	(29)
EBV-positive lymphoma	EBV-specific T cells	Resist inhibitory effects of TGF-beta *in vitro* and *in vivo*, enhanced antitumor activity *in vivo*	(27)
Advanced prostate cancer	TCR-modified T cells	Complete and sustained tumor regression, enhanced cell survival, restored differentiation of prostate epithelium	(30)
Prostate cancer	PSMA CAR T cell	Increased proliferation, enhanced cytokine secretion, resistance to exhaustion, *in vivo* persistence, induction of tumor eradication in aggressive prostate cancer	(31)

IL10, IL13, and IL4. Another approach to reversing the immunosuppressive effects of these cytokines are chimeric cytokine receptors (CcR) (37). CcR's use the extracellular binding domain of an immunosuppressive cytokine bound to the intracellular signaling domain of an immune-activating cytokine to reverse its signaling effects. The first use of a chimeric IL4 cytokine receptor was described by Wilkie et al. where a fusion of IL4 receptor alpha ectodomain was fused to the subunit used by IL-2 and IL-15; this resulted in expansion and enhanced killing of MUC1 CAR T cells (38). In another study combining the extracellular domain of IL-4 cytokine receptor and the intracellular signaling domain of IL-7 cytokine receptor, CcRs restored the anti-tumor cytotoxicity of autologous T cells against EBV-transformed B cell tumors *in vivo* (37). In this study, CcR expression induced phosphorylation of STAT5 (part of the native signaling cascade in IL7 signaling) after ligation with tumor-secreted IL4, and restored T cell proliferation in the presence of the cytokine (37). This chimeric cytokine receptor also showed efficacy in a pancreatic cancer model: T cells modified to express a chimeric antigen receptor targeting prostate stem cell antigen (PSCA), found in pancreatic tumors, maintained their antitumor activity in an IL4-rich tumor microenvironment when they are co-transduced with the IL4/IL7 CcR (39). Another example uses a tumor-derived cytokine, CSF-1, to stimulate T cells by modifying these cells to express CSF-1R. Acquired responsiveness to CSF-1 allowed for improved chemotaxis and proliferation (40).

A simpler construct involves overexpression of a native cytokine receptor to allow for improved persistence following exogenous administration of the cytokine. One of the major challenges in T cell therapies is enhancing persistence of the cells *in vivo*. Previously, IL2 was administered to maintain T cell proliferation and activity (41), but IL2 is also associated with adverse effects (42)—limiting its applicability. IL7, on the other hand, provides the same effects without the unwanted toxicities. T cells, however, lose expression of the IL7 receptor after prolonged culture. In one study, genetic modification of EBV-specific CTLs to forcibly express IL-7 receptor α chain (IL-7Rα) led to restoration of CTL responsiveness to IL-7, and their antitumor activity sustained *in vivo* and *in vitro* without the unwanted toxicities related to IL-2 administration (43). In another study, cytokine feedback loops were used to improve efficacy of T cells by modifying these cells to express IL-7 and IL-21 (44).

TABLE 2 | Examples of clinical trials using various DNR-expressing T cells for the treatment of malignancies (35).

Trial ID	Disease	Product
NCT02065362	EBV-positive Nasopharyngeal carcinoma	TGFβ-resistant EBV-specific Cytotoxic T-lymphocytes +/– Cyclophosphamide and Fludarabine
NCT01955460	Metastatic melanoma	TGFβ-resistant T cells + Cytoxan, Fludarabine, Mesna, and Interleukin-2
NCT00368082	Relapsed EBV+ Lymphoma	TGFβ-resistant LMP-specific Cytotoxic T-lymphocytes (CTLs)
NCT00889954	HER2+ malignancies	TGFβ-resistant HER2/EBV-T cells

Cytokines

Select cytokines, like IL2, IL15, and IL12 perform stimulatory functions for T cells; in theory, autocrine secretion of these cytokines should help keep these cells persisting *in vivo*, even in the face of a hostile tumor environment (20).

In an example of this approach, CD19-CAR-specific T cells were modified to secrete IL15, and its anti-tumor efficacy evaluated using a xenogeneic model of lymphoma (45). In this study, IL-15 modified CD19 CAR T cells secreted IL15 following antigen stimulation, showed enhanced survival as a result of the transgenic cytokine, expanded better *in vivo*, and have better *in vivo* anti-tumor activity (45).

Other cell therapies incorporating cytokine secretion are listed in **Table 3**. One study, by Koneru et al. looked at MUC-16 specific T cells secreting IL12. Promising preclinical results (enhanced lysis of tumors and persistence *in vivo*) (49) led to its subsequent use in a phase I clinical trial for recurrent platinum-resistant ovarian cancer (50).

Chemokine Receptors

One relatively underappreciated requirement for improving T cell therapies is successful migration to the site of disease (51). In the setting of malignancies, a possible avenue for improvement relies on the fact that tumors secrete chemokines that can potentially be harnessed to lead T cells to the tumor site. Chemokine receptors corresponding to the chemokines that are secreted by tumor cells have been introduced into T cells, which maximized efficacy of these therapies by improving localization (51).

The first chemokine receptor-engineered T cells redirected cells using CXCR2: these allowed cells to migrate toward the Gro-alpha chemokine gradient, and induced interferon gamma secretion from transduced T cells (52).

Moon et al. transduced the chemokine receptor CCR2b into mesoCAR T cells to treat tumors that express CCL2 and mesothelin (53). These modified T cells improved tumor localization, a limitation of CAR-based approaches, and showed enhanced anti-tumor activity. Craddock et al showed that in neuroblastoma cell lines derived from six patients, modified activated T cells showed a 60% increase in the expression of CCR2b and co-expressed CCR2b and GD2-CAR showed a 10-fold improvement in migration to the tumor site compared to CCR2 negative activated T cells (54). Using the transgenic adenocarcinoma of mouse prostate (TRAMP) model, Garetto et al. (55) showed that expressing chemokine receptors on T cells tailored for chemokines that are strongly secreted in the tumor milieu can be used to improve targeting of T cells.

Additional examples are listed in **Table 4**.

Checkpoint Inhibitors

In addition to T cell therapies, the introduction of checkpoint inhibitors has been responsible for the interest in immunotherapies. These molecules, typically antibodies directed against checkpoint receptors expressed on T cells, inhibit negative regulation of these cells—removing the "brakes" to their activity. Combinations of T cell therapies and checkpoint inhibitors are therefore particularly attractive. Administration of a PD-1 blocking antibody enhanced CAR T cell function against established tumors (58).

One way to coordinate spatiotemporal activity of these therapeutics is to have T cells directly secrete these inhibitors. One group engineered CD19 CAR T cells to secrete single chain variable fragments targeting PD1. T cells were shown to secrete functional anti-PD1 scFv (∼600 ng/mL), capable of reversing PD1/PDL1 interactions and their negative effects on T cell function. This allowed for enhanced T cell expansion and effector function *in vitro* and *in vivo* (59). Another group also modified various CAR T cells to secrete PD1 blocking scFV and showed improved antitumor activity, as well as bystander tumor-specific T cell activity, in syngeneic and xenogeneic murine models of tumors expressing PDL1 (60). Other groups knocked down expression of PD-1 (61) or components of PD-1 signaling, to improve function of adoptively transferred cells (62).

OTHER IMMUNE CELLS

Although the specific, direct actions of gene-modified T cells are mostly responsible for the promising clinical results—indirect effects mediated through other immune cells also contributed to efficacy. In addition, there is an increasing body of evidence that suggests engagement of multiple arms of immunity are key toward longer lasting resolution of tumor.

The use of other immune cells as immunotherapies for cancer is therefore a necessary adjunct to the existing T cell therapies. Some of the more commonly studied cells include gamma-delta (γδ) T cells, invariant natural killer T (iNKT) cells, natural killer (NK), and dendritic cells. We limit this section to these endogenously occurring cells, though acknowledge that other cells that can be expanded *ex vivo*—e.g., cytokine induced killer cells (CIK)—may form a potentially efficacious immune therapeutic (**Figure 2**).

Gamma-Delta T Cells

γδ T cells are a small subset of cells, whose functions make them attractive candidates for potential immunotherapies. γδ T cells have many innate like properties, and similar to other innate cells, such as NKs, γδ T cells express NK receptor NKG2D and

TABLE 3 | Examples of preclinical research evaluating T cells expressing cytokines for the treatment of malignancies.

Cytokine	Effector cell	Observed effects	References
IL2	SWM specific T cell line 14.1	Autocrine growth without tumor formation, antigen specificity retained	(46)
IL12	Pmel-1 T cells	Enhanced lysis of established melanoma, no toxicities	(47)
IL15	Activated CD4+ and CD8+ lymphocytes	Continued proliferation after cytokine withdrawal, resistance to apoptosis	(48)

TABLE 4 | Examples of preclinical research evaluating T cells expressing chemokine receptors for the treatment of malignancies.

Chemokine receptor	Effector cell	Observed effects	References
CX3CR1	Activated T cells	Enhanced lymphocyte migration and tumor trafficking, significant inhibition of tumor growth	(56)
CCR2	WT1-TCR-modified T cells	CCL2-tropic tumor trafficking, cytocidal reactivity against WT1-expressing cells, augmentation of TCR signaling	(57)
CCR4	CD30 CAR T cells	Enhanced migration to Hodgkin lymphoma cells, sustained cytotoxic function and cytokine secretion *in vitro*, enhanced tumor control *in vivo*	(51)

show cytotoxicity to tumor cells (63). Two groups of γδ T cells are recognized, based on the TCR V delta usage: V delta 1 cells are located in mucosal tissue, and V delta 2 cells are located in the peripheral blood (64). V delta 2 cells are a source of proinflammatory cytokines once activated, including TNF- α and IFN-γ (64). The mechanisms by which γδ T cells recognize cancer are not fully understood. They can recognize tumor antigens via their TCRs and NK receptors, but it is unclear what specific antigens they respond to (65). γδ2 T cells typically recognize pyrophospate antigens produced by bacteria, while γδ1 T cells recognize MHC class I related molecules like MICA/MICB (64). In the cancer setting, it is thought that γδ T cells recognize stress induced self-like antigens, typically expressed by malignant cells and found to infiltrate tumors in some cases (66). These cells appear to mediate a graft vs. tumor response without eliciting GVHD (67).

In pre-clinical studies, γδ T cells have been expanded and have demonstrated cytotoxicity to a variety of tumor cell lines derived from lung carcinoma, liver cancer, and breast cancer, in an MHC-unrestricted manner (66). Deniger et al. demonstrated that they were able to see a 107-fold increase in γδ T cell numbers, despite a small starting population, suggesting it is possible to expand to clinically relevant numbers (68). Another study by Liu et al. show that γδ T cells have the ability to recognize and kill some forms of prostate cancer *in vitro* via innate mechanisms (69). In other preclinical studies, it was demonstrated that γδ T cells could be transduced to generate CAR-T cell products that maintained their natural tumor infiltration and killing abilities (70).

Some clinical trials using these cells are already underway In a Phase I study, autologous γδ T cells were infused in combination with IL-2 into 10 patients with metastatic renal cell carcinoma (mRCC) (71). This trial demonstrated safety, as infusions were tolerated with few serious adverse events related to the immunotherapy, with six patients showing stable disease. In another study, patients with hepatocellular carcinoma were given an injection of γδ T cells (NCT00562666).

Although γδ T cells have been well-tolerated in cancer patients, they are limited by difficulties in their isolation (65), and some questions surround their potential tumor-promoting effects (effects on angiogenesis and secretion of IL-17) (72, 73).

Natural Killer Cells

Natural killer cells were initially identified for their ability to target and kill tumor cells (74). They exhibit cytolytic function through the release of perforin and granzyme B as well as through FasL-TRAIL-mediated pathways, and NK cell activity is governed by a balance of signals from both activating and inhibitory receptors (75–78). NK cells are an possible option for adoptive immunotherapy because they do not require prior antigen exposure to elicit cytotoxicity. In addition, NK cells have limited persistence *in vivo*, a feature that appeals to clinicians and scientists alike. There is preclinical and clinical evidence that NK cells do not cause graft vs. host disease (GVHD) (79–83) or result in systemic toxicities associated with "cytokine storms" seen in T cell therapies (84–86). Similar to other new immunotherapies, an initial roadblock to the clinical use of NK cells was the inability to expand NK cells to clinically relevant numbers.

An additional challenge facing NK cells for adoptive therapy is the immunosuppressive tumor microenvironment, which directly nullifies the cytotoxicity of NK cells (87). Specifically, there is an abundance of immunosuppressive cell types such as myeloid-derived suppressor cells (MDSC) (88–90), tumor-associate macrophages (TAM) (91), and regulatory T cells (Treg) (26, 92–95), as well as cytokines such as transforming growth factor beta (TGFβ) and indoleamine 2,3 dioxygenase (IDO) (26, 96), that have been shown to interact with NK cells and cause phenotypic and functional dysfunction. Many groups have performed preclinical work in order to exploit the anti-tumor, cytotoxic, capabilities of NK cells, while addressing the challenges faced by adoptive cell therapy. For instance, Mentlik et al. focused on these combining NK cell therapy with monoclonal antibodies, boosting NK cell's ability to conduct ADCC (97); these combination therapies with antibodies or cytokines are the focus of other preclinical efforts (98).

Extensive effort has been put into generating and characterizing NK cells for adoptive cell therapy from both primary donor and immortalized NK line donor sources, with mixed results (**Table 5**). As with T cells, there is tremendous appeal for equipping cytotoxic cells with the ability to specifically recognize and kill a given tumor target—as such, there have been multiple attempts at generating CAR-NKs, that retain

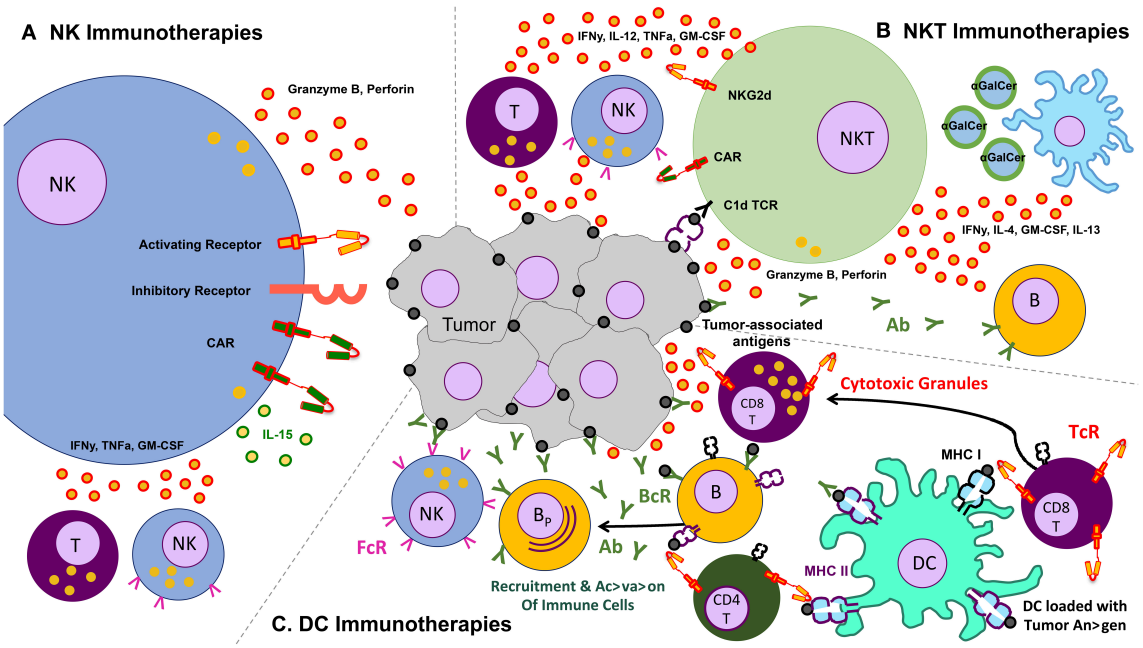

FIGURE 2 | Schematic of various other cells and their effects on the tumor – clockwise from left: **(A)** natural killer (NK) cells, which lyse tumors without the need for identifying a known antigen, working through a balance of inhibitory and activating receptors (and also amenable to transduction with chimeric antigen receptors), as well as secrete cytokines that activate other components of the immune response, **(B)** natural killer T (NKT cells, which recognize lipid antigens and also help orchestrate the immune response, **(C)** dendritic cells (DC) which present antigen to T cells and help jump start immune responses like a vaccine.

their cytotoxicity but are instead directed toward a specific antigen (122). CAR-NKs targeting B cell malignancies have demonstrated impressive *in vivo* cytolytic efficacy (123–126), and represent a promising transition of the technology to the clinic. Other modifications have been incorporated in NK cells—in one such study, cord blood NK cells engineered to express IL15 and a CD19 CAR showed marked increase in survival in a xenograft lymphoma model (127).

To date, three trials with genetically modified primary NK cells, and are currently active (NCT03056339, NCT00995137, NCT01974479). Existing clinical CAR-NK therapies borrow directly from the manufacturing schemes in the CAR-T cell field. One new approach involves substitution of the CD3ζ domain, which initiates TCR-based activation in T cells, with an intracellular domain that is specifically involved in NK cell activation. Indeed, NK-specific activation domains DNAX Activating Protein 10 (DAP10) and 12 (DAP12) have been introduced as the intracellular component in a CAR-NK in preclinical work, and promising results have demonstrated enhanced NK activation and function with this modification (128, 129).

In addition to the abovementioned CAR-NK clinical efforts, multiple clinical trials are underway using infusions of either autologous or allogenic NKs, with more promising results occurring in patients treated with allogenic NKs [reviewed in (130, 131)]. A study by Burns et al. using *ex vivo* activated NKs for treating patients with Hodgkin's and renal cell carcinoma was unable to demonstrate clinical efficacy (132), perhaps due to the autologous donor source. Furthering this claim were the results from multiple groups that demonstrated enhanced NK cell cytotoxicity occurring in patients if there was a killer immunoglobulin receptor-human leukocyte antigen (KIR-HLA) mismatch between donor and recipient cells (83, 93). One of the outstanding challenges for the use of adoptive NK cell therapy pertains to the cells' innate sensitivity to the freeze-thaw process. Indeed, preclinical reports have demonstrated impaired viability and cytotoxicity following cryopreservation (133, 134).

In addition to improving the manufacture end of NK cells therapies, developments are underway that aim to enhance the functionality and persistence of these therapies. For instance, focus for NK cell as well as other cell therapies has shifted toward modulating the suppressive tumor microenvironment concurrently with cell therapy in order to enhance efficacy (26). Moreover, a class of immunomodulatory drugs, such as thalidomide, have been found to modify the NK cells in the tumor environment by upregulating surface expression of TRAIL, which may increase NK-mediated apoptosis of target tumor cells (135–137). Miller et al. are developing bi-specific killer engagers (BiKEs) and tri-specific killer engagers (TriKEs) that can address many of the challenges facing NK cell therapy all in one construct (138–141). They have developed a platform by which NK cells are rendered specific for a given target antigen, while simultaneously increasing NK cell potency and persistence by incorporating CD16 single chain variable fragment (to increase ADCC-associated signaling) and an IL15 moiety (to increase NK activation and thus persistence). These findings that BiKE and TriKE-modified NK cells delivered potent anti-tumor responses in the setting of AML, ALL, and CLL, as well

TABLE 5 | NK cell production methods.

Donor source	Cell population	NK purity	Cell expansion method	References
Umbilical cord blood	CD34+ hematopoietic stem cells	>90%	Differentiation and expansion in bioreactor with GM-CSF, G-CSF, IL-6, IL-7, SCF, IL-15, and IL-2	(99, 100)
Umbilical cord blood	CD34+ hematopoietic stem cells		Differentiated and cultured with IL-15 and MS-5 or OP9 stromal feeder cells	(101)
Umbilical cord blood	CD34+ hematopoietic stem cells	>90%	Cultured with StemRegenin-1,IL-7, SCF, IL-15, and IL-12	(102)
Umbilical cord blood	CD34+ hematopoietic stem cells	>80%	Cultured with high dose cytokine cocktail and OP9 or M2-10B4 feeder cells	(103)
Umbilical cord blood	Total mononuclear cells	90%	Cultured with K562 Clone 9.mbIL21 feeder cells, IL-2, IL-12, IL-15, and IL-18	(104)
Umbilical cord blood	CD56+ NK cells		Cultured with irradiated feeder cells (K562-C9), IL-15, and IL-2	(26)
Peripheral blood	Total PBMC	>80%	Cultured with irradiated feeder cells (K562-C9)	(105)
Peripheral blood	Total PBMC	>55%	Cultured with IL-2 and OKT3	(106–109)
Peripheral blood	Total PBMC	90%	Cultured with OK432, IL-2, and modified FN-CH296 induced T cells	(110)
Peripheral blood	Total PBMC	>80%	Cultured with low dose IL-2 and RPMI 8866 feeder cells	(111, 112)
Peripheral blood	Total PBMC	>90%	Cultured with IL-2 and LAZ388 feeder cells	(113)
Peripheral blood	CD56+ NK cells		Cultured with IL-15 and IL-2	(114)
Peripheral blood	CD5 and CD8 depleted PBMC	>88%	Cultured with high dose IL-2	(115, 116)
NK-92 Cell line	Immortalized NK cell line	100%	Cultured with IL-2	(117, 118)
KHYG-1 Cell line	Immortalized NK cell line	100%	Cultured with IL-2	(119)
NK-YS Cell line	Immortalized NK cell line	100%	Cultured with IL-2 and SPY3-2 feeder cells	(120)
haNK Cell Line	Immortalized NK cell line (based on NK-92)	100%	Cultured with IL-2	(121)

as the extensive number of ongoing clinical trials are only one example of how the field of immunotherapy is rapidly expanding to include a variety of non-T cell-based immunotherapies.

Natural Killer T Cells

NKTs represent an important link between the innate and adaptive immune system, as they can be activated by both antigen dependent and antigen-independent mechanisms. Divided into invariant (iNKT) or diverse (dNKT) subsets, they have a highly restricted TCR repertoire, only recognizing antigen in the context of the MHC class I-like CD1d molecule (142), and are uniquely classified by their ability to rapidly produce regulatory cytokines such as IFNγ, IL4, IL10, IL13, IL-17, GM-CSF, and TNFα in large quantities (143). These characteristics together contribute to the appeal of this cell subset as a form of immunotherapy. Although populations of iNKT cells isolated from cancer patients have been found to be decreased in quantity and defective (144–146), many groups have shown that this impaired phenotype is in fact reversible *ex vivo* (147–150). Additionally, preclinical studies have supported the promise of NKT therapy as a multimodal platform—the glycolipid alpha-galactosylceramide (αGalCer) can reactivate impaired NKTs *ex vivo* to result in restored cytokine production and anti-tumor responses (151–154). Further, inhibition of tumor progression has been demonstrated in models of colon carcinoma, lymphomas, sarcoma, melanoma, prostate cancer, and lung cancer, leading to resurgence of optimism in iNKT cells as agents of immunotherapy.

NKTs are of particular interest as a possible cell for CAR modification for two main reasons: first, because clinical data has indicated better outcomes occurring in patients with higher NKT cell tumor infiltrate (155, 156), and second because the CD1d restricted nature of NKT antigen recognition is able to limit the potential off-target toxicity and increase potential applicability in both the autologous and allogeneic setting (157). Because NKTs secrete a wide range of regulatory cytokines, they are able to both activate antigen presenting cells such as dendritic cells as well as cytotoxic cells such as CD8+ T cells and NK cells—further increasing their value as an agent of immunotherapy (**Figure 2**) (158–163). Heczey et al. generated CAR-modified NKT cells to target neuroblastoma (aGD2 CAR) and lymphoma (aCD19 CAR), with marked success. They found that their CAR NKT cells had highly potent and selective cytotoxic activity against tumor target antigen-expressing cells, and were able to efficiently proliferate and produce large amounts of cytokines in the tumor environment, thus mediating their efficacy (164). Rotolo et al. generated CAR CD19-modified NKT cells to better target CD19-expressing lymphomas that also express CD1d, the ligand for NKT (165).

Many attempts have been made to directly target and restore function to patients' endogenous NKT cells, and current trials are summarized in **Table 6**. This avenue has focused on the infusion of NKT cell activating or stimulating agents, largely αGalCer (166), or by combining these agents with APCs such as dendritic cells to enhance immune activation at the suppressed tumor site (167–173). Dendritic cells can be pulsed with glycolipid and reintroduced into patients, a strategy regularly used in vaccine development,

TABLE 6 | Examples of clinical trials with iNKT cells (35).

Trial ID	Disease	Product
NCT00003985	Solid Tumors	KRN7000 (alpha gal-cer)
NCT00698776	Myeloma	Combination of Lenalidomide and dendritic cells loaded with KRN7000 (alpha gal-cer)
NCT03093688	Advanced Solid Tumors	Infusion of iNKT cells and CD8+T cells
NCT02562963	Non-small cell lung cancer, gastric cancer, hepatocellular carcinoma, colorectal cancer	NKT cells expanded from PBMC
NCT01801852	Breast Cancer, Glioma, Hepatocellular Cancer, Squamous Cell Lung Cancer, Pancreatic Cancer, Colon Cancer, Prostate Cancer	Autologous NKT cells
NCT03198923	Non-small cell lung cancer	NK and NKT cells
NCT03294954	Neuroblastoma	NKT cells genetically modified to express a GD2-CAR
NCT00909558	Breast Cancer, Glioma, Hepatocellular Cancer, Squamous Cell Lung Cancer, pancreatic Cancer, Colon Cancer, Prostate Cancer	Autologous NK or NKT cells
NCT01235845	Malignant Glioma	DC-activated NKT cells and DCs

TABLE 7 | Examples of clinical trials with dendritic cells (35).

Trial ID	Disease	Product
NCT01875653	Metastatic melanoma	Autologous dendritic cells loaded with irradiated autologous tumor cells in GM-CSF
NCT00005947, NCT01133704	Metastatic prostate cancer that has not responded to hormone therapy	Sipuleucel-T
NCT00065442	Metastatic prostate cancer that has not responded to hormone therapy	Sipuleucel-T
NCT00779402	Early stage, non-metastatic prostate cancer	Sipuleucel-T
NCT01582672	Renal cell carcinoma	AGS-003 (Autologous dendritic cell product)
NCT00045968	Glioblastoma multiforme	DCVax-L
NCT01067287	Multiple myeloma	Pidilizumab (CT-011) + Dendritic cell-myeloma fusion vaccine
NCT01096602	Acute myelogenous leukemia (AML)	Dendritic cell-AML fusion vaccine
NCT01441765	Renal cell carcinoma	Pidilizumab (CT-011) + Dendritic cell-renal cell carcinoma fusion vaccine

which has been proven to induce activation and restore function to endogenous NKT cells in a range of cancer types (167, 174–176).

The largest challenge facing the advancement of CAR and non-CAR NKT cell therapies is that of persistence; tumor progression negatively correlates with NKT cell functionality. Attempts to subvert this impairment in NKT function include efforts where autologous NKTs are expanded *ex vivo* with αGalCer prior to reinfusion, as previously described (177). Attempts have been made to classify the phenotype of NKT cells during tumor progression, and CD62L has been identified as a potential indicator of NKT cells most likely to demonstrate enhanced anti-tumor activity (178). Moreover, new approaches to drug or glycolipid delivery systems are currently in development, which aim to package agents causing activation of NKTs in enhanced nanoparticle-based constructs. Examples of this novel immunotherapy "associated agent," such as αGalCer packaged into microspheres or liposomes, have demonstrated enhanced NKT functional responses as compared to the agent alone (179–182). These modifications to CAR and non-CAR NKTs speak to the tremendous promise of generating enhanced clinical NKT therapies.

Dendritic Cells

Dendritic cells (DCs), one of the professional antigen-presenting cells of the immune system, efficiently process antigens for presentation to T cells in order to activate the adaptive immune system (183). DCs naturally play a role in the control of immune responses and immune tolerance, both critical in anti-tumor immunity (183–185). Pre-clinical *in vivo* mouse models of cancer have demonstrated that DCs have the ability to home to tumor sites and capture tumor-associated antigens for processing. These DCs subsequently travel to nearby lymph nodes, where they present tumor antigens to T cells, generating tumor-specific T cells that can lead to clearance or tumor rejection (184, 185). Furthermore, DCs have the unique role of interacting with several subsets of the immune system, including both CD4 and CD8 T cell subsets in lymph nodes, resulting in downstream B cell activation into antibody-secreting cells, as well as activation of NKs and phagocytes. For example, in a murine model of melanoma, it was demonstrated that DCs interact with both cytotoxic T cells and NK cells to mediate tumor elimination (186, 187). However, NK depletion resulted in no tumor elimination, emphasizing the importance of DC-NK interactions in anti-tumor immunity (186, 187). This ability to interact with

and regulate multiple immune cells make DCs an interesting candidate cell subset to be used in immunotherapy trials.

Due to their natural role in antigen processing and presentation, dendritic cells have been used in multiple Phase III clinical trials as an adjuvant or therapeutic vaccine for certain cancers including metastatic melanoma (NCT01875653), prostate cancer (below), renal cell carcinoma (NCT01582672), and glioblastoma multiforme (NCT00045968) (188, 189) (**Table 7**). The main objective of these studies was to deliver tumor antigens via DCs to stimulate and activate anti-tumor antigen-specific T cells, which subsequently eliminate cancerous cells and provide immunological memory to prevent tumor relapse. Furthermore, it has been demonstrated that the induction of anti-tumor T cell responses from DC-immunotherapies concurrently enhances natural killer immunity (187), underscoring the importance of DCs in regulating multiple immune cell subsets (**Figure 2**).

One of the significant advantages with DC-based immunotherapies is the demonstration of safety across multiple clinical trials (188–191), with promising efficacy shown in certain cancer settings. For example, in a Phase 3 IMPACT study for prostate cancer, DC-based therapy, sipuleucel-T, demonstrated significantly better survival by 4 months, for patients with metastatic hormone-resistant prostate cancer compared to the placebo group (191). Multiple Phase 3 prostate cancer studies (NCT00005947, NCT00065442, NCT00779402, NCT01133704) with DC-immunotherapy sipuleucel-T have shown induction of antigen-specific immune responses correlate with better survival in patients (190–194) (**Table 7**). Because of observed improvements in survival, sipuleucel-T was FDA approved in 2010. It is interesting to note that this coincides with <5% patients achieving an objective response, or tumor reduction over time.

Current clinical strategies are looking to optimize DC immunotherapy through combinations with other agents, in an effort to improve tumor burden. For example, the immunosuppressive tumor environment may prevent DCs from effectively activating cytotoxic T cells and NK cells to eliminate the tumor. Consequently, immune checkpoint inhibitors such as pidilizumab, are currently being explored in combination with DC immunotherapies (NCT01067287, NCT01096602, NCT01441765) for multiple myeloma, acute myelogenous leukemia (AML), and renal cell carcinoma, in an effort to enhance activation of tumor-specific cytotoxic T cells by DCs (195, 196) (**Table 7**). Ultimately, DC immunotherapies have shown promise in certain cancer settings, and have the advantage of interacting with numerous immune cell subsets to mediate anti-tumor immunity. The efficacy of these DC immunotherapies may be improved upon through combination strategies with other agents and the targeting of immunosuppressive barriers to tumor eradication.

CONCLUSIONS

CAR T cell therapies have demonstrated the clinical benefits of harnessing our body's own defenses to combat tumor cells. Similar research is being conducted on lesser known modifications and gene-modified immune cells. Promising preclinical and clinical results point to a likely establishment of these therapeutics as another treatment modality against cancer. Because the field is a recent one, it is necessarily disjointed: different groups focus on their preferred immune effector and seldom compare efficacy with others, much less look at potential combinations. By presenting this review, the authors hope that researchers become more familiar with what is out there—and hope that more efforts at head-to-head comparisons between therapies and combination therapies (which is how the immune system is supposed to act) be explored.

AUTHOR CONTRIBUTIONS

SP and CC conceptualized the review and made edits to the manuscript. SP, RB, EC, AP, SV, NH, SM, and CC wrote the body of the text. PH and CC made final edits to the manuscript.

FUNDING

Part of the work was supported by the Alex's Lemonade Stand Foundation A Award, given to CC.

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Conflict of Interest Statement: PH and CC are co-founders of Mana Therapeutics, a biotech startup focusing on cell therapies. PH is a member of the board, and CC is a member of the scientific advisory board.

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

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ORIGINAL RESEARCH
published: 11 April 2019
doi: 10.3389/fimmu.2019.00761

NIH3T3 Directs Memory-Fated CTL Programming and Represses High Expression of PD-1 on Antitumor CTLs

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Memory CD8$^+$ T cells have long been considered a promising population for adoptive cell therapy (ACT) due to their long-term persistence and robust re-stimulatory response. NIH3T3 is an immortalized mouse embryonic fibroblast cell line. We report that NIH3T3-conditioned medium (CM) can augment effector functions of CTLs following antigen priming and confer phenotypic and transcriptional properties of central memory cells. After NIH3T3-CM-educated CTLs were infused into naïve mice, they predominantly developed to central memory cells. A large number of NIH3T3-CM-educated CTLs with high functionality persisted and infiltrated to tumor mass. In addition, NIH3T3-CM inhibited CTLs expression of PD-1 *in vitro* and repressed their high expression of PD-1 in tumor microenvironment after adoptive transfer. Consequently, established tumor models showed that infusion of NIH3T3-CM-educated CTLs dramatically improved CTL mediated-antitumor immunity. Furthermore, NIH3T3-CM also promoted human CD8$^+$ T cells differentiation into memory cells. These results suggest that NIH3T3-CM-programmed CTLs are good candidates for adoptive transfer in tumor therapy.

Keywords: NIH3T3-CM, cytotoxic T lymphocytes, memory precursor, memory CD8$^+$ T cells, adoptive cell therapy

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Edited by:
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Reviewed by:
Graham Robert Leggatt, University of Queensland, Australia
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: *26 October 2018*
Accepted: *21 March 2019*
Published: *11 April 2019*

Citation:
Qin Y, Lee Y, Seo J, Kim T, Shin JH and Park S-H (2019) NIH3T3 Directs Memory-Fated CTL Programming and Represses High Expression of PD-1 on Antitumor CTLs. Front. Immunol. 10:761. doi: 10.3389/fimmu.2019.00761

INTRODUCTION

Adoptive cell therapy (ACT) using autologous tumor reactive T cells has emerged as a potentially curative therapy for cancers (1–3). Cytotoxic T lymphocytes (CTLs) are considered as potent lymphocytes that can perform direct lysis of target tumor cells precisely. Nevertheless, a significant limitation for ACT is that repeated stimulation alters functional capabilities of CTLs which can result in defects in survival and function after transfusion (4, 5). Many studies have suggested that memory T cells are superior to effector T cells in antitumor activity due to their long-term persistence and more robust effector functions in response to tumor antigens (6–9). One prominent notion that has been accepted is that once naïve CD8$^+$T cells are primed, the majority of effector CTLs will die via differentiation into short-lived effector cells (SLECs) while only a small subset will differentiate into memory precursor effector cells (MPECs) destined to become long-lived memory cells (10–13). In this regard, exploring a proper culture condition to direct the differentiation of tumor-specific CD8$^+$ T cells to MPECs may be a promising approach to develop a curative antitumor therapy upon adoptive transfer.

Fibroblasts are heterogeneous tissue connecting cells that play critical roles in wound healing. Fibroblasts are also responsible for the production of extracellular matrix molecules that can act as co-stimuli for T lymphocyte activation (14, 15). Soluble factor(s) secreted by fibroblasts from malignant or non-malignant tissue can enhance T cell IFN-γ and IL17A production (16). Fibroblasts derived factor(s) can also inhibit activation-induced apoptosis of T cells (17, 18). Given these comprehensive effects of fibroblasts on T cells, altering the fate or intrinsic functions of T cells by fibroblasts might have potential to be utilized in an *in vitro* culture system for ACT. Our previous report has shown that soluble factor(s) derived from mouse embryonic fibroblast (MEF) can strongly enhance the effector function of $CD8^+$ T cells (19). NIH3T3 is an immortalized embryonic fibroblast cell line. NIH3T3 cells are widely used as feeders to support long-term survival and self-renewal of tissue progenitor cells (20, 21). In this regard, we sought to investigate whether NIH3T3 could affect the function or the fate of $CD8^+$ T cells during antigen priming in co-culture conditions. We found that NIH3T3-conditioned medium (NIH3T3-CM) directed $CD8^+$ T cells toward differentiation of potent memory-fated effector clones. NIH3T3-CM not only strengthened effector functions of $CD8^+$ T cells, but also conferred characteristics of memory cells. Using adoptive transferred model, we experimentally demonstrated that NIH3T3-CM could program CTLs with high capacity in development of long-lived memory cells. In addition, using established tumor model, we found that adoptive transfer of NIH3T3-CM-educated CTLs exhibited dramatical therapeutic effects. This is not only attributed to high persistence and functions of CTLs, but also due to their low expression of PD-1.

MATERIALS AND METHODS

Mice and Cells

Wild type C57BL/6 mice (WT B6, $Ly5.2^{+/+}$) and ovalbumin $(OVA)_{257-264}$-specific TCR (Vα2 and Vβ5) transgenic mice (OT-1) maintained on B6 background were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). $Ly5.1^{+/-}$ ($Ly5.1^+Ly5.2^+$) OT-1 mice were obtained from OT-1 mice that were crossed to congenic $Ly5.1^{+/+}$ B6 mice. $Ly5.1^{+/-}$ OT-1 mice were backcrossed with B6 ($Ly5.1^{+/+}$) to obtain $Ly5.1^{+/+}$OT-1 mice. All mice were 7–9 weeks old at the beginning of each experiment. They were raised in a specific pathogen-free environment at Korea University. Experimental protocols adopted in this study were approved by the Institutional Animal Care and Use Committee of Korea University.

NIH3T3 cells were purchased from ATCC. EG.7 tumor cells expressing chicken OVA were provided by Dr. M. Mescher (University of Minnesota, Minneapolis, MN, USA). Human peripheral blood mononuclear cells (PBMCs) were purchased from ImmunoSpot. T2 cells were obtained from ATCC. NIH3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco). EG.7 cells, T2 cells, and primary lymphocytes were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco). Both culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco), 2 mM L-glutamine, 1% penicillin-streptomycin, 10 μg/mL gentamycin, and 50 μM β-mercaptoethanol (Gibco-BRL). NIH3T3-conditioned medium (CM) was obtained by seeding NIH3T3 cells at density of 1.25×10^5 cells/ml in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin, 10 μg/mL gentamycin, and 50 μM β-mercaptoethanol and cultured for 2–3 days. CM was then collected by centrifuging at 400 g for 5 min followed by filtration through a 0.22 μm pore size filter. It was then stored at −85°C.

In vitro T Cell Activation

$CD8^+$ T cells were sorted from OT-1 or WT splenocytes with a MACS column using anti-mCD8α magnetic beads (Miltenyl Biotec). The purity of sorted OT-1 cells was >95%. For K^b-OVA beads preparation, 1 μg of $OVA_{257-264}$ (Genscript) loaded biotinylated recombinant MHC class I molecules (H2-K^b), 0.3 μg of biotinylated anti-CD28 antibodies, and 0.05 μg of streptavidin magnetic beads [NEB, S1420S] were incubated at 4°C overnight with rotation. Then $0.5–1 \times 10^5$ enriched OT-1 $CD8^+$ T cells were stimulated with K^b-OVA beads in the presence or absence of NIH3T3-CM (v/v, 50%) in 96-well plates at indicated time points for *in-vitro* analysis. For adoptive transfer, 3×10^5 OT-1 $CD8^+$ T cells were stimulated with K^b-OVA beads in the presence or absence of NIH3T3-CM (v/v, 50%) in 48-well plates and 3×10^5 WT $CD8^+$ cells were stimulated with plate bounded anti-CD3/CD28 in 48-well plates. After 3 days of culture, cells were harvested and washed twice with PBS for adoptive transfer. For whole splenocyte activation, 2×10^5 splenocytes were stimulated with 100 ng/ml $OVA_{257-264}$ peptides for 2 days in 96-well plates. Goligistop was used for treatment for 4 h before intracellular staining. Human naïve $CD8^+$ T cells were sorted from PBMCs using a naïve $CD8^+$ T cell isolation kit (Miltenyi Biotec). Then 1×10^5 purified naïve $CD8^+$ cells (purity >95%) were activated by plate-coated anti-CD3 (OKT3, 1 μg/ml) and anti-CD28 (CD28.2, 3 μg/ml) in the presence or absence of NIH3T3-CM in the 96-well plates. On day 3, 20 U/ml hIL-2 (animal-free, PeproTech) was added. Purified naïve $CD8^+$ cells (HLA-A*0201) (2×10^5) were activated by 1×10^6 CMV-pp65 peptide (NLVPMVATV)-loaded autologous PBMC (2000 rad γ irradiation) in 96-well in the presence or absence of NIH3T3-CM. On day 3, 100 U/ml hIL-2 was added. Expanded cells were moved to 48-well plate on day 5. Medium was refreshed every 2 to 3 days. Cells were collected on day 12 and FACS analysis was performed.

Trans-well Assay

A trans-well insert (Corning) with diameter of 6.5 mm and pore size of 0.4 μm was utilized to physically separate OT-1 $CD8^+$ T cells (1×10^5, upper chamber) from NIH3T3 (1×10^5, lower chamber) while soluble factors were allowed to diffuse into the upper chamber. OT-1 $CD8^+$ T cells together with NIH3T3 were added to the upper chamber. After 2 days of stimulation with K^b-OVA beads, IFN-γ and granzyme B producing cells were detected by flow cytometry after intracellular staining.

In vitro Cytotoxicity Assay

Specific killing of target tumor EG.7 was measured using CFSE/7-AAD based flow cytometry assay as described previously (22).

Briefly, effector cells (splenic OT-1 $CD8^+$ T cells) were activated by K^b-OVA beads for 3 days in the presence or absence of NIH3T3-CM (50%, v/v) and labeled with CFSE. CFSE labeled effector cells were then incubated with target cells at effector: target (E:T) ratios of 8:1, 4:1, 2:1, 1:1, or 0:1 at 37°C incubator with 5% CO_2. After 4 h of incubation, cells were washed and stained with 7-AAD to assess dead cells on CFSE negative cells via flow cytometry. The percentage of specific lysis was calculated as follows: %lysis = 100 × (% sample lysis–% basal lysis) /(100–% basal lysis), where basal lysis was lysis of target cells in the absence of effectors. For T2 killing assay, 1×10^6 T2 cells as targets were loaded with 5 μg/ml CMV peptides followed by 30 μM calcein-acetyoxymethyl dye (Invitrogen) treatment. Effector cells obtained from CMV-pp65 specific expansion were incubated with CMV-pp65 peptide-loaded T2 cells at E:T ratio of 10:1, 5:1 or 2.5:1 at 37°C in an incubator with 5% CO_2 for 4 h.. The fluorescence of sample supernatant was measured. The percentage of specific lysis was calculated as follows: %lysis= 100 × (sample release-SP) /[MAX + (MM -MT)–SP], where target cells treated with 2% Triton X-100 represented MAX (Maximum release), SP (Spontaneous release) represented target cell alone, MM represented background of medium, and MT represented background of Triton X-100.

Antibodies and Flow Cytometric Analysis

For flow cytometric analysis, cells were incubated with anti-Fcγ receptor antibody (2.4G2) generated from mice acsite and then labeled with the following antibodies: anti-mouse antibodies TCRβ-Fluorescein isothiocynate (FITC, H57-597), CD25-FITC (7D4), CD69-FITC (H1.2F3), Ly5.2- Phycoerithrin (PE, 30-F11) or FITC (30-F11), TCRVα2-PE (B20.1), CD127-PE (A7R34), CD27-PE (LG.7F9), Tim3-PE (RMT3-23), CD8α-PerCP-Cyanine5.5 (53-6.7), B220-PerCP-Cyanine5.5 (RA3-6B2), KLRG1-PE.Cy7 (2F1), TCRVβ5.1,5.2-Allophycocyanin (APC, MR9-4) or FITC (MR9-4), CD44-APC (1M7) or PE (1M7), CD62L-APC (MEL-14) or APC.Cy7 (MEL-14), streptavidin-PE or APC or APC.Cy7, CD28-biotin (37.51), CD122-biotin (5H4), PD-1-biotin (29F.1A12), Ly5.1-biotin (A20) or FITC (A20), and K^b-$OVA_{257-264}$-biotin-streptavidin-PE (Lab made); anti-human antibodies CD3-APC (RE613), CD45RA-PE-vio770 (T6D11), CD45RO-FITC (UCHL1), and CCR7-APC efluor780 (3D12).

Intracellular molecule expression was determined following fixation and permeabilization with either Cytofix/Cytoperm (BD Biosciences) or FoxP3/Transcription Factor Staining Buffer Set (eBioscience) with anti-mouse granzyme B-PE (NGZB), Eomes-PE (Dan11mag), Bcl-6-PE (K112-91), IFN-γ-APC (XMG1.2), T-bet-APC (4B10, also react to human), Blimp-1-Alexa Fluor 647 (5E7), or anti-human Eomes PE (WD1982). Flow cytometry was performed using FACSVerse or FACSCalibur device (BD Biosciences). Data were analyzed using FlowJo_V10 (FlowJo LLC).

Adoptive Transfer Studies

For cell persistence assay, $Ly5.1^{+/+}$ OT-1 CTLs (2.4×10^6) generated in RPMI-1640 medium and $Ly5.1^+Ly5.2^+$ OT-1 CTLs (1.6×10^6) generated in the presence of NIH3T3-CM (50%, v/v) were intravenously (i.v.) co-transferred to $Ly5.2^{+/+}$ B6 WT mice. The persistence of transferred cells in peripheral blood was detected on days 3, 7, 15, and 30. On day 30, frequencies of transferred OT-1 cells that migrated to spleen, inguinal lymph node, bone marrow, and lung were measured.

Tumor Rejection Assay

To evaluate tumor reactivity of memory $CD8^+$ T cells, OT-1 CTLs generated in the presence or absence of NIH3T3-CM (50%, v/v) were i.v. injected to WT B6 mice (1×10^6/mouse). One month later, OVA-expressing EG.7 tumor cells (2×10^5/mouse) were subcutaneously (s.c.) transferred to mice and tumor growth was monitored.

To detect tumor reactivity to established tumors, EG.7 tumor cells ($0.5–0.7 \times 10^5$/mouse) were s.c. inoculated to WT B6 mice. When tumor grew to 30–50 mm^3 (usually between 12 to 14 days), CTLs were i.v. transferred to tumor-bearing mice. Tumor sizes were measured every 2 to 4 days. IL-2 co-administration was performed as described previously (23). Briefly, OT-1 CTLs were i.v. transferred to tumor-bearing mice and 2 μg hIL-2 (animal-free, PEPROTECH) was i.p. (intraperitoneal injection) injected to mice once on the day of CTL injection and twice a day on the two following days. Tumor sizes were calculated by determining the length of short (l) and long (L) diameters (tumor volume = $l^2 \times L/2$). Experimental endpoints were reached when tumor volume exceeded 2,500 mm^3. For transferred CTLs detection, EG.7 tumor cells ($1–1.5 \times 10^5$/mouse) were s.c. transferred to WT B6 mice. When tumor grew to 50–100 mm^3, 1×10^6 *in vitro*-generated $Ly5.1^{+/--}$ OT-1 CTLs in the presence or absence of NIH3T3-CM (50%, v/v) or combination of $Ly5.1^{+/+}$ OT-1 CTLs (medium alone) and $Ly5.1^+Ly5.2^+$OT-1 CTLs (NIH3T3-CM, 50%, v/v) at a ratio of 1:1 were i.v injected to tumor-bearing mice. At 6 days after T cell transfer, mice were sacrificed and analyzed.

Statistical Analysis

Statistical analysis was performed using Prism software (GraphPad Prism6.0). Statistically significant differences were assessed by either unpaired *t*-test or one-way ANOVA or two -way ANOVA with Bonferroni's multiple comparison test. Log-rank (Mantel-Cox) test was used to analyze mouse survival curves. Bars in all graphs are expressed as mean ± SEM. "ns" denotes no significance. Significance was marked by asterisk (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and ****$P < 0.0001$).

RESULTS

NIH3T3-CM Enhances Effector Functions of $CD8^+$ CTLs

Fibroblasts with different subsets are functionally heterogeneous (either stimulatory or inhibitory) on T lymphocytes (24). Our previous report has shown that MEFs can significantly enhance effector functions of CTLs through up-regulation of IFN-γ and granzyme B expression (19). Therefore, we determined whether NIH3T3 enhanced effector functions of CTLs upon TCR stimulation. Co-culturing $CD8^+$ T cells with NIH3T3 using trans-wells showed that NIH3T3-enhanced IFN-γ and granzyme B expressions of $CD8^+$ T cells were independent of direct contact (**Supplementary Figure 1**). Thus, NIH3T3-CM was focused. To further analyze the impact of NIH3T3-CM on $CD8^+$ T cells during priming, kinetic patterns of IFN-γ

and granzyme B productions were evaluated (**Figure 1A**). We found that NIH3T3-CM strongly promoted antigen induced IFN-γ expression in an acute manner within 24 h. The level of IFN-γ peaked at 24 h and then declined thereafter. It seemed that NIH3T3-CM could expedite kinetic IFN-γ production of CTLs compared to that of medium alone-cultured CTLs which peaked at 72 h (**Figure 1A**, top panel). Contrast to acute enhancement in IFN-γ production, NIH3T3-CM continuously enhanced granzyme B expression. The level of granzyme B in NIH3T3-CM-cultured CTLs peaked at 48 h after TCR stimulation and declined thereafter. The frequency of granzyme B producing CTLs peaked at 48 h in the condition of medium alone culturing while granzyme B levels per cell fluctuated modestly (**Figure 1A**, bottom panel). Next, the expressions of typical surface molecules associated with T cell differentiation in NIH3T3-CM-cultured CTLs were compared with those in medium-alone-cultured CTLs. After 3 days of TCR stimulation, expressions of CD25 (IL2Rα), common β receptor CD122 (IL-2β, IL-15β), and CD28 were increased significantly while expressions of CD69 (early inducible activation marker) and CD44 increased modestly (**Figure 1B**). The level of "lymph homing receptor" CD62L (L-selectin), a naïve and central memory marker, was decreased on day 1 after TCR stimulation. It was then up-regulated thereafter. Surprisingly, the level of CD62L was much higher on NIH3T3-CM-cultured CTLs (**Figure 1B**). We also examined whether NIH3T3-CM could enhance the proliferative capacity of CTLs. After 3 days of stimulation, cell numbers of NIH3T3-CM-cultured CTLs were higher than those of medium-cultured CTLs (**Figure 1C**). Enhanced cytotoxic activity was also observed in NIH3T3-CM-cultured CTLs (**Figure 1D**). Taken together, these results suggest that NIH3T3-CM can strengthen effector functions of CTLs following antigen priming.

NIH3T3-CM Improves the Intrinsic Quality of CTLs Which May Acquire Memory-Fated Potential

As shown in **Figure 1B**, CD62L was expressed much higher on NIH3T3-CM-cultured $CD8^+$ T cells. Prompted by this result, we investigated whether NIH3T3-CM directed the differentiation of antigen-primed $CD8^+$ T cells toward memory type cells. First, we determined whether NIH3T3-CM might have dose-dependent effects on surface expression level of CD62L on CTLs. Results showed that expression levels of CD62L were directly correlated with treated amount of NIH3T3-CM (**Figure 2A**). Consequently, higher frequency of $CD44^{++}CD62L^{++}$ population representing the phenotype of central memory cells was observed in NIH3T3-CM-cultured CTLs (**Figure 2B**). Consequently, NIH3T3-CM enhanced effector functions of CTLs along with enhancement of central memory markers such as CD44, CD62L (**Figure 2B**), and CD122 (**Figure 1B**). In this context, we questioned whether NIH3T3-CM-programmed CTLs were memory-fated effector clones. Accumulating evidences have established that T cells can integrate modulated signals during priming to determine their fate (MPECs or SLECs) (10, 12, 13). Hence, we investigated whether NIH3T3-CM-cultured CTLs were also phenotypically consistent with MPECs [$CD27^{++}$CD127(IL-7Rα)$^{++}$KLRG1 (markers of senescent $CD8^+$T cells)$^-$]. Phenotypic analysis showed that NIH3T3-CM-cultured CTLs displaying $CD27^{++}CD127^{+/--}KLRG1^-$ were partially in line with MPECs. However, NIH3T3-CM did not significantly regulate expression of CD127, CD27, or KLRG1 as their expression levels were comparable with those of medium alone-cultured CTLs (**Figure 2B**). Collectively, these results suggest that high level of central memory markers (CD44 CD62L and CD122) and high level of CD27 as well as intermediate level of CD127 might give NIH3T3-CM-cultured CTLs potential in differentiation of memory cells, although the phenotype of NIH3T3-CM-cultured CTLs is not totally consistent with what is generally considered MPECs.

Next, we explored whether NIH3T3-CM influenced transcriptional programming in $CD8^+$ T cells following antigen priming. T-box transcription factors, T-bet, and eomesodermin (Eomes) play essential roles in inducing $CD8^+$ T cells acquisition of effector functions and formation of memory pools (25–28). To investigate whether NIH3T3-derived factor(s) influenced the expression of T-box transcription factors, we examined expression levels of T-bet and Eomes during 72 h of TCR stimulation (**Figure 2C**). After 72 h of stimulation, NIH3T3-CM strongly induced CTLs expression of Eomes. In striking contrast, medium alone-cultured CTLs expressed Eomes at low levels without showing significant difference between 24 h and 72 h of stimulation. Compared to strongly induced Eomes by NIH3T3-CM in CTLs, there was no significant enhancement of T-bet level in response to NIH3T3-CM treatment. Given the notion that Eomes expression appears to preferentially increase relative to T-bet in memory cell differentiation and homeostasis (12, 25, 28, 29), we performed analysis of mean fluorescence intensity (MFI) for Eomes and T-bet. Results showed that NIH3T3-CM-cultured CTLs had much higher ratio of Eomes to T-bet compared to medium cultured CTLs (**Figure 2C**, right panel). These data indicate that Eomes might play a role in NIH3T3-CM-induced enhancement of effector function as well as in conferring potentials of memory cell differentiation. To investigate whether NIH3T3-CM regulated other transcriptional factors involved in the control of memory cell differentiation, we analyzed another pair of antagonistic transcription factors: Bcl-6 and Blimp-1. It has been well-characterized that Bcl-6 controls memory cell differentiation while Blimp-1 is crucial for effector function acquisition, including cell proliferation, cytotoxicity, and cytokine production (30–33). After 72 h of TCR stimulation, NIH3T3-CM slightly enhanced levels of Bcl-6 and Blimp-1 (**Figure 2D**). Collectively, these results indicate that NIH3T3-CM can affect intrinsic transcriptional events of CTLs which helps differentiation, effector function, and memory development of CTLs.

Vigorous response of CTLs after re-encountering antigens reflects the potential of CTLs in memory cell differentiation. Therefore, we examined the re-stimulatory activity of CTLs programmed by NIH3T3-CM following initial priming (**Figure 2E**). The production level of granzyme B was

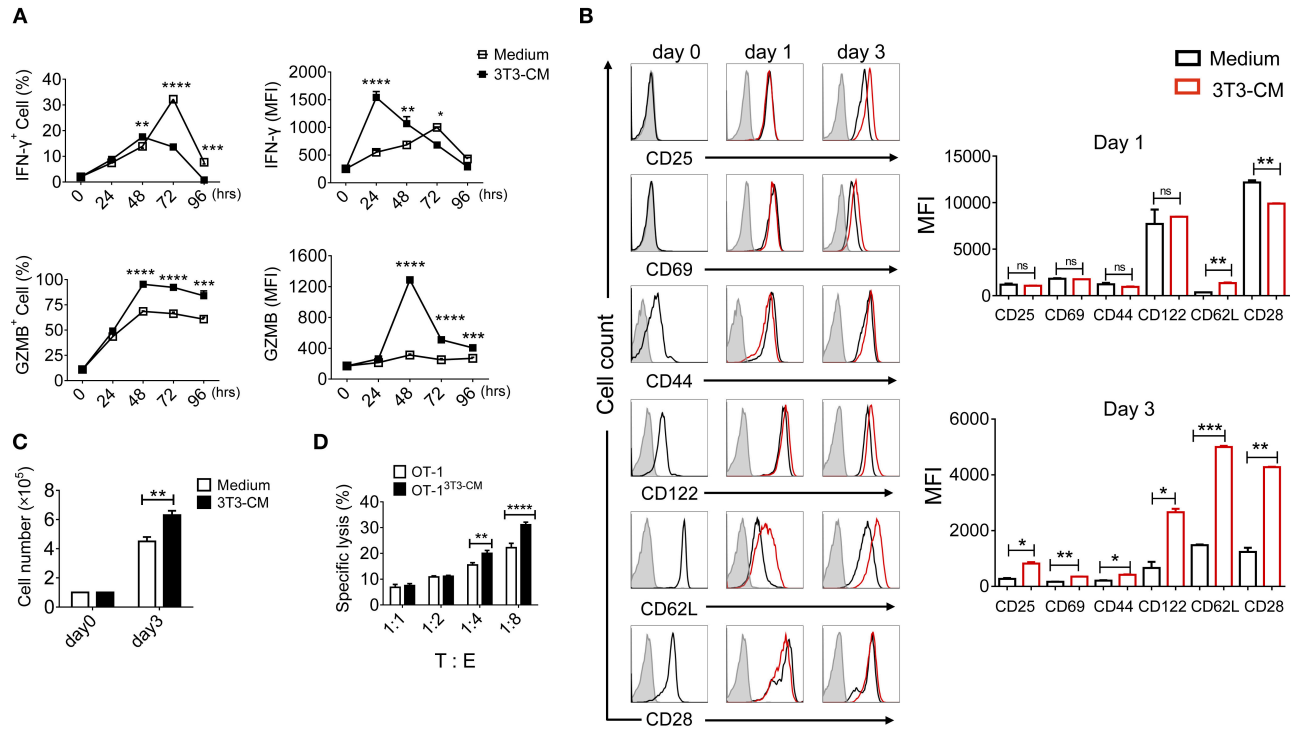

FIGURE 1 | NIH3T3-CM strengthens effector functions of CTLs. OT-1 $CD8^+$ T cells were stimulated with K^b-OVA beads in the presence or absence of NIH3T3-CM (50%, v/v). **(A)** Intracellular flow cytometric determination of expression levels of IFN-γ and granzyme B (GZMB) at indicated time points. Frequencies of IFN-γ or GZMB positive $CD8^+$ T cells are shown (left panel). MFI (mean fluorescence intensity) of IFN-γ or GZMB was plotted for IFN-γ^+ or $GZMB^+$ cells (right panel). **(B)** Levels of surface molecules were determined by flow cytometry at indicated time points. Left, representative histograms. Right, MFI values. The gray histogram as a background represents cells that are not stained. **(C)** Cell numbers were counted after 3 days of stimulation. **(D)** Specific cytotoxicity of OT-1 CTLs generated in the presence or absence of NIH3T3-CM against EG.7 cells was evaluated at the indicated effector: target (E:T) ratios. All data are representatives of at least three independent experiments. Data are presented as mean ± SEM; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Two-way ANOVA with Bonferroni's multiple comparison tests.

comparable between the two different pre-cultured T cells. However, NIH3T3-CM-educated CTLs dramatically produced higher level of IFN-γ than medium alone-cultured CTLs after re-encountering antigens (**Figure 2E**, right). Consequently, high frequency of IFN-γ^+ granzyme B^+ cells were obtained in NIH3T3-CM-educated CTLs (**Figure 2E**, left).

Based on these findings, we conclude that CTLs derived with NIH3T3-CM displaying a phenotype of $CD44^{++}CD62L^{++}CD122^{++}CD25^{++}CD27^{++}CD127^{+/-}$ and $KLRG1^-$ as well as transcriptional program of $Eomes^{++}T\text{-}bet^{++}Bcl\text{-}6^+Blimp\text{-}1^+$ possess high effector function and vigorous re-stimulatory activity. These characteristics implicate that NIH3T3-CM can enhance intrinsic properties of CTLs and direct development of memory precursors following antigen priming.

NIH3T3-CM Educated CTLs Can Develop to Long-Lived Memory Cells

To determine whether NIH3T3-CM-programmed effector clones with memory characteristics could further differentiate to long-lived memory cells, we co-transferred both NIH3T3-CM-educated OT-1 CTLs and medium alone-cultured OT-1 CTLs into WT B6 recipients and analyzed the persistence potential of infused cells using their congenic markers Ly5.1 and Ly5.2. Kinetic frequencies of transferred cells in peripheral blood were detected. During 30 days of examination, frequencies of NIH3T3-CM-educated CTLs showed gradual decline. In striking contrast, frequencies of medium alone-cultured CTLs showed rapid decline (**Figures 3A,B**). Consequently, very low number of medium alone-cultured CTLs survived in peripheral blood whereas higher frequency of NIH3T3-CM-cultured CTLs persisted until 30 days after cell transfer (**Figures 3A,B**). The persistence of co-transferred OT-1 cells in other tissues was also analyzed. Consistent with the higher frequency of NIH3T3-CM-educated CTLs that persisted in blood, much higher frequencies of NIH3T3-CM-educated CTLs also homed to lymphoid organs (lymph node, spleen and bone marrow) and non-lymphoid organ (lung) compared to those of medium alone-cultured CTLs (**Figures 3C,D**). Phenotypic analysis showed that the majority of persisted cells displayed central memory markers ($CD44^{++}CD62L^{++}$) (**Figures 3C,E**). To determine the stimulatory activity of memory cells, whole splenocytes were re-stimulated *in vitro*. In response to $OVA_{257-264}$ peptide stimulation, we observed high levels of IFN-γ and granzyme B productions in memory cells derived from NIH3T3-CM-educated OT-1 cells. The number of memory OT-1 cells deriving

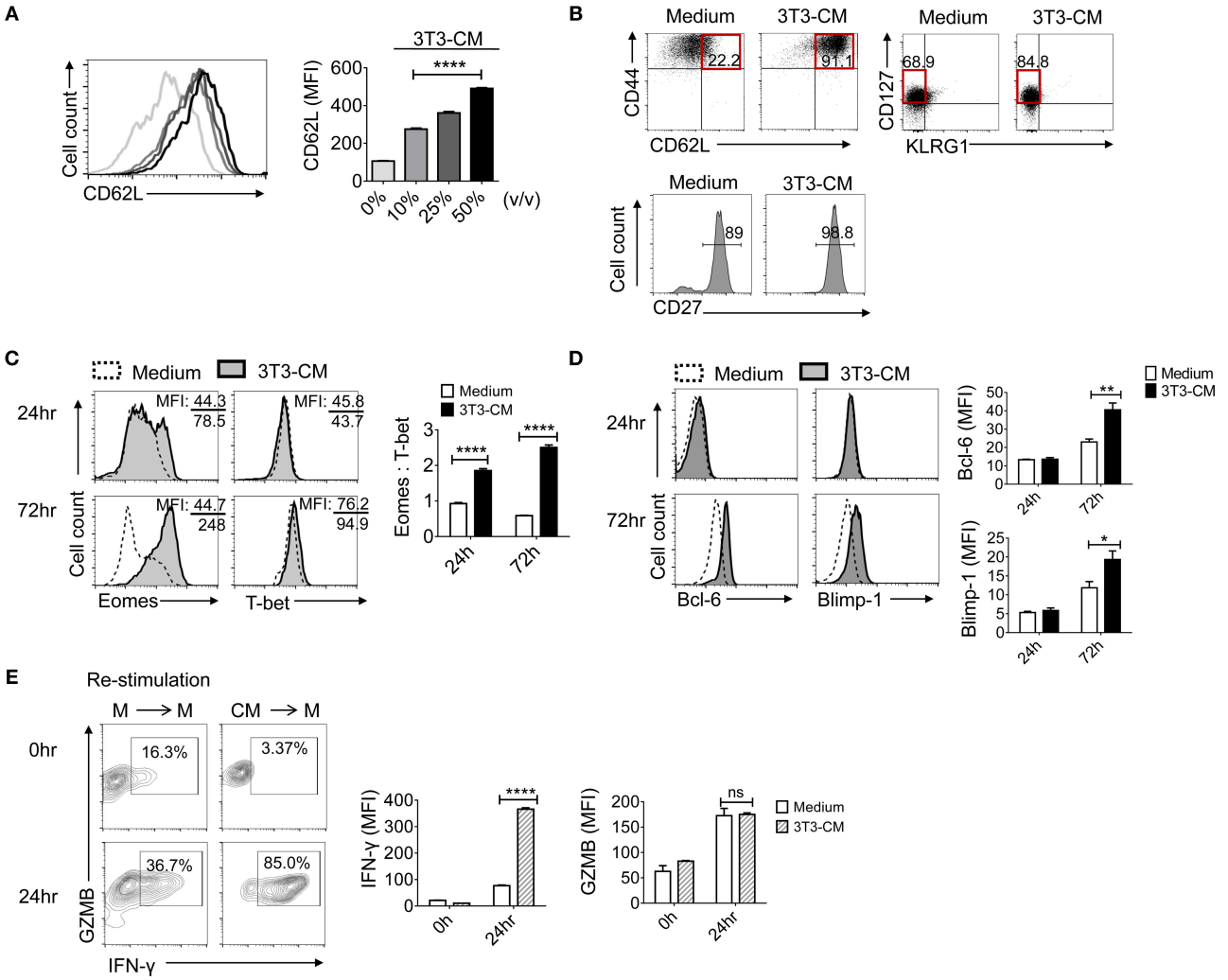

FIGURE 2 | NIH3T3-CM confers potential of memory cell differentiation to CTLs**. (A)** Sorted OT-1 CD8$^+$ cells were stimulated with different volumes of NIH3T3-CM and CD62L levels on cell surface were measured on day 3. **(B)** Typical surface markers representing central memory cells and MPECs were evaluated after 3 days of stimulation. **(C,D)** Transcriptional factors were examined by intracellular FACS analysis after 24 h and 72 h of stimulation. Representative FACS data are shown (**C**, left). The ratio of Eomes to T-bet was calculated by their MFI (**C**, right). Representative FACS data are shown (**D**, left). Quantification of Bcl-6 and Blimp-1 was plotted respectively (**D**, right). **(E)** OT-1 CD8$^+$ cells were stimulated in the presence or absence of NIH3T3-CM for 4 days. CTLs were then washed and re-stimulated in the absence of NIH3T3-CM for another 24 h followed by intracellular FACS analysis. M, RPMI-1640 medium; CM, NIH3T3-CM. Representative FACS data are shown (left panel). Quantification of IFN-γ and GZMB was plotted respectively (right panel). All data are representatives of at least three independent experiments. Data are presented as mean ± SEM; $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. One-way **(A)** or Two-way **(C–E)** ANOVA with Bonferroni's multiple comparison tests.

from control medium-cultured CTLs was below detectable level (**Figure 3F**). The hallmark of memory CD8$^+$ T cells is their ability to generate protective immune response when re-exposed to antigens (34). To further assess the anti-tumor immunity of memory CD8$^+$ T cells derived from NIH3T3-CM-educated CTLs responding to tumors, OT-1 CTLs generated in the presence or absence of NIH3T3-CM were transferred to B6 WT mice. One month later, large dose of tumor cells were inoculated to mice. It was observed that tumor growth was further inhibited in the group that received NIH3T3-CM-educated OT-1 cells compared to the group received medium alone-cultured OT-1 cells (**Figure 3G**). Taken together, these two adoptive transfer experiments demonstrated that NIH3T3-CM-programmed CTLs had high potential to develop into long-lived memory cells which could efficiently protect mice from tumor growth.

NIH3T3-CM-Educated CTLs Regress Tumor Growth Effectively

Long-term persistence and function of transferred cells are crucial factors in ACT for cancers (4, 5). Therefore, we assessed therapeutic effects of NIH3T3-CM-educated CTLs using an established tumor model. *In vitro*-activated OT-1 cells generated in the presence or absence of NIH3T3-CM were

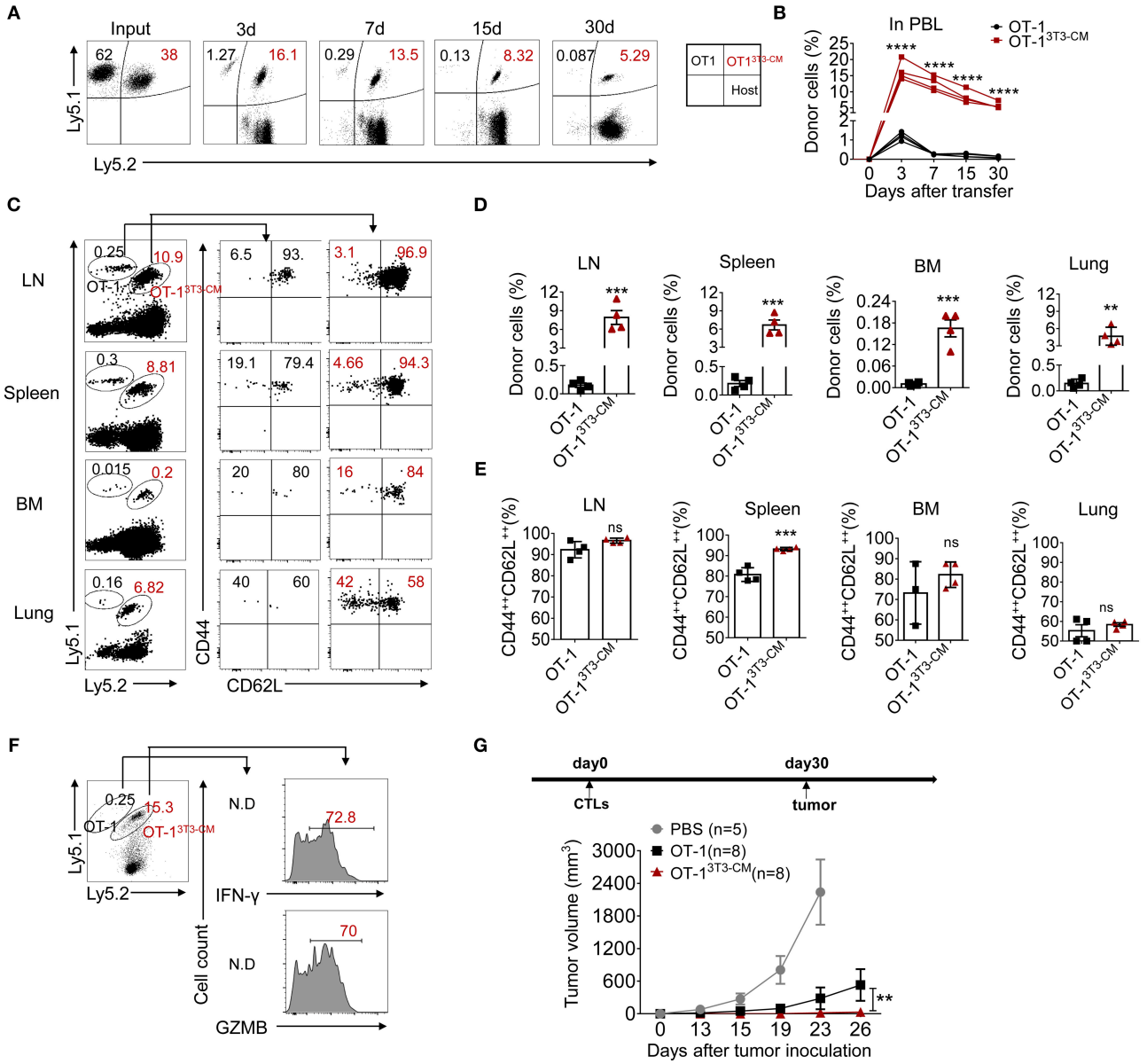

FIGURE 3 | NIH3T3-CM-educated CTLs develop into functional central memory cells. **(A–F)** Congenic OT-1 CTLs were obtained by 3 days activation in the presence or absence of NIH3T3-CM. 2.4 × 10^6 Ly5.1$^{+/+}$OT-1 CTLs (RPMI-1640 medium) and 1.6 × 10^6 Ly5.1$^+$ Ly5.2$^+$ OT-1 CTLs (NIH3T3-CM, 50% v/v) with ratio of 6:4 were i.v. co-transferred to WT C57BL6/c mice (Ly5.2$^{+/+}$, $n = 4$). **(A,B)** Frequencies of transferred cells in peripheral blood were determined at indicated time points. **(A)** Representative FACS data show frequencies of donor cells. **(B)** Graphs show the summary of dot plots of **(A)** with each dot representing for a recipient. **(C–E)** Frequencies of transferred OT-1 cells that migrated to spleen, inguinal lymph node (LN), bone marrow (BM), and lung were evaluated after 30 days of T cell transfer. The phenotype of transferred cells that displayed central memory cells (CD44^{++}CD62L^{++}) and effector memory cells (CD44^{++}CD62L$^-$) was evaluated. **(C)** Representative FACS data are shown. Histograms represent frequencies of transferred cells **(D)** and frequencies of central memory cells in transferred cells **(E).** Each dot represents a single mouse. **(F)** Whole splenocytes were re-stimulated with $OVA_{257-264}$ peptides for 2 days *in vitro*. Levels of IFN-γ and GZMB were evaluated by intracellular FACS analysis. Representative FACS data are shown. **(G)** Effector OT-1 cells (1×10^6/mouse) generated in the presence or absence of NIH3T3-CM were transferred to WT C57BL6/c mice. One month later, EG.7 tumor cells (2 × 10^5/mouse) were s.c inoculated to mice. Tumor growth was monitored. PBS group ($n = 5$), OT-1 group ($n = 8$), OT-1^{3T3-CM} group ($n = 8$). All data are representatives of two independent experiments. Data are presented as mean ± SEM; $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Two-way ANOVA with Bonferroni's multiple comparison tests **(A, G)**; Unpaired *t*-test **(A,E)**.

respectively, transferred into WT B6 mice bearing EG.7 tumors. As expected, NIH3T3-CM-educated OT-1 CTLs dramatically regressed tumor growth (**Figure 4A**). Because timely preparation of sufficient number of *in vitro*-expanded CTLs with high functionality is a limitation for ACT (5), antitumor efficiencies of NIH3T3-CM-educated CTLs with serially diluted numbers were

compared with those of determined number of medium alone-cultured CTLs. Results showed that NIH3T3-CM-educated CTLs effectively regressed tumor growth even at a quarter number of medium alone-cultured CTLs (**Supplementary Figure 2**). This implies that NIH3T3-CM-educated CTLs can have therapeutic benefit even with a limited number of CTLs for ACT. To determine whether survived mice could maintain tumor reactive memory OT-1 cells, the same dose of EG.7 tumor cells was used for re-challenge. Tumor growth was observed in no tumor-experienced mice. However, tumors failed to be established on first-round survivors (**Figure 4B**). In the spleen of survivors, significant numbers (> 2% of all lymphocytes) of tumor reactive OT-1 cells were detected. Most of them displayed a central memory phenotype (**Figure 4C**). The functionality of these maintained OT-1 cells was further confirmed by measuring IFN-γ production level after *in vitro* re-stimulation (**Figure 4D**). These data demonstrate that NIH3T3-CM-educated CTLs not only exert superior antitumor effects, but also establish long-term protective immunity to prevent tumor relapse.

Adoptive T cell therapy together with exogenous IL-2 administration is considered a relatively effective method that has been extensively used in clinical trials (35). Despite clinical successes, IL-2 treatment has fatal defect in that high dose of IL-2 administration can induce severe dose-limiting toxicities in patients (35, 36). In this regard, we compared the therapeutic effect of NIH3T3-CM-educated CTLs with that of medium alone-cultured CTLs with co-administration of exogenous IL-2 (**Figure 4E**). As expected, co-administration of IL-2 significantly improved the antitumor activity of medium alone-cultured OT-1 cells. However, it exerted lower antitumor effects compared to adoptive transfer of NIH3T3-CM-educated OT-1 cells without IL-2 co-administration (**Figure 4E**, left). In consistence with regressed tumor growth, NIH3T3-CM-educated OT-1 cells prolonged mice survival than medium alone-cultured OT-1 cells with concomitant IL-2 therapy (**Figure 4E**, right).

As superior antitumor immunity was observed in the group of NIH3T3-CM-educated CTLs treatment, we examined the frequency of transferred cells in tissues of tumor-bearing mice. In line with efficient tumor regression by NIH3T3-CM-educated CTLs, significantly higher frequencies of NIH3T3-CM-educated CTLs persisted in peripheral blood, spleen, and tumor site in comparison with those of medium alone-cultured CTLs (**Figures 5A,B**). Because tumor cell-mediated exhaustion of CTLs could affect the number and overall functionality of tumor-specific CTLs, we questioned whether NIH-3T3-education could affect expressions of immune check point molecules on CTLs. Thus, we analyzed expression levels of PD-1 and Tim-3 on NIH3T3-CM-educated CTLs or medium alone-cultured CTLs that migrated to tumors. Results showed that about half frequency of NIH3T3-CM-educated CTLs did not express PD-1 whereas nearly all of medium alone cultured-CTLs expressed high level of PD-1 (**Figures 5C,D**, top panel). Expression levels of another exhaustion marker Tim3 were comparable between these two cultures. In consistent with patterns of PD-1 and Tim3 expression levels observed on TILs, down-regulation of PD-1 expression and comparable levels of Tim3 expression were also found on NIH3T3-CM educated CTLs that migrated to the spleen (**Figures 5C,D**, bottom panel). Next, effector function of transferred cells was compared between the two groups through re-stimulation *in vitro*. We observed that NIH-3T3-educated CTLs displaying lower level of PD-1 expression significantly increased IFN-γ production (**Figures 5E,F**). Expression levels of TNF-α and granzyme B were comparable between the two groups. This implies that increased IFN-γ production of PD-1^{low} CTLs might enhance inflammatory response in tumor tissues. Taken together, these results demonstrate that NIH3T3-CM-educated CTLs exhibiting low PD-1 expression exert superior persistence and function, thus contributing to their strong antitumor effects following transfusion.

NIH3T3-CM-Programmed CTLs Express Low Levels of PD-1

In **Figures 5C,D**, we observed that NIH3T3-CM-educated CTLs expressed low levels of PD-1 in both tumor and spleen. Therefore, we wondered whether the decreased PD-1 expression might be an intrinsic characteristic of NIH3T3-CM-programmed CTLs. We first stimulated OT-1 cells *in vitro* in the presence of different volumes of NIH3T3-CM and determined PD-1 expression. Results showed that NIH3T3-CM down-regulated CTLs expression of PD-1 during antigen priming in a dose-dependent manner (**Figure 6A**). It is known that PD-1 is transiently expressed on T cells after priming and down-regulated after antigen clearance (37). Thus, we assessed whether PD-1 expression on CTLs was down-regulated after transfer into antigen-free mice (**Figure 6B**). Without antigen exposure, both medium alone and NIH3T3-CM-cultured CTLs decreased PD-1 expression. After 7 days of cell transfer, most of NIH3T3-CM-educated CTLs abrogated PD-1 expression whereas a certain number of medium alone-cultured CTLs still maintained PD-1 expression (**Figure 6B**). No significant increase in Tim3 level was observed on both cultured CTLs (data not shown). Because NIH3T3-CM-educated CTLs exhibited more potent ability of tumor rejection, the tumor microenvironment of the mouse that received NIH3T3-CM-educated CTLs might have established more inflammatory status compared to that of mouse that received medium alone-cultured CTLs. Difference in tumor microenvironment might also affect expression levels of PD-1 on CTLs surrounded by such environment. To test whether the relatively lower level of PD-1 expression on NIH3T3-CM-educated TILs shown in **Figures 5C,D** was influenced *in situ* or was an intrinsic characteristic conferred by NIH3T3-CM during *in vitro* culture, we co-transferred these two cultured OT-1 CTLs to EG.7 tumor-bearing mice (**Figures 6C,D**). After 6 days of T cell transfer, transferred OT-1 cells in tumor, lymph node, and spleen were analyzed. We found that NIH3T3-CM-educated OT-1 ($Ly5.1^{+}Ly5.2^{+}$) expressed low levels of PD-1 whereas medium alone-cultured OT-1 ($Ly5.1^{+/+}$) expressed high levels of PD-1 regardless of analyzed tissues (**Figures 6C,D**, top panel). Different from results of PD-1 expression, expression pattern of Tim3 was comparable between the two cultures (**Figures 6C,D**, bottom panel). Taken together, these results suggest that low PD-1 expression is an intrinsic characteristic of NIH3T3-CM-programmed CTLs.

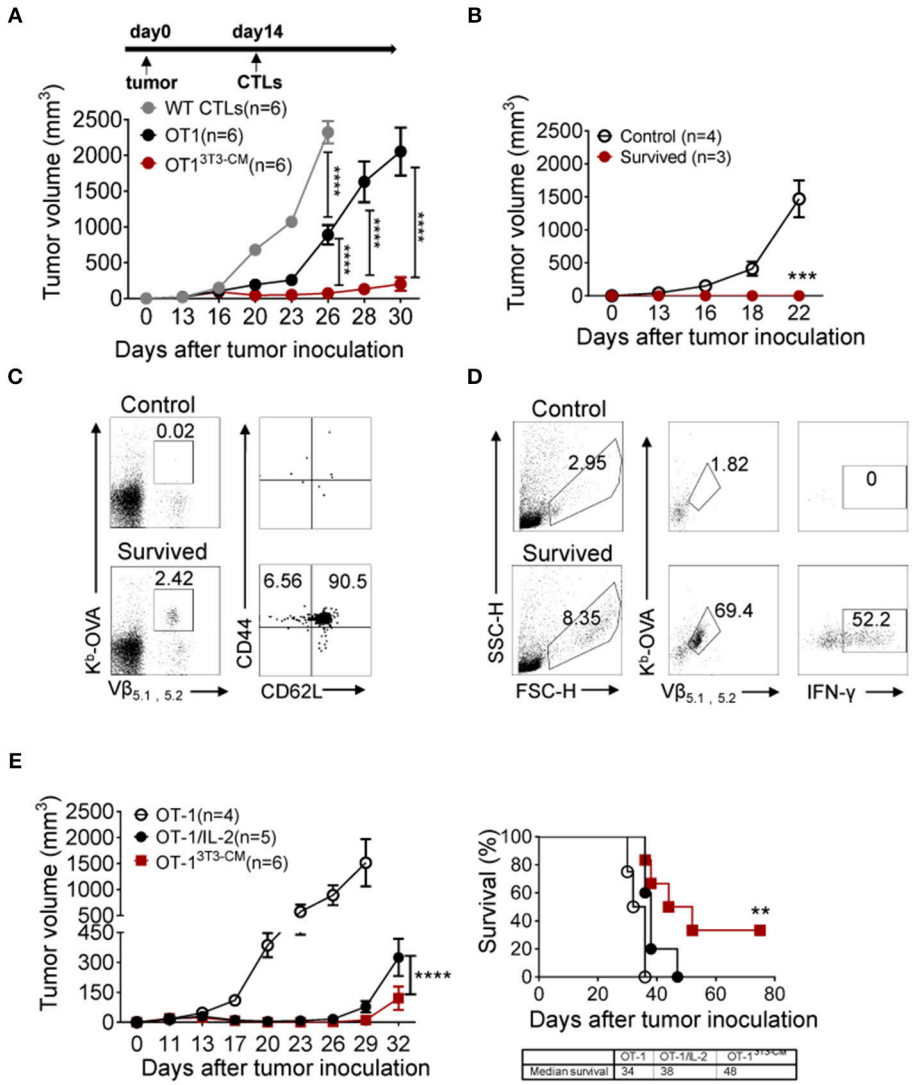

FIGURE 4 | NIH3T3-CM-educated OT-1 CTLs effectively regress EG.7 tumor growth along with memory cell generation. **(A)** EG.7 tumor cells ($5–7 \times 10^4$/mouse) were s.c. inoculated to WT C57BL6/c mice. Then 30–50 mm^3 tumor was established (usually 12–14 days after tumor inoculation). WT CTLs (anti-CD3/CD28 stimulation), medium alone, or NIH3T3-CM cultured OT-1 CTLs (K^b/OVA beads stimulation, 1×10^6/mouse) were i.v. injected into tumor-bearing mice ($n = 6$/group). Tumor growth was then monitored. Serial tumor measurements were obtained. **(B)** At day 60 after the first tumor challenge, after removing mice that showed any sign of tumor growth, survived mice that have received NIH3T3-CM-cultured OT-1 CTLs were re-challenged with E.G7 tumor cells ($5–7 \times 10^4$/mouse). No tumor experienced mice as control also received the same dose of tumor cells. Tumor growth was then monitored. **(C)** At 22 days after tumor re-challenge, mice were sacrificed and tumor specific $CD8^+$ T cells in spleen were analyzed. K^b-OVA tetramer staining was used to detect tumor specific $CD8^+$ T cells. Memory phenotype of tumor specific T cells was analyzed by anti-CD44 and anti-CD62L antibody. **(D)** Whole splenocytes were re-stimulated with $OVA_{257-264}$ peptides for 2 days *in vitro*. Activated antigen specific T cells and the expression of IFN-γ in antigen specific T cells were determined. **(E)** As described in A, after 12 days of tumor inoculation, OT-1 CTLs were transferred to tumor-bearing mice. For the group of IL-2 administration, 2 μg IL-2 once on the day of CTL injection and twice a day on the two following days were i.p. injected to mice. Tumor growth and survivals were monitored ($n = 4$ in OT-1 group; $n = 5$ in OT-1/IL-2 group; $n = 6$ in OT-1^{3T3-CM} group). Data are representatives of three independent experiments **(A)** or two experiments **(B–E)**. Error bars indicate SEM. $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Two-way ANOVA with Bonferroni's multiple comparison tests; Comparison of survived curves with Log-rank (Mantel-Cox) tests.

NIH3T3-CM Confers Potential of Human Memory $CD8^+$ T Cell Differentiation

To determine whether murein-derived NIH3T3-CM can also have the same effect on human $CD8^+$ T cells, naïve $CD8^+$ T cells isolated from peripheral blood lymphocytes (PBL) were polyclonally expanded with increasing amount of NIH3T3-CM and surface expression levels of markers CD45RA (naïve T cells) and CD45RO (antigen experienced T cells) were analyzed. We observed that NIH3T3-CM increased CTLs expression of CD45RO along with decrease of CD45RA expression (**Figure 7A**). Levels of T-box transcription factors, T-bet, and Eomes were upregulated by NIH3T3-CM (**Figure 7B**). Numbers of CTLs expanded by anti-CD3 antibody were also dramatically increased when NIH3T3-CM was treated (**Figure 7C**). To

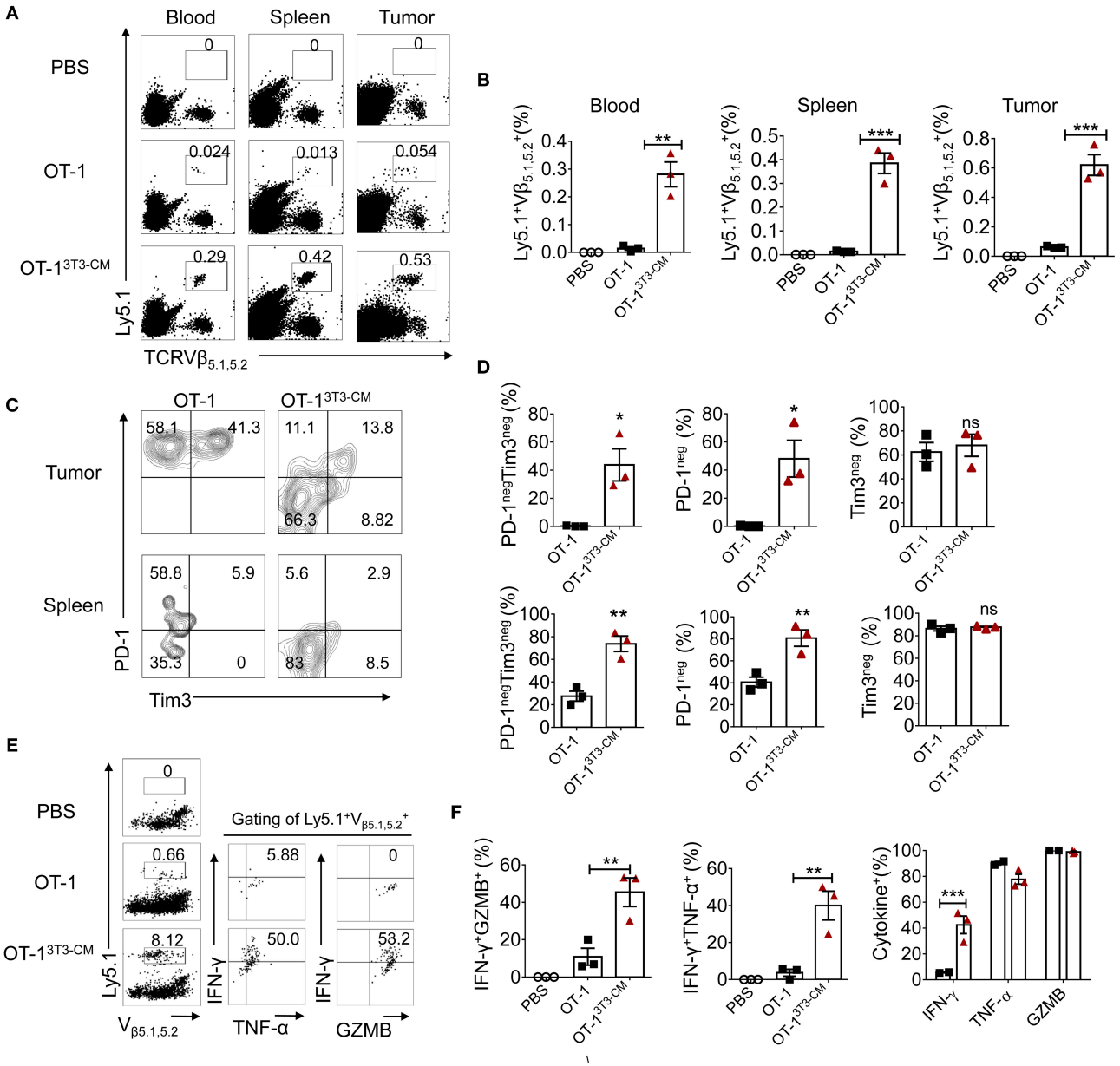

FIGURE 5 | High persistence and low PD-1 expression of NIH3T3-CM-educated OT-1 CTLs in tumor-bearing mice. 1×10^5 EG.7 tumor cells were s.c inoculated to WT C57BL6/c ($Ly5.2^{+/+}$) mice (1×10^5/mouse). After tumor growth reached 50∼70 mm^3, 1×10^6 congenic $Ly5.1^{+/-}$ OT-1 CTLs generated in the presence or absence of NIH3T3-CM ($n = 3$/group) were i.v transferred to tumor-bearing mice. **(A,B)** Frequencies of transferred $Ly5.1^+$ OT-1 cells in blood, spleen, and tumor were analyzed at 6 days after T cell transfer. **(A)** Representative FACS data are shown. **(B)** Summarized results of **(A)**. Each dot represents a single mouse. **(C–D)** FACS analysis of expression level of PD-1 and Tim3 on $Ly5.1^+$ OT-1 cells in tumor and spleen. **(C)** Representative FACS data and **(D)** summarized frequencies of populations from **(C)** are shown. Each dot represents a single mouse. **(E,F)** Whole splenocytes were treated with $OVA_{257-264}$ peptides *in vitro* for 2 days. Expression levels of IFN-γ, TNF-α, and GZMB were evaluated by intracellular FACS analysis. **(E)** Representative FACS data and **(F)** summarized frequencies of cytokine positive cells from **(E)** are shown. Each dot represents a single mouse. Two independent experiments were carried out and similar results were obtained. Data are presented as mean ± SEM $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. Two-way ANOVA with Bonferroni's multiple comparison tests **(B,F)**; Unpaired *t*-test **(D)**.

test their effects on antigen-specific clones, human naïve $CD8^+$ T cells were expanded by CMV-pp65 peptide-loaded PBMC in the presence or absence of NIH3T3-CM. After 12 days of expansion, NIH3T3-CM significantly enhanced levels of CD45RO, consistent with conditions of polyclonal expansion (**Figure 7D**). Higher frequency of antigen experienced clones with similar phenotype of memory stem T cells ($CD45RA^+CD45RO^+CCR7^+$) was obtained in NIH3T3-CM-cultured CTLs (**Figures 7D,E**). NIH3T3-CM also elevated CCR7 expression in $CD45RA^-CD45RO^+$ cells which dramatically

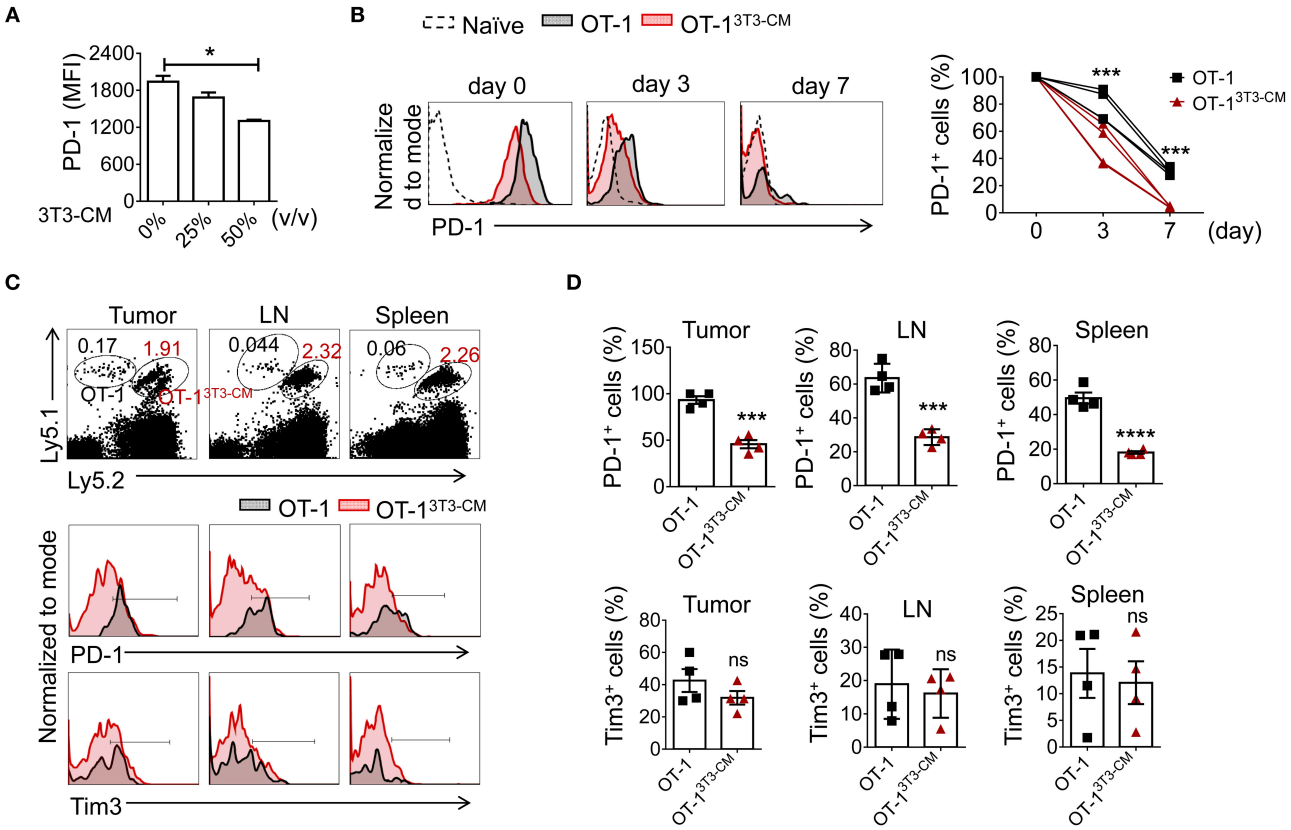

FIGURE 6 | NIH3T3-CM -programmed CTLs intrinsically express low levels of PD-1. **(A)** OT-1 $CD8^+$T cells were stimulated with different volumes of NIH3T3-CM for 3 days and levels of PD-1 on CTLs were analyzed. **(B–D)** Congenic OT-1 CTLs [$Ly5.1^{+/+}$ OT-1 cells cultured in medium alone, $Ly5.1^+$ $Ly5.2^+$ OT-1 cells cultured in NIH3T3-CM (50%, v/v)] were co-transferred with ratio of 1:1 to **(B)** naïve WT C57BL6/c ($Ly5.2^{+/+}$) mice ($n = 4$) or **(C,D)** EG.7 tumor (50∼100 mm^3)-bearing WT C57BL6/c ($Ly5.2^{+/+}$) mice ($n = 4$). **(B)** Levels of PD-1 on transferred cells in peripheral blood lymphocytes (PBL) of naive mice were analyzed at indicated time points using FACS analysis. **(C,D)** Levels of PD-1 and Tim3 on transferred cells in tumor mass, inguinal lymph node, and spleen of tumor-bearing mice were analyzed at 6 days after T cell transfer. **(C)** Representative FACS analysis and **(D)** frequencies of $PD\text{-}1^+$ cells or $Tim3^+$ cells in transferred cells are shown. Each dot represents a mouse. Data are representatives of three independent experiments **(A)** or two experiments **(B–D)**. Data are presented as mean ± SEM. $^*P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$. One-way **(A)** or Two-way **(B)** ANOVA with Bonferroni's multiple comparison tests; Unpaired *t*-test **(D)**.

enhanced the frequency of central memory phenotypic cells ($CD45RA^-CD45RO^+CCR7^+$) (**Figures 7D,E**). These results suggest that NIH3T3-CM not only confers memory characters of mouse $CD8^+$ T cells, but also modulates differentiation of human memory $CD8^+$ T cells. To anticipate tumor reactivity of NIH3T3-CM-educated human CTLs with memory characters, their cytotoxic activities were determined. NIH3T3-CM-educated CTLs had higher cytotoxicity than medium alone-cultured CTLs (**Figure 7F**). Mouse embryonic fibroblasts (MEFs) can provide various growth factors and suitable attachment substrates. They are widely used as feeders to support human embryonic stem cell self-renewal and growth (38). Mouse 3T3 cells also have been used as feeders to support long-term survival of human epithelial cells or tissue progenitor cells (20, 21, 39). Together with our results, these facts support the idea that factor(s) secreted from primary or immortalized MEFs can be cross-reactive to human cells.

DISCUSSION

Features of infused cells such as sufficient number of antigen specific T cells, capabilities of proliferation and long-term persistence, successful tumor infiltration, and overcoming the immunosuppressive tumor microenvironment are associated with clinical benefit from ACT. In the current study, we provided a novel method to generate potent $CD8^+$ T cell clones using NIH3T3-CM. NIH3T3-CM augmented effector functions of CTLs during initial priming and conferred memory associated-characteristics to direct long-lived memory cell differentiation. Furthermore, NIH3T3-CM programmed CTLs to reduce PD-1 expression in response to tumor antigens. Consequently, adoptive transfer of NIH3T3-CM-programmed CTLs exerted great therapeutic effects on solid tumors. Although NIH3T3 cells are murine derived cells, NIH3T3-CM could promote the differentiation of human $CD8^+$ T cells into central memory and/or memory stem T

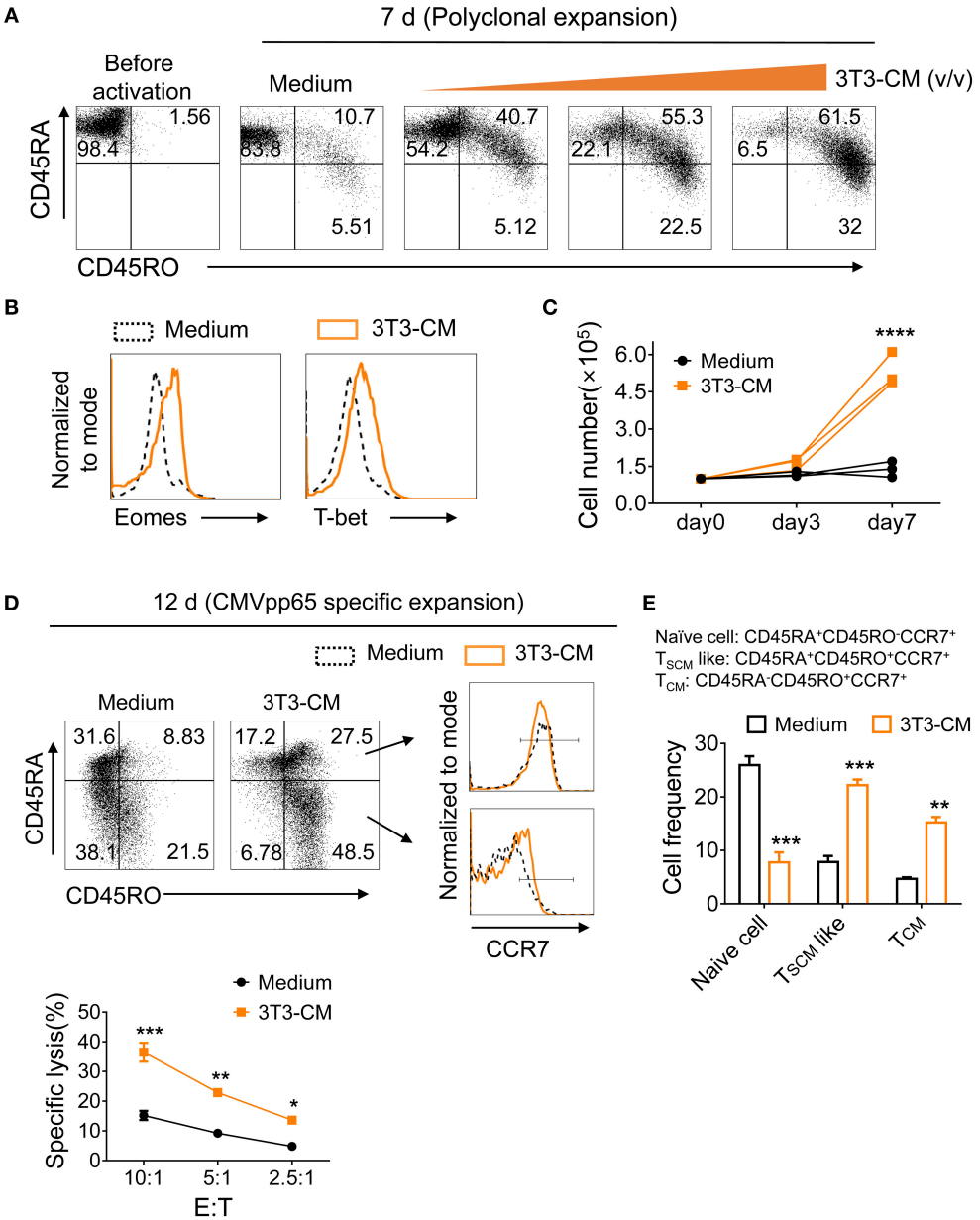

FIGURE 7 | NIH3T3-CM confers potential of human memory CD8$^+$ T cell differentiation. **(A–C)** Purified human naïve CD8$^+$ T cells were expanded by OKT3/anti-CD28 in the absence or presence of increased volumes of NIH3T3-CM. After 7 days of expansion, surface phenotypic changes **(A)** and transcription factors **(B)** were analyzed by FACS analysis. **(C)** Counted cell numbers at indicated time points. Each line in the same color represents a donor. **(D–F)** Purified human naïve CD8$^+$ T cells were expanded by CMVpp65-peptide-loaded PBMCs (irradiated) in the presence or absence of NIH3T3-CM. After 12 days of expansion, surface phenotype of CD8$^+$ T cells **(D),** frequencies of naïve and memory CD8$^+$T cells **(E)**, and specific cytotoxicity of the expanded CD8$^+$ T cells **(F)** were determined. Representative results of three donors. Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. Two-way ANOVA with Bonferroni's multiple comparison tests **(C,E,F)**.

like cells during antigen priming. NIH3T3-CM modulated CTLs with functionality superior to medium alone-cultured CTLs. Therefore, NIH3T3-CM provides a new insight to *ex-vivo* culture system to generate potent T cell clones for ACT.

In this study, we found that NIH3T3-CM augmented antigen induced acquisition of potent effector CTL clones by characterizing levels of effector molecules production, phenotypic expression, transcriptional regulation, and cytolytic function. Although many studies have reported that fibroblasts and fibroblast like mesenchymal stem cells play immunosuppressive roles on T lymphocytes (40, 41), our findings support the idea that embryonic fibroblasts also have the ability to strengthen effector function of T

cells (19). Our results are partially in accordance with some groups suggesting that conditioned medium from lung tumor associated fibroblasts or normal (skin or lung) fibroblasts can enhance IFN-γ production of $CD8^+$ T cells (16).

During initial TCR stimulation, we found that NIH3T3-CM provided signals that could enhance effector function of CTLs and modify intrinsic properties of CTLs to develop into memory-fated precursors. We further demonstrated that NIH3T3-CM-programmed CTLs, compared to medium alone-cultured CTLs, had higher capacity in long-term persistence and conversion into central memory cells upon transfer into naïve mice (**Figure 3**). Although phenotypic analysis showed that both medium alone and NIH3T3-CM-cultured CTLs displaying surface markers ($CD127^{+/-}CD27^{++}KLRG1^-$) were partially associated with MPECs ($CD127^{++}CD27^{++}KLRG1^-$) (**Figure 2**), NIH3T3-CM-cultured CTLs expressed higher levels of central memory markers (CD62L, CD44, and CD122). This supports the idea that *ex-vivo* expanded T cells acquiring phenotypic properties of central memory cells have high potential of memory pool formation (8, 42, 43). Furthermore, expression of CD62L on effector $CD8^+$ T cells plays an important role in facilitating cells entry into secondary lymphoid or inflamed tissues (44). Consistent with this notion, high levels of CD62L may promote NIH3T3-CM-cultured CTLs homing to peripheral lymphoid organs where they reside to further develop to long-lived central memory cells under antigen free condition or they are rapidly stimulated after antigen re-encounter. Transcriptional programs that control fates of effector and memory $CD8^+$ T cell were also observed. Consistence with the notion that Eomes are preferentially increased relative to T-bet in memory cell development (12, 28, 45), we observed that NIH3T3-CM dramatically enhanced levels of Eomes whereas medium alone-cultured CTLs always kept very low levels of Eomes during TCR stimulation (**Figure 2C**). In addition, NIH3T3-CM elevated Bcl-6 levels (**Figure 2D**). These results indicate that NIH3T3-CM-induced transcriptional programming plays a crucial role in the development of memory-fated clones.

We also validated that murine derived NIH3T3-CM could direct differentiation of human $CD8^+$ T cells into memory-phenotype cells which showed increased expression of CCR7 and CD45RO. Primary mouse embryonic fibroblasts or their immortalized cell lines including 3T3 cells are widely utilized as feeders to facilitate self-renewal of human primary cells (20, 21, 38, 39). Our findings are consistent with previous studies because self-renew is a significant character of memory cells.

Although adoptive cells after transfusion tenaciously have survived and infiltrated to tumors, another obstacle to successful cancer immunotherapy is overcoming the immunosuppressive environment of the tumor. Exhaustion markers PD-1 and Tim3 are highly expressed on tumor infiltrating cells, leading to promotion of immune evasion of tumor cells (46, 47). In this study, we found that NIH3T3-CM-educated CTLs effectively regressed tumor growth due to their ability of survival and their prominent tumor reactivity. In tumors, we also observed that NIH3T3-CM-educated CTLs expressed low levels of PD-1 whereas almost all medium alone-cultured CTLs expressed high levels of PD-1. The same pattern was also observed on transfused cells that migrated to the spleen (**Figures 5C,D**). These results indicate the superior antitumor immunity of NIH3T3-CM-educated CTLs is also contributed by their features of low expression of PD-1. Another significant point of our study is that NIH3T3-CM could directly suppress PD-1 expression. It has been reported that Blimp-1 can repress CTLs expression of PD-1 during acute viral infection (48). Therefore, NIH3T3-CM-mediated suppression of PD-1 expression might be influenced by up-regulation of Blimp-1. Consistence with the notion that PD-1 is transiently expressed on CTLs after early activation and then down-regulated after antigen clearance (37), we observed that PD-1 was rapidly decreased on NIH3T3-CM-educated CTLs following transfer to antigen-free mice while medium alone-cultured CTLs showed slow PD-1 down-regulation. Moreover, NIH3T3-CM-educated CTLs kept low levels of PD-1 whereas medium alone-cultured CTLs expressed high levels of PD-1, although they were in the same tumor microenvironment (**Figures 6C,D**). These results imply that NIH3T3-CM-programmed CTLs with characters of memory cells might have potential of resistance of PD-1 expression whereas medium cultured CTLs with low ability of memory cell differentiation are inclined to express high level of PD-1 in tumor microenvironment. In addition, a recent report has shown that $CD8^+$ TILs fail to infiltrate to tumor islands due to PD-1/PD-L and FAS/FAS-L induced apoptosis by tumor associated fibroblasts (TAFs) (49). This indicates that NIH3T3-CM-programmed CTLs with the intrinsic characteristic of low PD-1 expression may escape TAF induced apoptosis.

Although we have not yet identified the effective factor(s) derived from NIH3T3, our current results on NIH3T3-CM together with our previous investigations of MEF-CM (19) suggest that the factor(s) (not cytokines) involved in the modulation of CTL differentiation may not be a single factor. MEF-CM and NIH3T3-CM and some adult fibroblast-CM (16) may share similar factor(s) that enhance CTL effector functions. Especially, factor(s) derived from MEFs might be mainly responsible for enhancement effector function of CTLs while unique factor(s) derived from NIH3T3 might direct memory programming of CTLs. Future studies focusing on growth factors, extracellular matrix proteins, and exosomes are needed to identify soluble molecules.

In conclusion, NIH3T3-CM-educated CTLs exhibited characteristics related to memory lineages which enabled their differentiation to long lived-central memory cells after adoptive transfer. NIH3T3-CM also could program CTLs to reduce expression of PD-1 in response to tumor. Three different therapeutic models powerfully demonstrated that NIH3T3-CM-educated CTLs could efficiently regress tumor growth with high potential for ACT even if the number of transferrable cells was limited. Although soluble factor(s) underlying modulation $CD8^+$ T cells remains to be identified, our findings provide a

promising strategy to establish highly efficient CTL clones for ACT.

ETHICS STATEMENT

The experimental protocols adopted in this study were approved by the Institutional Animal Care and Use Committee of Korea University

AUTHOR CONTRIBUTIONS

YQ and S-HP: conception and design, development of methodology, analysis and interpretation of data, and writing, review, and/or revision of the manuscript. YQ, YL, and JS: acquisition of data. JS, JHS, and TK: administrative, technical, or material support. S-HP: study supervision.

FUNDING

This work was supported by a grant (NRF-2018R1A2A2A05023297) of the Basic Science Research Program of the National Research Foundation of Korea.

ACKNOWLEDGMENTS

We thank crews of Geyrim Experimental Animal Resource Center for their assistance in animal handling and maintenance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.00761/full#supplementary-material

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

frontiers in Oncology

REVIEW
published: 16 April 2019
doi: 10.3389/fonc.2019.00279

Turning the Tide Against Regulatory T Cells

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Regulatory T (Treg) cells play crucial roles in health and disease through their immunosuppressive properties against various immune cells. In this review we will focus on the inhibitory role of Treg cells in anti-tumor immunity. We outline how Treg cells restrict T cell function based on our understanding of T cell biology, and how we can shift the equilibrium against regulatory T cells. To date, numerous strategies have been proposed to limit the suppressive effects of Treg cells, including Treg cell neutralization, destabilizing Treg cells and rendering T cells resistant to Treg cells. Here, we focus on key mechanisms which render T cells resistant to the suppressive effects of Treg cells. Lastly, we also examine current limitations and caveats of overcoming the inhibitory activity of Treg cells, and briefly discuss the potential to target Treg cell resistance in the context of anti-tumor immunity.

Keywords: immune regulation, Treg cells, T cells, tumor immunity, immune therapy

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Edited by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *24 January 2019*
Accepted: *26 March 2019*
Published: *16 April 2019*

Citation:
Han S, Toker A, Liu ZQ and Ohashi PS (2019) Turning the Tide Against Regulatory T Cells. Front. Oncol. 9:279. doi: 10.3389/fonc.2019.00279

INTRODUCTION—REGULATORY T CELL IN CANCER

Challenges in Immune-Oncology—Immunosuppressive Cells

The concept of utilizing the T cells, to recognize and eliminate cancer cells has contributed to the advancement of immunotherapy against multiple malignancies. Recent advances in checkpoint inhibitors (in particular CTLA-4 and PD-1 inhibitors) and cell-based therapy such as Chimeric Antigen Receptor (CAR)—T cell therapy demonstrate promising clinical responses in various cancer types in a subset of patients. However, despite the attempts to modulate anti-tumor T cell responses, a proportion of patients still do not respond to these immune therapies (1–3). The mechanisms of resistance against immune therapy is currently a key area of investigation. Some of these mechanisms include the presence of immunoregulatory cells in the tumor microenvironment such as tumor-associated macrophages (TAMs), myeloid-derived suppressor cells (MDSCs) and regulatory T (Treg) cells which could play an important role in restricting T cell immunity (4–6). Thus, overcoming the effects of these immunosuppressive cells remain a challenge for those seeking to enhance anti-tumor immune response.

Evidence for a Role for Regulatory T Cells in Anti-tumor Immunity

Treg cells are one of the integral components of the adaptive immune system that contribute to maintaining tolerance to self-antigens and preventing autoimmune diseases (7, 8). It is postulated that these cells have an important role in regulating immune surveillance and promoting tumor progression. However, their precise role in regulating anti-tumor immunity and the mechanism of how Treg cells could suppress T cells in tumor is still unclear (9). Early studies used $CD4^+CD25^+$ markers to identify Treg cells with the caveat that activated helper T cells would also express these markers (10). Woo et al. (11) provided evidence for the presence of regulatory T in patients with

early-stage non-small cell lung cancer and late-stage ovarian cancer. Numerous other manuscripts have also noted the presence of potential $CD4^+CD25^+$ Treg cells in multiple types of cancer including melanoma, pancreatic cancer and breast cancer (12–14).

In 2003, studies reported that the transcription factor FoxP3 was critical for Treg development (15–17), Subsequently, Curiel et al. (18) examined $CD4^+CD25^+FoxP3^+$ cells and found that increased infiltration of Treg cells correlated with disease progression in ovarian carcinoma, and infiltration of these cells in each stage of cancer served as a good metric for survival prediction. Similarly, studies demonstrated that the presence of Treg cells in breast cancer correlated with reduced overall survival (19, 20). In contrast, several reports suggested that infiltration of Treg cells can be a favorable prognostic factor (21–24). Such discrepancies may result from the inability to precisely identify regulatory T cells within the heterogenous pool of $FoxP3^+$ expressing $CD4^+$ T cells (25). Alternatively, considering high infiltration of Treg cells also correlate with high infiltration of $CD8^+$ T cells in a specific tumor subtypes (24), regulatory T cells may be recruited in response to an inflamed tumor microenvironment. Part of the controversy could also be due to the finding that FoxP3 can be transiently upregulated in activated human T cells, and is therefore not an exclusive marker for Treg cells (25, 26). The expression level of other markers such as CD45RA (27) and Treg-specific DNA demethylation status within the *FoxP3* locus can increase the accuracy of identifying functionally active Treg cells (28, 29). However, it is not always possible to perform these in depth analysis. Studies have also utilized *ex vivo* Treg suppression assays to demonstrate the presence of regulatory T cells within tumor tissue (18, 30, 31).

In mice, the role of Treg cells in regulating anti-tumor immunity has been investigated through ablation of Treg cells (using $FoxP3^{DTR}$ mice or antibodies targeting receptors highly expressed on Treg cells, such as CD25, GITR, and folate receptor 4) in transplantable tumor models (32–35). In these models, depletion of regulatory T cells in conjunction with modulation of T cell immunity improves anti-tumor immunity. In contrast, co-adoptive transfer of $CD8^+$ T cells with Treg cells prevented effective adoptive cell therapy against B16-F10 melanoma (36). In summary, although the presence of Treg cells in tumors cannot be used as an accurate prognostic factor, the literature suggests that Treg cells are a potent regulator of anti-tumor immunity.

Immune Therapy and Treg Cells

One potential mechanism that may reduce the efficacy of cancer immunotherapy is suppression mediated by the Treg cell population. In addition, the therapeutic modalities such as anti-PD-1 may potentially alter Treg cell function and/or frequency, either directly or indirectly by changing the immune microenvironment (37–39). Thus, the potential effect of Treg cells on tumor-specific T cells should not be neglected even in therapeutic arena.

One of the most predominantly utilized checkpoint inhibitors in clinical and translational studies involve therapeutic blockade of PD-1 (nivolumab and pembrolizumab) or PDL-1 (atezolizumab and duravalumab) (40). There is a limited number of clinical studies thoroughly documenting changes in the quantity and quality of Treg cells in response to these PD-1/PD-L1 inhibitors. To date, studies either report an increase or no change in the frequency of Treg cells in response to nivolumab or pembrolizumab (39, 41). It is also important to note that PD-1 and PD-L1 can be expressed by Treg cells, thus direct modulation of Treg cell function should not be excluded as a possibility (31, 42–44). A few reports demonstrate that PD-1 blockade attenuates Treg cell suppression *in vitro*, based on the effect of PD-1 inhibitor on T cell proliferation in the presence of Treg cells (39, 45, 46). However, the effect of these inhibitors on Treg cells have not been clearly discriminated against its effect on T cells. A few reports including a study conducted by Toor et al. (47, 48) suggest that PD-1 blockade does not modulate Treg cell phenotype or function, but instead targets activated T cells. A murine study conducted by Chen et al. (49) demonstrates that PD-1 has no influence over the development and suppressive effects of thymically-derived Treg cells, however PD-1 appears to be crucial for differentiation of naïve $CD4^+$ T cells into iTregs. Similarly, PD-L1 blockade can interfere with the induction and maintenance of iTreg cells in mice (50). Collectively, the precise effect of PD-1 blockade on Treg cells is poorly understood. Nevertheless, PD-1 inhibition synergizes with therapeutic strategies which reduce the quantity of Treg cells in mice (35, 51, 52), suggesting that enhanced anti-tumor immunity in response to PD-1 blockade may still be limited by Treg cells. Extensive studies have been performed evaluating the clinical potential of interfering with immune checkpoint receptors beyond PD-1, including CTLA-4, LAG-3, and TIM-3. However, the effect of each checkpoint inhibitors on Treg cells is also poorly understood and are beyond the scope of this review.

Adoptive cell therapies using TCR transduced T cells, CAR-T cells and Tumor-infiltrating Lymphocytes (TIL) are capable of directly recognizing and targeting tumor cells (3, 53). However, whether or not these T cell products are susceptible to regulation by Treg cells in humans is yet to be elucidated. In a few cases, the frequency of lymphocytes resembling Treg cells increases with adoptive T cell therapy (37, 38, 54). In the context of TIL therapy, Yao et al. (37) has demonstrated that the quantity of Treg cells reconstituted after non-myeloablative chemotherapy, which correlates with the number of administered doses of IL-2, is associated with patient responsiveness to TIL therapy. Supportive of this finding, administration of high-dose IL-2 (often utilized in conjunction with TIL therapy) can result in expansion of immunosuppressive $ICOS^+$ Treg cells, which may be predictive of clinical outcomes in patients with metastatic melanoma (55). Baba et al. (56) utilized a murine model of fibrosarcoma to suggest that rapid reconstitution of Treg cells post-lymphodepletion suppress anti-tumor immunity, and targeting these regulatory T cells using neutralizing antibodies significantly reduced tumor growth. In the context of CAR-T cell therapy, the effect of the treatment on Treg cells may vary. For instance, clinical infusion of EGFRvIII-directed CAR-T cells for the treatment of glioblastoma resulted in influx of $CD4^+CD25^+FoxP3^+$ cells in the tumor (38), whereas CD19-targeted CAR-T cells against B-cell lymphoma and leukemia did not increase the frequency of Treg cells (57). Lymphodepletion,

known to transiently reduce the frequency of Treg cells, improves persistence of CAR-T cells as well as therapeutic outcome (58), however the direct effect of Treg cells on CAR-T cells is unknown. In summary, the role of regulatory T cells in the context of adoptive T cell therapy is currently unknown, however the literature suggests that Treg cells may limit the outcome of these therapeutic modalities.

Mechanisms of Treg Suppression

The general mechanisms of T cell suppression by Treg cells, mostly evaluated through *in vitro* experiments, suggest that Treg cells may exploit diverse contact-dependent and cytokine-mediated mechanisms to limit T cell function (59, 60). One of the proposed mechanisms involve the ability of Treg cells to downregulate CD80/86 expression on dendritic cells (61–63). In a study conducted by Wing et al. (62, 64) and Onishi et al. (63), Treg-specific deletion of CTLA-4, which binds to CD80/86, results in reduced suppressive effects of Treg cells *in vivo* and failed to downregulate CD80/CD86 expression on dendritic cells (DCs) *in vitro*. Qureshi et al. (65) also demonstrate that CTLA-4 can reduce CD80/CD86 expression on DCs through trans-endocytosis and subsequent degradation of the co-stimulatory molecules. Furthermore, *in vitro* engagement of CTLA-4 with cognate receptors on DCs reduces the secretion of cytokines by DCs such as IL-6 and TNF, while increasing the expression of IDO, an immunosuppressive tryptophan catabolizing enzyme (66, 67). However, evidence also suggests that Treg cells can maintain suppressive functions without CTLA-4. For example, Paterson et al. (68) demonstrated that conditional ablation of CTLA-4 in adult mice do not result in systemic autoimmunity as observed in germline CTLA-4 deficiency, and also suggested that these Treg cells deficient in CTLA-4 are functional both *in vitro* and *in vivo*. Several other potential mechanisms of T cell suppression have been proposed, including (1) increased interaction between Treg cells and dendritic cells through high expression of LFA-1 on Treg cells resulting in reduced T cell priming (63, 69), (2) perforin and granzyme-mediated lysis of effector T cells (70–72), and (3) CD39 and CD73-mediated metabolic disruption of T cells (73). Through *in vitro* experiments, Deaglio et al. (73) suggested that CD39 and CD73 (ectonucleotidases used for hydrolysis of phosphate residues) expression by Treg cells can induce hydrolysis of extracellular ATP to adenosine, which triggers A2A receptor on T cells and elevates intra-cellular cAMP for T cell inhibition. However, most of these proposed mechanisms have not been explored *in vivo*.

Treg cells may also attenuate the T cell response via the production of chemokines and inhibitory cytokines. Treg cells can secrete TGF-β, IL-10, and IL-35 in a context-dependent manner, and reduce effector T cell function (74–77). For example, TGF-β can be a potent regulator of CTL function *in vitro* and *in vivo* (76, 78, 79), and reduce anti-tumor immunity in a transplantable tumor model (76, 79, 80). Although the secretion of TGF-β by Treg cells appears to be an important mechanism of suppression, an *in vitro* study conducted by Piccirillo et al. (81) also suggests that blockade of TGF-β produced by regulatory T cells do not reduce the suppressive effects of Treg cells. The role of IL-10 on T cells is unclear due to evidence of IL-10 serving as either stimulatory or inhibitory cytokine in a context-dependent manner, however evidence suggests that IL-10 plays an important role in Treg cell-mediated suppression of T cells (82, 83). For instance, Chaudhry et al. (82) suggests that IL-10 signaling acts on Treg cells to attenuate pathogenic T_h17 response, however, the molecular mechanism of T cell suppression is still unclear. Similarly, the precise mechanism of T cell inhibition by IL-35 is also unclear, but studies suggest that IL-35 restricts T cell proliferation and induces "infectious tolerance" by inducing Treg cells from naïve $CD4^+$ T cells (84, 85). Lastly, in conjunction with previously described cytokine-driven suppressive mechanisms, it has been recently demonstrated in EAE and islet allograft models that secretion of the chemokines CCL3 and CCL4 by Treg cells plays an important role in the recruitment of effector T cells to close proximity of Treg cells where they become susceptible to suppression (86).

Lastly, *in vitro* Treg suppression assays suggest that Treg cells compete with other T cells for IL-2, and that the decreased availability of IL-2 reduces T cell proliferation and function (87–89). In this particular system, Treg cells constitutively express a high level of high-affinity IL-2 receptors whereas stimulated naïve T cells do not express high-affinity IL-2 receptors at an earlier time point; this may further contribute to preferential acquisition of IL-2 by Treg cells. Furthermore, IL-2 provides STAT5 signaling in Treg cells that is necessary to further enhance their immunosuppressive function (90, 91). This particular mechanism of suppression can also be observed *in vivo*. A study conducted by Chinen et al. (91) suggest that the ability of Treg cells to capture and compete for IL-2 is critical for controlling $CD8^+$ T cell expansion and function. The general consensus for those investigating Treg cell-mediated suppression of T cells is that each suppressive mechanism likely acts in a context-dependent manner and more than one mechanism could be employed simultaneously to inhibit T cell function (7, 59). Thus, the ability of Treg cells to compete for IL-2 likely works in tandem with other suppressive mechanisms to regulate T cell immunity.

It remains unclear which of the previously described mechanisms are relevant for regulatory T cells residing in the tumor. Treg cells found in the tumor often display a distinct phenotype in comparison to those circulating the periphery, which is exemplified through their unique transcriptional signatures and the expression of markers including PD-1 (31, 43, 44, 92, 93). In the context of head and neck squamous cell carcinoma, tumor-infiltrating $CD4^+CD25^{hi}Foxp3^+$ T cells produce a higher level of TGF-β and reduced T cell proliferation more effectively than Treg cells from the periphery in Treg suppression assays (30, 94). These correlative studies suggest that intra-tumoral Treg cells display highly immunosuppressive phenotype *in vitro*, suggesting that they may regulate anti-tumor immunity. However, it is still unclear precisely "when," "where" and "how" these distinct Treg cells exert their suppressive effect in cancer biology. Most *in vivo* and *in vitro* experiments performed to elucidate the cellular and molecular mechanism of T cell suppression by Treg cells in mice were performed using Treg cells from secondary lymphoid organs such as spleen and lymph nodes, and therefore may not fully recapitulate the interaction between intra-tumoral Treg

cells and T cells. Nevertheless, evidence acquired from studies using non-tumor derived Treg cells may provide insights in understanding how intra-tumoral Treg cells could potentially limit anti-tumor T cells.

Potential Strategies to Interfere With Immune Suppression by Regulatory T Cells

Acknowledging the significance of Treg cells and their potential role in inhibiting anti-tumor immunity, multiple strategies have been proposed to deplete Treg cells *in vivo*. However, one major challenge associated with Treg cell depletion is the lack of a Treg cell-specific marker. Most surface molecules expressed on Treg cells are also present on activated T cells, although the level of expression may be different. Similarly, FoxP3 is expressed by both activated T cells and Treg cells in humans (25, 26). Despite such challenges, several potential strategies have been proposed to reduce the suppressive effects of Treg cells (**Figure 1**). First, several non-specific anti-cancer drugs have been shown to reduce Treg cell activities. Low-dose cyclophosphamide (CTX), a common chemotherapeutic agent known to target rapidly dividing cells, significantly reduced Treg cells owing to their higher rate of proliferation, leading to enhanced anti-tumor immunity (95–98). In these studies, investigators have noted that CTX reduced the levels of intra-tumoral Treg cells while maintaining or elevating the level of $CD8^+$ T cells in the tumor (96, 97). In contrast, several studies have reported contradicting data where CTX either increased the level of Treg cells or did not enhance anti-tumor immunity (99, 100). Additional studies showed that treatment with CTX was further improved in its selectivity and efficacy through combination therapy with OX40 agonist or anti-PD-1, demonstrating increased intra-tumoral Teff/Treg cell ratio and subsequent regression of B16 and TC-1 tumors (101, 102). Several other FDA-approved anti-cancer agents including tyrosine kinase inhibitors sunitinib, sorafenib, and imatinib also reduced the levels of intra-tumoral Treg cells (101, 103–105).

While specific targeting of tumor-infiltrating Treg cells can be challenging, several agents including daclizumab (CD25 blocking antibody), denileukin diftitox (Ontak, IL-2-diphtheria toxin conjugate protein), and several other antibodies have been proposed to target Treg cells and enhance anti-tumor immunity (106, 107) (**Figure 1**). First, the use of CD25 to target and deplete Treg cells has resulted in improved anti-tumor immunity in some cases (108, 109). However, this strategy has raised a number of concerns based on inconsistent *in vivo* responses and lack of specificity. Similar to the effects of anti-CD25 in mice (clone PC-61), the use of denileukin diftitox for depleting Treg cells and eliciting a stronger anti-tumor immune response remains controversial, due to varying clinical responses (110, 111). For instance, treatment of patients with renal cell carcinoma using denileukin diftitox effectively relieved inhibition by Treg cells to promote anti-tumor immunity, but the opposite trend was observed in patients with metastatic melanoma (110, 111). Tumor heterogeneity, the existence of $CD25^-$ Treg cells and CD25 expression on other immune cells, such as T cells, B cells, and NK cells (112, 113), may explain seemingly opposite outcomes in this particular approach. However, recent studies have further modified and improved strategies targeting CD25 and suggest that it may still be a viable option to restrict Treg cell activities. Arce Vargas et al. (35) demonstrated that Fc-optimized antibodies against CD25 could effectively reduce the frequency of intra-tumoral Treg cells and improve tumor control. Furthermore, CD25-targeted near-infrared photoimmunotherapy (NIR-PIT) has been developed in a murine model. By conjugating anti-CD25 with a photoactivatable silica-phthalocyanine dye sensitive to near-infrared light, and localizing near-infrared irradiation specifically on tumors, NIR-PIT achieved reduction of intra-tumoral Treg cells (114).

Beyond CD25 as a target molecule, regulatory T cells constitutively express receptors such as GITR, CTLA-4, and folate receptor 4. In the tumor microenvironment, Treg cells further upregulate a large number of receptors including ICOS, OX40, GITR, TIGIT, PD-1, and CTLA-4 (31, 115). Antibodies targeting some of these receptors expressed by Treg cells such as GITR and folate receptor 4 reduce the amount of Treg cells and enhance anti-tumor immunity in mice (32, 33, 116). Similarly, checkpoint inhibitors designed to block inhibitory signals on T cells may also play an important role in regulating Treg cell activities. With Treg cells expressing a high level of CTLA-4 (27), administration of an anti-CTLA-4 antibody has resulted in a major reduction in the frequency of intra-tumoral $CTLA\text{-}4^+FoxP3^+$ Treg cells which was dependent on Fcγ receptor-expressing cells in the tumor microenvironment (117–121). This is consistent with the correlation of decreased frequency of tumor-infiltrating Treg cells with the usage of ipilimumab in patients with bladder cancer and advanced melanoma (122–124). Lastly, a study conducted by Sugiyama et al. (125) demonstrated that a high proportion of Treg cells express CCR4 in tumor-infiltrating lymphocytes (TILs) acquired from melanoma patients. CCR4 expression was specific to $CD4^+CD45RA^-FoxP3^{hi}$ Treg cells, a terminally differentiated and highly suppressive subset of Treg cells that preferentially accumulates within tumors, whereas CCR4 is not expressed on $CD4^+CD45RA^+FoxP3^{lo}$ naïve T cells. In agreement with these findings, administration of anti-CCR4 (Mogamulizumab) in patients with Adult T-Cell Leukemia-Lymphoma (expressing NY-ESO-1) resulted in reduction in $CD4^+CD45RA^-FoxP3^{hi}$ Treg cells and enhanced NY-ESO-1-specific $CD8^+$ T cell response (125). Although anti-CCR4 antibodies target a specific subset of Treg cells that are highly abundant within tumors, this particular strategy does not selectively deplete intra-tumoral Treg cells since a large proportion of Treg cells in peripheral blood are $CD4^+CD45RA^-CCR4^+FoxP3^+$ Treg cells (8, 27, 125).

Interestingly, studies published within the last few years suggest that promoting the conversion of Treg cells into immune-stimulatory cells could be an alternative approach to enhancing anti-tumor immunity (**Figure 1**). $FoxP3^+$ regulatory T cells are comprised of heterogenous sub-populations of cells some of which display functional plasticity. Depending on the environmental cues, these Treg cells remain uncommitted and become susceptible to being re-programmed to $FoxP3^-$ helper T cells or $FoxP3^+$ cells which display properties of

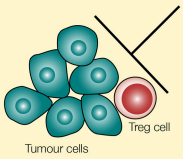
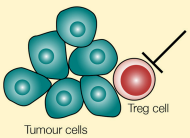
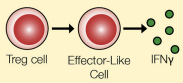
Strategies	Examples
 **Non-Specific Anti-Cancer Drugs** Certain chemotherapeutic agents were found to reduce the frequency of Treg cells and enhance anti-tumor immunity.	Cyclophosphamide Tyrosine kinase inhibitors sunitimab sorafenib imatinib
 **Treg Cell-Depleting Agents** Treg cells express numerous receptors which can be targeted to reduce the cell frequency and subsequently enhance anti-tumor immunity.	Daclizumab (anti-CD25 antibody) Denileukin difitox (IL-2-diptheria toxin conjugate) Fc-optimized anti-CD25 antibody CD25-targeted NIR-PIT MK-4166, DTA-1 (anti-GITR antibody) Anti-folate receptor 4 antibody Ipilimumab (anti-CTLA4 antibody) Mogamulizumab (anti-CCR4 antibody)
 **Conversion of Treg Cells to Immune-Stimulating Cells** Disrupting the stability of intra-tumoral Treg cells using antibody or epigenetic modifiers have found to be an alternative approach to enhance anti-tumor immunity.	Anti-neuropilin-1 antibody EZH2 inhibitor BET bromodomain inhibitor (JQ1) Ep300 inhibitor

FIGURE 1 | Regulatory T cells can be targeted through chemotherapeutic agents, neutralizing antibodies and epigenetic modifiers. Chemotherapeutic agents reduce the quantity of Treg cells and synergize with immune-modulatory drugs to enhance anti-tumor immunity. However, these approaches are not necessarily specific to Treg cells. Surface markers expressed on Treg cells (such as CD25, GITR, folate receptor 4, CTLA-4, and CCR4) can be targeted to more reliably reduce the quantity of Treg cells. Recent approaches involve conversion of Treg cells into "effector-like" $CD4^+$ T cells through the use of neutralizing antibodies as well as epigenetic modifiers.

a helper T cell (126–129). Similarly, there are heterogenous populations of highly suppressive Treg cells in the tumor microenvironment. Although the composition and function of these tumor-infiltrating Treg cells is still a topic of debate, evidence suggest that both thymically-derived natural Treg cells, characterized by high expression of neuropilin-1, and induced Treg cells play important role in regulating anti-tumor immunity (130). Peripherally-derived regulatory T cells, which display greater plasticity, can be targeted to enhance anti-tumor immunity (130, 131). Furthermore, despite the initial assumption that thymically derived Treg cells undergo a strict lineage commitment, Overacre-Delgoffe et al. (132) demonstrated that targeting neuropilin-1 on Treg cells induces IFNγ production and "functional fragility" which can in turn enhance anti-tumor immunity. A recent approach of converting Treg cells into immune-stimulatory cells in the context of tumor immunity involve epigenetic modification of intra-tumoral Treg cells to disrupt their lineage and functional stability. For example, Wang et al. (133) have demonstrated that the histone H3K27 methyltransferase enhancer of zeste homolog 2 (EZH2) activities are increased in tumor-infiltrating Treg cells in both murine and human cancers, and molecular targeting of EZH2 promoted conversion of Treg cells into IFNγ producing cells that were capable of remodeling the tumor microenvironment and enhancing anti-tumor immunity. Several other epigenetic modifiers such as Bromodomain and Extra-Terminal (BET) family proteins and histone acetyltransferase Ep300 can also be targeted to disrupt Treg cell function and improve anti-tumor immune response (134, 135). However, these epigenetic modifiers possess other biological functions, and molecular targeting of these proteins could potentially induce off-target effects.

Despite these alternative approaches to Treg cell blocking or depletion strategies, limitations still exist, including the lack of a Treg cell-specific biomarker and potential induction of autoimmunity as a consequence of systemic Treg cell depletion (136, 137). Lastly, depletion of Treg cells can be followed by their rapid reconstitution, often resulting in a higher frequency in comparison to the level of Treg cells prior to depletion (138, 139). Alternatively, another approach to enhance anti-tumor immunity would be to modify tumor-specific T cells to be resistant to the suppressive effects of Treg cells. This approach may be relevant when adoptive T cell therapies are used including TCR transduction with tumor specific TCR or CAR-T cells.

REPORTED CASES OF TREG RESISTANCE

Since the early 2000s, evidence suggests that there are a variety of molecular pathways and cellular mechanisms which render T cells resistant to the suppressive effects of Treg cells. Numerous surface receptors, intracellular signaling molecules and cytokines have been implicated in T cell resistance to Treg cells (**Figure 2**).

Intracellular and Receptor Targets Controlling Treg Resistance

E3 Ubiquitin Ligase Cbl-b

The inhibition of E3 ubiquitin ligase Cbl-b has shown promising results based on the ability of T cells to resist the suppressive effects of Treg cells both *in vitro* and *in vivo* (140, 141). Through

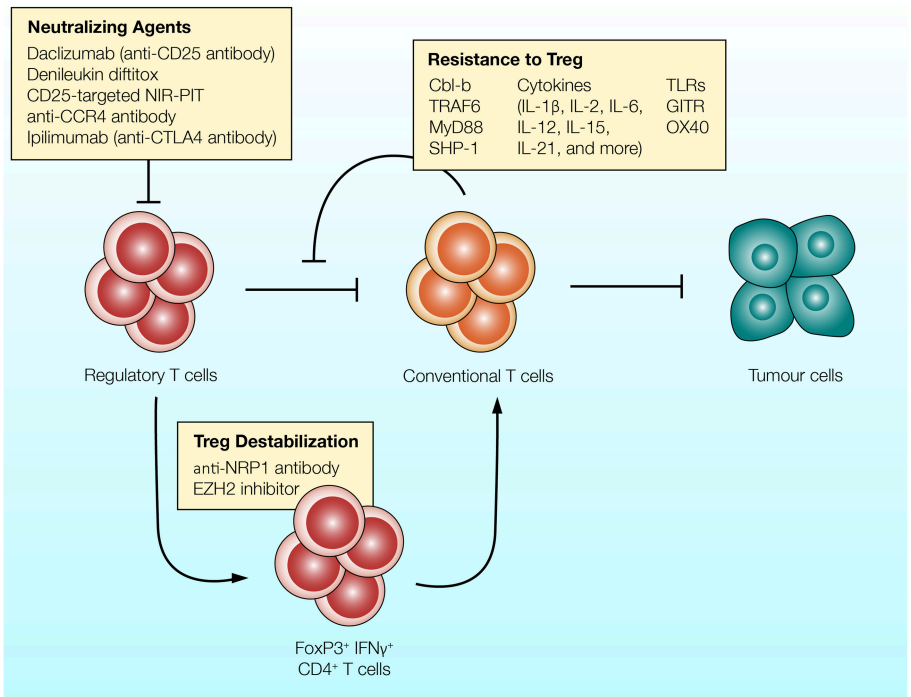


FIGURE 2 | Different strategies can be utilized to overcome the suppressive effects of Treg cells. (1) Antibodies targeting CD25, CCR4, or CTLA-4 expressed on Treg cells can be used to reduce the frequency of regulatory T cells and enhance anti-tumor immunity. (2) Regulatory T cells can convert into T cell stimulatory cells in response to inhibition of EZH2 epigenetic modifier or NRP-1-targeting antibody. Treg cells treated with these agents upregulate IFNγ and enhance anti-tumor immunity (132, 133). (3) T cells can be rendered resistant to the suppressive effects of Treg cells. Intracellular molecules which govern T cell activation (such as Cbl-b and TRAF-6), co-stimulatory receptors (such as TLRs and GITR) and various T cell stimulatory cytokines reduce the ability of Treg cells to suppress T cells.

ubiquitination (and in many cases, subsequent ubiquitin-mediated degradation) or phosphorylation of proteins involved in the TCR signaling pathway, Cbl-b serves as a negative regulator of antigen-induced T cell activation (142). Several molecular targets have been identified, including PKCθ, Nedd4, PLC-γ1, Vav1, LAT, and p85, along with several other TCR signaling molecules that play an important role in T cell activation (143–147). Consequently, through the regulation of these molecules, Cbl-b can control a diverse repertoire of intracellular mechanisms associated with the early phase of T cell activation, such as calcium influx, cytoskeletal rearrangement, immune synapse formation, cytokine secretion as well as proliferation (148, 149). Amongst several signaling pathways downstream of TCR activation, reports highlight the role of PI3K/Akt signaling pathway in T cell resistance to Treg cell-mediated suppression (150, 151). Interestingly, it has become evident that that PI3K and Cbl-b are indirectly regulated by each other to control T proliferation (**Figure 3**). Fang et al. (143) has suggested that Cbl-b regulates the PI3K signaling pathway by binding and ubiquitinating a PI3K regulatory subunit p85. However, a study conducted by Guo et al. (146) offers an alternative explanation where Cbl-b does not directly inhibit PI3K, but instead inhibits the Nedd4-mediated ubiquitination of PTEN, a negative regulator of PI3K activity. Adding to the complexity of the interaction between PI3K/Akt pathway and Cbl-b, Akt also negatively regulates Cbl-b protein level through inactivation of GSK-3, a protein kinase which enhances Cbl-b activity by catalyzing the phosphorylation at Ser476 and Ser480 (152).

In addition to the ability of Cbl-b to regulate molecular pathways associated with TCR signaling, evidence suggests Cbl-b is intertwined with multiple T cell inhibitory signaling pathways. Early studies demonstrated that Cbl-b can be re-expressed in response to CTLA-4 signaling, and CTLA-4 deficient T cells display reduced Cbl-b expression (153). Recent studies suggest that T cells deficient in Cbl-b are less susceptible to PD-1 inhibitory signaling *in vitro* (154, 155). These findings are consistent with a study suggesting that SHP-1, which plays an important role in downstream PD-1 and CTLA-4 signaling pathway, controls Cbl-b activity through direct phosphorylation (156). Furthermore, a study conducted by Mercadante and Lorenz (157) utilizes an *in vitro* Treg suppression assay and homeostatic *in vivo* Treg suppression assay to demonstrate that SHP-1 deficient T cells are less responsive to the suppressive effects of Treg cells. These studies suggest that Cbl-b is linked with key negative regulatory pathways in T cells. Lastly, Cbl-b is also intertwined with TGF-β receptor signaling. Gruber et al. (158) demonstrated that Cbl-b directly ubiquitinates and subsequently downregulates SMAD7, an attenuator of TGF-β receptor signaling. Consistent with this finding, $CD4^+$ T cells

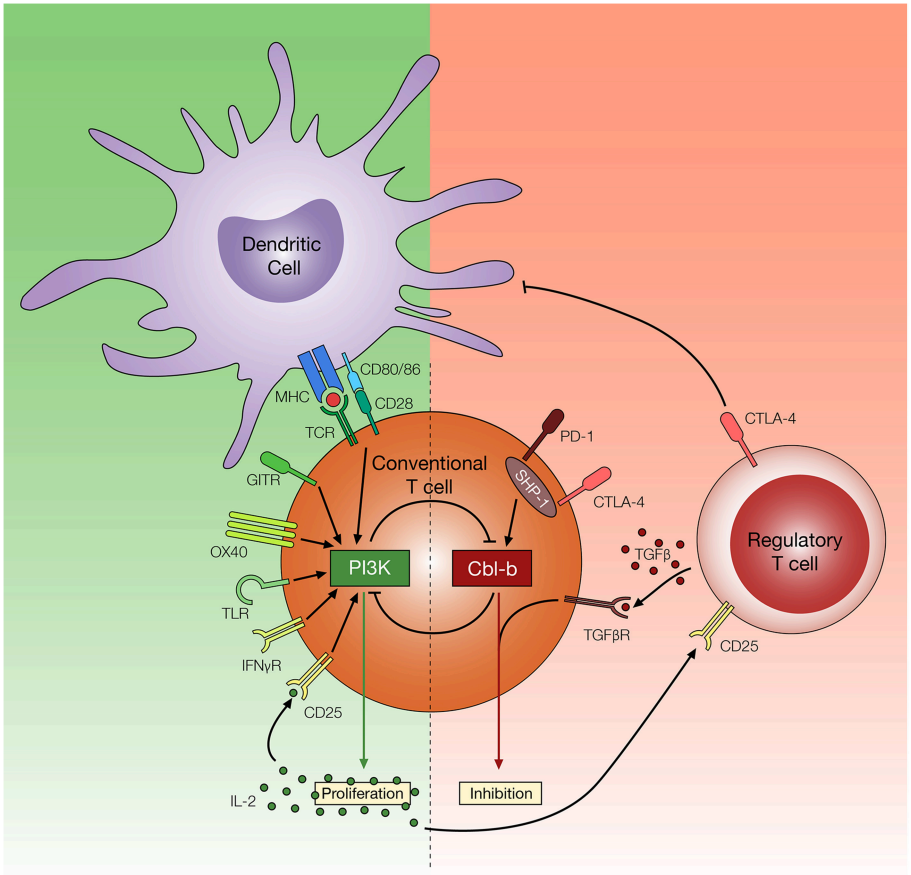


FIGURE 3 | Potential mechanisms of T cell resistance to Treg cells. Regulatory T cells utilize multiple inhibitory mechanisms to limit T cell activation and proliferation, such as downregulation of CD80/86 on DCs, secretion of TGF-β, and consumption of IL-2. Reports suggest amplified PI3K signaling, through TCR, co-stimulatory and cytokine receptors, may render T cells resistant to these effects of Treg cells. In contrast, Cbl-b plays an important role in regulating diverse arms of the TCR signaling pathways and promoting T cell inhibition. Cbl-b deficient T cells are refractory to Treg cell-mediated suppression, but the mechanism of Treg cell resistance remains yet to be elucidated.

deficient in Cbl-b display reduced sensitivity to TGF-β mediated inhibition (140, 141, 158, 159). The multi-faceted role of Cbl-b in regulating TCR signaling pathways as well the inhibitory signaling pathway enables Cbl-b deficient T cells to acquire TCR sensitivity, CD28-independent stimulation, increased cytokine production, and context-dependent TGF-β insensitivity (141, 160), all of which potentially contribute to T cell resistance to Treg cell-mediated suppression (**Figure 3**).

Cbl-b deficient $CD4^+$ and $CD8^+$ T cells resist Treg cell-mediated suppression in an *in vitro* Treg suppression assay, where naïve Cbl-$b^{-/-}$ T cells stimulated with anti-CD3 and irradiated APCs are capable of overcoming the suppressive effects of splenic Treg cells (140, 161). However, (1) the ability of Cbl-$b^{-/-}$ T cells to resist potentially "activated" Treg cells (such as those found in tumors) has not been explored, and (2) *in vitro* Treg suppression assay cannot recapitulate the complex interaction between T cells and Treg cells *in vivo* (60), especially since the Cbl-$b^{-/-}$ mice do not have the same phenotype as Treg deficient mice (17, 162–164). Despite these limitations, many of the *in vitro* observations have been consistent with *in vivo* properties of Cbl-$b^{-/-}$ T cells. For example, T cells deficient in Cbl-b also display a hyperactive T cell status *in vivo*. Gronski et al. (165) has demonstrated the role of Cbl-b in regulating T cell activation threshold, as mice deficient in Cbl-b were more sensitive to antigen-induced T cell stimulation resulting in autoimmunity. Lastly, Adams et al. (141) has demonstrated the role of Cbl-b in $CD4^+$ T cell resistance to Treg cells *in vivo* through a graft-vs.-host disease model, where adoptively transferred Treg cells fail to suppress Cbl-$b^{-/-}$ $CD4^+$ T cells *in vivo*. However, the mechanism by which Cbl-$b^{-/-}$ T cells resist Treg cell suppression has not been investigated in these studies.

T cells deficient in Cbl-b have also been studied in the context of enhancing tumor immune surveillance and anti-tumor immunity. Cbl-b deficiency augments anti-tumor T cell responses in both genetically engineered and transplanted tumor models (161, 166–168). Loeser et al. (161) and Chiang et al. (166) provide evidence showing a greater infiltration of $CD8^+$ T cells using TC-1 and EL4/EG7 transplantable tumors in Cbl-b deficient mice. In both circumstances, $CD4^+$ effector T cell infiltration did not increase. Interestingly,

despite the increased infiltration of Treg cells in the tumors from Cbl-b deficient mice, T cells were able to either reject or attenuate tumor growth. A similar observation has been made when Cbl-b deficient mice were crossed with ataxia telangiectasia mutated (ATM) deficient mice, which attenuated the spontaneous development of lymphoid tumors and increased overall survival, demonstrating a robust anti-tumor immunity against genetically engineered tumor model (166). Although further investigation is required to understand how Cbl-b deficient T cells enhance anti-tumor immunity, one of the proposed mechanisms include insensitivity to TGF-β receptor signaling. Gruber et al. (158) suggested that Cbl-b deficiency promotes spontaneous rejection of TC-1 tumors, whereas Cbl-$b^{-/-}$ mice crossed with $CD4^{Cre}$- $SMAD7^{fl/fl}$ mice abrogates anti-tumor immunity, thus highlighting the importance of Cbl-b deficient T cells in anti-tumor immunity and the ability of these T cells to potentially overcome TGF-β receptor signaling. Lastly, in all of the previously described studies, whether Cbl-b deficient T cells resist the suppressive effects of Treg cells to enhance anti-tumor immunity has not been shown *in vivo*.

TLR—MyD88—TRAF6 Axis

Evidence suggests that TLR signaling also play an important role in T cell resistance to Treg cells. Pasare and Medzhitov (169) suggested that TLR4 and TLR9-mediated stimulation of DCs and the subsequent increase in IL-6 production by DCs render T cells resistant to the effects of Treg cells. However, this particular study presumed that TLR signaling was restricted to DCs. TLRs can be expressed by effector T cells and Treg cells, and play an important role in their cellular activation and survival (170, 171). Although our understanding of TLR signaling pathways in T cells is rather limited, TLRs expressed on T cells likely function similar to co-stimulatory receptors which trigger the downstream MyD88 signaling pathway as well as the PI3K/Akt signaling pathway (172). TLR signaling in T cells may also play an important role in rendering T cells refractory to Treg cell-mediated suppression. For example, TLR9 stimulation of murine T cells enhances the PI3K/Akt signaling pathway and MyD88-dependent IL-2 production; TLR9 signaling also renders T cells resistant to the suppressive effects of Treg cells (173, 174). Downstream of TLRs, MyD88 interacts with IRAK1 and IRAK4, modulating the activities of an E3 ubiquitin ligase TRAF6 which may contribute to NFκB signaling (175). However, the role of TRAF6 in T cells is far more complex and contradictory, which is exemplified through a study suggesting that TRAF6 also serves as a negative regulator of T cell function (176). In this study, T cells deficient in TRAF6 display enhanced T cell activation, CD28-indpendent stimulation and resistance to Treg cell-mediated suppression (176). Although TLR signaling can promote T cell resistance to Treg cells, the precise molecular mechanism remains yet to be elucidated. It is worth noting that TLR stimulation of T cells increases cytokine production (173, 177), thus future studies should delineate the effect of TLR-MyD88 signaling vs. subsequently induced cytokines in generating resistance to Treg cells. Lastly, it is also crucial to evaluate the effect of TLR signaling on regulatory T cells which also express TLRs (170). The role of TLR signaling on Treg cell function requires further investigation and clarification since it can both abrogate and enhance Treg cell functions (170, 177–179). A recent study suggested that TLR signaling on regulatory T cells induces PI3K/Akt/mTORC1 signaling which subsequently increases glycolysis and GLUT1 expression, which in turn interferes with FoxP3 expression and the suppressive ability of Treg cells (180). However, increased Treg cell function observed in several studies could also occur indirectly as a result of enhanced T cell stimulation and IL-2 secretion, which can subsequently promote Treg cell function.

Although TLR agonists can improve anti-tumor immune responses by enhancing T cell function and/or stimulating APC maturation, they may also act on other immune cells and cancer cells to impact anti-tumor immunity (181, 182). Therefore, it would be difficult to specifically target TLRs to promote resistance to Treg cells.

TNF Family Members

TNF family members such as GITR, OX40, and 4-1BB on T cells can also be targeted to induce T cell resistance to Treg cells (183–188). Evidence suggests that amplification of GITR signaling through the use of agonistic antibody, DTA-1, enhances T cell stimulation in the presence of Treg cells both *in vitro* and *in vivo* (184, 189, 190). However, GITR is also highly expressed on Treg cells and studies suggests that a GITR agonist attenuates Treg cell stability (191, 192). In contrast, *in vivo* administration of non-depleting Fc-GITR-L induces context-dependent modulation of Treg cell activities (193). Further work is required to precisely understand the effect of GITR signaling on Treg cells. Although the role of GITR agonist in the interaction between T cell and Treg cell is unclear *in vivo*, Stephens et al. (184) suggested that GITR signaling directly acts on T cells to resist the suppressive effects of Treg cells *in vitro*. Lastly, a GITR agonist antibody (DTA-1) has demonstrated its potential in enhancing $CD8^+$ T cell response and reducing intra-tumoral Treg cell activities using transplantable tumor models including the B16 melanoma model (190, 192, 194). In summary, administration of TNF-family receptor agonists such as those targeting GITR promote T cell response in the presence of Treg cells and contribute to enhanced anti-tumor immunity. However, the mechanism behind how TNF family receptor signaling renders T cells refractory to Treg cell-mediated suppression is poorly understood.

Cytokine Networks

Most intracellular molecules and surface receptor targets which render T cells resistant to inhibition by Treg cells often promote the secretion of a high quantity of T cell stimulatory cytokines. This is demonstrated by the early study conducted by Pasare and Medzhitov (169), which showed that LPS stimulation of DCs leads to increased IL-6 which plays an important role in T cell resistance to regulatory T cells (169, 195). Similarly, inhibition of Cbl-b or activation of GITR signaling increases IL-2 production by T cells both *in vitro* and *in vivo* (167, 168, 183). Increased cytokine production is often perceived as an indicator of Treg resistance. However, evidence suggests that various cytokines themselves can directly drive T cell resistance to Treg cells (195–199). This raises a question—to what extent do cytokines play a role in Treg resistance? Both T cells and Treg cells are susceptible

to cytokine receptor-mediated signaling, and therefore the effect of cytokines in both cell compartment must be considered.

Soluble mediators such as cytokines can modulate a powerful receptor-mediated T cell signaling required for cellular proliferation, survival, and resistance to Treg cell-mediated suppression. Cytokines including interferons (IFNγ and IFNα), those binding to receptors that include the common γ-chain (IL-2, IL-4, IL-7, IL-15, IL-21, and TSLP), gp130 receptor cytokines (IL-6) and IL-1 receptor cytokines (IL-1β and IL-18) employ diverse combinations of intracellular signaling pathways such as the JAK/STAT signaling pathways to promote T cell differentiation and effector functions (200–202). Many studies have also highlighted the role of T cell stimulatory cytokines, in particular IL-1β, IL-2, IL-4, IL-6, IL-7, IL-15, and IL-21, as central drivers of T cell stimulation in the presence of Treg cells (87, 195–198, 203–205). Some of these T cell stimulatory cytokines may induce T cell proliferation and survival in the presence of Treg cells by common mechanisms, because their receptors share overlapping downstream signaling pathways, but the mechanism by which each of these cytokines support T cell proliferation in the co-cultures has not been fully clarified.

One of the first cytokines reported to enhance T cell proliferation in the presence of Treg cells *in vitro* is IL-2 (199). Upon high-affinity quaternary IL-2-IL2R complex formation, tyrosine kinases JAK1, and JAK3 also initiate a STAT1, STAT3, and STAT5-dependent response, along with the induction of the PI3K signaling pathway (201, 202). Although IL-2 serves as a potent inducer of T cell proliferation in Treg suppression assays, there is no strong evidence suggesting that the signaling pathways downstream of IL-2 directly attenuates the inhibitory signals induced by Treg cells. Instead, excess IL-2 could enable T cells to overcome Treg cell-mediated cytokine deprivation (87, 199), which, despite being somewhat controversial, may be an important suppressive mechanism utilized by Treg cells (89, 91). Lastly, many T cell stimulatory cytokines including IL-2, IL-7, and IL-15 play an important role in enhancing anti-tumor immunity (206–208), but whether or not these cytokines render T cells resistant to the suppressive effects of Treg cells in the context of anti-tumor immunity is unclear.

When evaluating the role of cytokines in rendering T cells resistant to Treg cells, the effect of cytokine signaling must also be evaluated on Treg cells. Under a circumstance where T cell stimulatory cytokine destabilizes Treg cell function, it becomes challenging to determine whether T cell resistance to Treg cells play an important role in the observed T cell proliferation in the presence of Treg cells. Although poorly understood, Treg cells display phenotypic and functional plasticity in response to certain cytokines; T cell stimulatory cytokines may mediate the downregulation of FoxP3 or conversion of Treg cells into conventional T cells (209, 210). This is exemplified through a study which demonstrates the ability of IL-4 to convert $FoxP3^+$ cells into effector $CD4^+$ T cells, thereby undermining oral tolerance (211). PI3K signaling pathway is regulated by PTEN expression in Treg cells to prevent loss of Treg cell stability (212, 213), however, IL-4 may disrupt this process by enhancing PI3K signaling. Several other cytokines including IL-21 also antagonize Treg cell proliferation and reduce the frequency of Treg cells (214). However, a study conducted by Attridge et al. (215) suggest that IL-21 may act on T cells to limit IL-2 production which subsequently impairs Treg cell homeostasis. Furthermore, a recent study conducted by Overacre-Delgoffe et al. (132) suggests that attenuating Nrp-1 signaling on intra-tumoral Treg cells induces increased secretion of IFNγ by the Treg cells, and IFNγ subsequently acts on nearby regulatory T cells to "destabilize" their suppressive phenotype. In contrast to the previously discussed examples which destabilize FoxP3 expression in Treg cells, a few cytokines binding to receptors that include the common γ-chain can enhance Treg cell proliferation and function. For instance, adding IL-2 enhances T cell proliferation, despite also stimulating Treg cells (87, 199).

Another possibility to be considered in cytokine-induced T cell resistance to Treg cells *in vitro* is proliferation and expansion of T cell quantity as the mechanism of Treg cell resistance, which should be distinguished from the ability to negate immunosuppressive signals. Especially in a murine *in vitro* system where Treg cell proliferation is limited, the capacity of T cells to proliferate may be independent of their ability to negate immunosuppressive signals by Treg cells. In other words, these T cells stimulated with cytokines may be equally susceptible to Treg cell-mediated suppression, but by increasing proliferation and quantity of T cells, the suppressive effect of Treg cells may become less apparent.

Observations From Current Clinical Studies

One of the primary objectives of cancer immune therapy is to modulate anti-tumor T cell properties to reduce the tumor burden. However, the presence of immunoregulatory cells such as Treg cells are likely to interfere with the anti-tumor T cell response (9, 60, 216). Thus, overcoming the suppressive effects of Treg cells to potentially enhance anti-tumor T cell response in patients is a strategy currently under investigation. Many of the current clinical studies involve targeting surface receptors on Treg cells such as CD25, CTLA-4, and CCR4 (110, 124, 217).

However, clinical studies have not focused on rendering T cells resistant to the suppressive effects of Treg cells. Interestingly, some of the existing treatment methods may already foster T cells resistant to Treg cells. For instance, high dose IL-2 is part of the protocol for adoptive TIL therapy against metastatic melanoma, despite actively expanding immunosuppressive $ICOS^+$ Treg cells (55, 218–221), supporting the possibility that high-dose IL-2 is successful because it may render TIL resistant to Treg cell suppression. Therefore, the dosage of systemic IL-2 administration in these studies may play an important role in promoting the T cell response against the tumor, since low dose IL-2 has been used to preferentially expand Treg cells to attenuate the progression of human autoimmune diseases (222, 223). To avoid IL-2-mediated expansion of immunosuppressive Treg cells, a pre-clinical study conducted by Charych et al. (224) suggested that NKTR-214, a biologic drug containing an IL-2 core conjugated to 6 releasable polyethylene glycol chains, can be utilized to preferentially induce IL-2 signaling on T cells while reducing the expansion of Treg cells. In this study, the ability of NKTR-214 to preferentially bind to IL-2Rβ over IL-2Rα induces a

greater $CD8^+$ T cell to Treg cell ratio, greater exposure to IL-2 in the tumor and a more robust anti-tumor immunity in comparison to aldesleukin. This particular approach is currently in clinical trials. Several other therapeutic strategies involving modified IL-2 biologics also suggest similarly promising results in their ability to preferentially enhance T cells over Treg cells (225, 226).

CONCLUDING REMARKS

Regulatory T cells can be potent regulators of anti-tumor immunity, and numerous strategies have been proposed to reverse the suppressive effects of Treg cells. One promising approach involves rendering T cells resistant to the suppressive effects of Treg cells. Resistance to Treg cells can be achieved through modulation of intracellular molecules, co-stimulatory surface receptors or cytokines, all of which may act through partially redundant or overlapping mechanisms. Concepts discussed in this review primarily focus on strategies to manipulate the balance between T cells and Treg cells. However, future studies should validate these concepts in the context of anti-tumor immunity and focus on recapitulating many of these observations using primary human T cells.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

Canadian Institute for Health Research Grant (Foundation Scheme - FDN 143220).

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

frontiers in Oncology

REVIEW
published: 30 April 2019
doi: 10.3389/fonc.2019.00322

A Metabolism Toolbox for CAR T Therapy

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The adoptive transfer of T cells expressing chimeric antigen receptors (CARs) through genetic engineering is one of the most promising new therapies for treating cancer patients. A robust CAR T cell-mediated anti-tumor response requires the coordination of nutrient and energy supplies with CAR T cell expansion and function. However, the high metabolic demands of tumor cells compromise the function of CAR T cells by competing for nutrients within the tumor microenvironment (TME). To substantially improve clinical outcomes of CAR T immunotherapy while treating solid tumors, it is essential to metabolically prepare CAR T cells to overcome the metabolic barriers imposed by the TME. In this review, we discuss a potential metabolism toolbox to improve the metabolic fitness of CAR T cells and maximize the efficacy of CAR T therapy.

Keywords: immunotherapy, metabolism, chimeric antigen receptor (CAR), tumor microenvironment (TME), anti-tumor immune response

OPEN ACCESS

Edited by:
Prashant Trikha, Nationwide Children's Hospital, United States

Reviewed by:
Pappanaicken R. Kumaresan, University of Texas MD Anderson Cancer Center, United States
Lenka V. Hurton, University of Texas MD Anderson Cancer Center, United States

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *24 January 2019*
Accepted: *10 April 2019*
Published: *30 April 2019*

Citation:
Xu X, Gnanaprakasam JNR, Sherman J and Wang R (2019) A Metabolism Toolbox for CAR T Therapy. Front. Oncol. 9:322. doi: 10.3389/fonc.2019.00322

CANCER CELL METABOLIC PROGRAM

Since cancer cells must constantly proliferate, they must also continuously generate new biomass. This in turn requires a substantially different metabolic program than that of non-proliferating somatic cells. Most non-proliferating cells utilize oxidative phosphorylation (OXPHOS) to efficiently extract ATP from pyruvate, while cancer cells reduce the majority pyruvate into lactate, a process termed aerobic glycolysis or "the Warburg Effect" (1). In 1956, Otto Warburg observed the tendency of cancer cells to metabolize glucose into lactate instead of carbon dioxide and concluded that cancer was a disease of damaged respiration (2). While not all of Warburg's conclusions have stood the test of time, it holds true that metabolism is a critical component of oncogenesis. Even if a cell has developed mutations to overcome the normal regulation of proliferation, it also requires a metabolic program that will allow the cell to synthesize all the molecules required for a new cell. Metabolism is so critical to oncogenesis that the most commonly mutated pathways, including Ras, Phosphoinositide 3-kinases (PI3K)/AKT/mammalian target of rapamycin (mTORC1), hypoxia inducible factor 1 (HIF-1), proto-oncogene MYC (c-MYC), and p53, are key metabolic regulators. HIF-1 and c-MYC in particular act in concert to express glucose and lactate transport proteins while diverting pyruvate away from OXPHOS and toward lactate production (3–5). While this method does not maximize the amount of ATP that can be extracted from glucose, it is none the less advantageous for proliferating cells. By keeping glucose derived carbon out of the TCA cycle, additional carbons are made available for lipid, protein and especially nucleotide synthesis (1, 6, 7). Sequential activation of PI3K, followed by Akt and mTORC1, are also able to aid cancer cells in capturing glucose from the environment, as well as catabolic metabolites from the mitochondria. Akt activates hexokinase and phophofructokinase-1, to retain glucose and commit it to further glycolysis, as well as ATP-citrate lyase, to convert mitochondrial citrate into cytosolic acetyl-CoA for lipid synthesis (7, 8). Finally, mTORC1 enhances mitochondrial biosynthesis to take

oxaloacetate and α-ketoglutarate (α-KG) and convert them into amino acids for protein synthesis (7). Alternative sources of carbon can support growth in cell size, but glucose is required for robust DNA synthesis and concomitant cell cycle progression (9). The other major carbon and energy source is glutamine. Upon being metabolized by the cell, glutamine is shuttled into the TCA cycle to make α-KG for the production of amino acids, or citrate for the production of lipids (1, 6). Loss of p53 additionally allows malate to leave the TCA cycle to be converted into pyruvate, generating additional NADPH, another necessity for nucleotide synthesis (10). Reduction of pyruvate into lactate serves to regenerate NAD^+ for further glycolysis while also conditioning the extracellular space (11).

The disorganized structure of a solid tumor means that it is not properly enervated by blood vessels. Cells located farther from the blood vessels experience hypoxia, nutrient deprivation and acidosis (3, 12). Cancer cells experiencing nutrient deprivation must rely on alternative metabolites or methods of nutrient acquisition. Ras expressing cancer cells may use macropinocytosis to scavenge nutrients from the surrounding milieu. Further, Ras driven cells utilize autophagy to degrade unnecessary cellular components into small molecule nutrients (12). Alternatively, hypoxia re-enforces glycolytic generation of lactate, while also shunting glutamine toward lipid genesis (3, 12). Finally, lactate is associated with several oncogenic processes, such as angiogenesis, cell migration and immune suppression (5). Not strictly a waste product, cells that experience chronic acidosis may take up lactate for gluconeogenesis and use in oxidative phosphorylation (11). Thus, as a tumor progresses, cancer cells condition the microenvironment, creating unique selection pressures and contributing to further heterogeneity and metabolic derangement.

T CELL METABOLIC PROGRAM

T cells play a key role in mounting a robust, antigen specific adaptive immunity against invading pathogens and tumor. Upon stimulation of antigen receptors, naïve T (T_n) cells rapidly transit from a quiescent to an active state that begins with a 24 h growth phase followed by massive proliferation, differentiation, and migration. To elicit a robust immune response, T cells can differentiate into diverse functional subsets. Depending on the cytokine milieu of the microenvironment, active $CD4^+$ T cells can differentiate into immune suppressive regulatory T (T_{reg}) cells or inflammatory T effector cells, such as T helper T_H1, T_H2, T_H9, T_H17 and follicular helper T (T_{fh}). On the other hand, active $CD8^+$ T cells mainly differentiate into $CD8^+$ effector T (T_{eff}) cells, also referred as cytotoxic T lymphocytes (CTL). Following pathogen clearance, the majority of the effector cells die through apoptosis while the remaining cells survive to form a population of long-lived memory T (T_{mem}) cells, responsible for immunity upon subsequent challenges to the same pathogen. T_{mem} cells are composed of distinct subsets including stem memory cells (T_{scm}), central memory cells (T_{cm}), and effector memory cells (T_{em}). T_{scm} cells exhibit a naïve like phenotype ($CD44^-CD62L^+/CD45RA^+$), express interleukin-2 receptor (IL-2R) β and the chemokine C-X-C motif receptor 3 (CXCR3), representing the earliest and long-lasting developmental stage of T_{mem} cells. T_{scm} cells have a capacity to self-renew and generate the entire spectrum of more differentiated cells. T_{cm} cells are $CD62L^+$, reside in lymph nodes and have limited or no effector function, but they proliferate and become effector cells upon secondary stimulation. These cells represent an intermediate population between T_{scm} cells and T_{em} cells. T_{em} cells are $CD62L^-$, are the progenitor cells prone to differentiate into T_{eff} cells upon secondary stimulation. Therefore, T_{em} are responsible for protective memory, and migrate into inflammatory tissues to elicit an immediate response against antigens (13).

T cell activation and differentiation are accompanied by dramatic shifts in cellular metabolic programs which fulfill their bioenergetic, biosynthetic and redox demands (14–19). Essentially, different phenotypic and functional T cell subsets are characterized by unique metabolic demands, which are tightly linked with immune modulatory signaling cascades. Specifically, quiescent T_n and T_{mem} cells rely on fatty acid oxidation (FAO) and OXPHOS to maintain their basic energy level, cellular function and viability (20, 21). In addition, heightened glycerol uptake and triglyceride synthesis also play an important role in promoting memory $CD8^+$ T cells (22, 23). Overall, active T cells predominantly engage in aerobic glycolysis, the pentose phosphate pathway (PPP) and glutaminolysis to drive proliferation and subsequent effector functions (20, 24–28). However, it remains unclear whether a shift in glucose metabolism promotes activated T cells to become long-lived T_{mem} cells. It has recently been suggested that persistently heightened glycolysis in T_{eff} cells compromises the formation of long-lived memory cells by driving T cells toward a terminally differentiated state, resulting in a failure to survive upon adoptive cell transfer, whereas a moderately dampened glycolysis supports the generation of long-lived memory $CD8^+$ T cells and enhances anti-tumor immune response (29, 30). $CD4^+$ effector T cells including T_H1, T_H2, T_H9, T_H17, and T_{fh} cells display heightened glycolysis, whereas FAO activity supports the differentiation and function of T_{reg} cells (31–37). Conversely, heightened aerobic glycolysis is eventually required to drive T_{eff} cells proliferation and differentiation into cytotoxic T cells (38).

Catabolism of glucose and glutamine generates energy, provides reducing power, and donates carbon and nitrogen to biosynthetic building blocks. During the sequential reactions of aerobic glycolysis, the breakdown of glucose into lactate generates ATP and intermediate metabolites, many of which are channeled into the biosynthesis of amino acids, lipids and nucleotide. Branching from glycolysis, the PPP starts from glucose-6-phosphate, an immediate metabolic product of glucose, and produces NADPH in the oxidative phase as well as five-carbon sugars in non-oxidative phase, the latter of which can feed back into glycolysis or provide precursors for nucleotides. Meanwhile, NADPH is required for modulating redox balance and cholesterol biosynthesis (17, 20, 39). During glutaminolysis, glutamine is converted to glutamate and subsequently to α-KG, which serves as an important anaplerotic substrate of the TCA cycle and fuels mitochondrial ATP production. As a major source

of carbon and nitrogen, the catabolic products of glutamine are funneled to support the biosynthesis of amino acids, hexosamine, polyamine, lipids and nucleotides during T cell proliferation and differentiation (40–43). Similar to glucose catabolism, glutamine catabolism is composed of multiple metabolic routes branched from glutaminolysis. Glutamate is the key branch point in glutaminolysis and can be committed toward the biosynthesis of glutathione (GSH) or toward mitochondrial oxidation to produce biosynthetic precursors and ATP. Glutamate is derived from glutamine in parallel through glutaminase, in mitochondria, as well as through glutamine utilizing enzymes, including amidotransferase, largely in cytosol (44–47). As such, the subcellular compartmentalization of glutamate may represent an important mechanism that enables a fine-tuned coordination between branched metabolic routes to fulfill bioenergetics, biosynthetic and redox demand in T cells (36, 48, 49).

T cell metabolic reprogramming during activation is strictly regulated by numerous kinase signaling cascades, including mitogen-activated protein kinase (MAPK)/extra-cellular signal-regulated kinase (ERK), mTOR kinase, AMP-activated protein kinase (AMPK), and PI3K/Akt (20, 24, 50–55). T cell activation requires co stimulation of CD28 and IL-2 signaling, which activates PI3K/AKT and mTOR to promote the uptake of glucose and amino acids to support CD4 T cell proliferation and differentiation (31, 56). AMPK and mTOR coordinate metabolic status with signaling transduction in regulating T cell differentiation. T_H1, T_H2, and T_H17 subsets, which predominantly rely on glycolysis, maintain high mTOR activity. Conversely, T_{reg} cells, which require FAO, maintain high AMPK activity. In addition, inhibition of mTOR complex 1 (mTORC1) by rapamycin or Wnt-β-catenin signaling in T cells drives the differentiation of T_{scm} cells by switching T cell metabolism toward FAO and increasing the long term survival (57, 58).

Beyond these key kinase-signaling cascades, (HIF-1) and the (c-Myc), are the key transcription factors that regulate the expression of metabolic enzymes in glucose and glutamine catabolism (20, 32, 59, 60). In addition, activating enhancer binding protein 4 (AP4), Activating transcription factor 4 (ATF4), interferon regulatory factor 4 (IRF4), Bcl-6 are involved in regulating the expression of various metabolic genes to promote glycolysis and glutaminolysis as well as T_{eff} function (61–63). On the other hand, *de novo* cholesterol biosynthesis is regulated by the dynamic regulation of nuclear receptor-liver X Receptor (LXR) Foxo1 protein (Foxo1) and the orphan steroid receptor, Estrogen-related receptor alpha (ERRα) (31, 33, 40, 64, 65).

METABOLIC ANTAGONISM IN THE TME

Emerging evidence suggests that various metabolites from various cellular compartments within the TME may serve as a complex form of intercellular communication which modulates tumor cell growth and response to therapy (66–72). T cell metabolic pathways are tightly and ubiquitously linked with T cell activation, proliferation, differentiation, and immune functions (24, 25, 27, 31, 39, 39, 51, 56, 73). Thus, the immune cells, particularly effector T cells, are intimately controlled by the metabolic communications in the TME.

Nutrients Depletion

In addition to lineage-specific metabolic requirements, which are associated with the metabolic network in the tissue-of-origin, cancer cells display a heightened ability to capture carbon and nitrogen sources from the TME and process these raw materials to meet the cell's fundamental requirements for energy, reducing power and starting materials for biosynthesis. These general metabolic features of cancer cells are required to support the needs imposed by proliferation and other neoplastic features, but at the same time often deplete the TME of nutrients (74, 75). In addition to the consumption of key carbon and nitrogen sources, glucose and glutamine, rapidly proliferating cancer cells and T effector cells have a strong demand for amino acids, some of which are not only required for protein synthesis, but are also coupled to other anabolic routes and therefore integrated into central carbon metabolism. However, both cancer and T effector cells are often dependent on the uptake of extracellular substrates from the TME, as opposed to *de novo* biosynthetic pathways, which are either defective or insufficient to fulfill the demands. It is well-documented that high expression of indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) by macrophages and cancer cells contributes to immune tolerance by mediating the conversion of tryptophan to kynurenine (76–79). Tryptophan depletion and kynurenine accumulation cooperatively suppress anti-tumor immunity by reciprocally impairing the growth and survival of T effector cells and enhancing the development and function of Tregs and myeloid-derived suppressor cells (MDSC) (80–85). Extracellular cysteine and arginine are also important nutritional resources, which both T and cancer cells compete over. Cysteine, alone with glycine and glutamate, are the substrates for the *de novo* synthesis of GSH, which is the most abundant cellular antioxidant, to ensure physiological levels of intracellular reactive oxygen species (ROS) (20, 36, 48, 49, 51, 73, 86, 87), While glucose and glutamine catabolism provide glycine, glutamate and reducing power though NADPH, proliferating cells largely obtain cysteine from the local microenvironment (20, 86, 88–101). Lack of cystathionase, the enzyme that converts methionine to cysteine, may render T cells particularly vulnerable to cysteine starvation compared to cancer cells (102). Supplementing T cells with arginine has been shown to promote the production of pro-inflammatory cytokines as well as a central memory phenotype *in vitro*, while also enhancing the T cell-mediated antitumor response *in vivo* (103–107). Conversely, the production of the arginine-degrading enzyme, arginase, in the TME has been known to causes arginine depletion and T cell anergy (104). Further, nitric oxide (NO), which is produced from arginine by nitric oxide synthases (NOS), may have cytotoxic effects on proliferating cells in the TME. However, mutated p53 may confer the cancer cells with enhanced resistance to NO-mediated cytotoxicity when compared to T effector cells (108–111).

Accumulation of Immune Suppressive Metabolic End-Products and By-Products

A fierce competition for limited carbon and nitrogen sources between tumor and T effector cells leads to the depletion of nutrients and accumulation of metabolic end-products and by-products, the latter of which also has a profound impact on T

effector cells. Accumulation of lactic acid and CO_2 results in the acidification of the TME, which suppresses T cell proliferation and impairs cytokine production and cytotoxic activity of T cells, while causing tumor radio resistance and promoting tumor cell migration and invasion (112–118). The acidification of the TME also profoundly impacts the cross-membrane transport of sodium ions and amino acids, as well as the pro inflammatory function of T effector cells (117–121). Additionally, tumor-derived potassium has been shown to potentially suppress T cell function (122). Stressed or damaged cells release ATP/ADP and their catabolic product adenosine into the TME, the latter of which elicits immune suppressive effects, partially through engaging cell-surface P2 purinergic receptors-mediated signaling in T cells (123–128). CD39 and CD73 are two ectonucleotidases that are widely expressed in the plasma membrane of cancer cells and cancer stromal cells and are responsible for the conversion of ADP/ATP to AMP and AMP to adenosine. As such, CD39 and CD73 play critical roles in determining the outcome of antitumor immunity through shifting ATP-driven pro-inflammatory effects to an anti-inflammatory milieu mediated by adenosine (129–131). Adenosine deaminase (ADA) converts adenosine to inosine, terminating adenosine-mediated immune suppressive effects (132). Consistent with this finding, the genetic loss of ADA results in an accumulation of adenosine, and leads to severe combined immunodeficiency (SCID) (133, 134).

The genetic context also plays a critical role in determining the composition of immunomodulatory metabolites in the TME, which differs dramatically in tumors with or without mutations in specific metabolic enzymes (135–137). R-2-hydroxyglutarate (2HG), which is enriched in tumors with gain-of-function isocitrate dehydrogenase 1/2 mutations, suppresses T cell activation and differentiation. Intriguingly, S-2HG (the other enantiomer of 2HG) supplement greatly enhances the anti-tumor capacity of adoptively transferred T cells (138). Tumors with succinate dehydrogenase (SDH) or fumarase mutations display elevated levels of succinate and fumarate, respectively. While the accumulation of intracellular 2HG, succinate and fumarate (often referred as onco-metabolites) may drive transformation in a tumor intrinsic manner, all these metabolites may indirectly contribute to tumorigenesis through their immunomodulatory effects (139, 140). A cell permeable form of fumarate, dimethyl fumarate (DMF), is the active ingredient of BG-12/TECFIDERA and FUMADERM, which have been widely used for treating autoimmune disorders (141–143). The anti-inflammatory activity of DMF has been partially attributed to its effect on suppressing T effector functions (36, 144, 145).

Depending on the nature of the metabolic stress imposed by the TME, cancer cells readily engage an array of signaling responses that are largely orchestrated by AMPK, mTOR or transcriptional factors, such as HIF1α, nuclear factor-like 2 (Nrf2), and general control non-derepressible 2 (GCN2). These stress responses are not only adaptive, but also cytoprotective and oncogenic, thus rendering cancer cells resistant to apoptosis and favoring the development of more aggressive, invasive and malignant phenotypes (146–150). Similarly, metabolic stresses favor the development of immune suppressive regulatory T (T_{reg}) cells and tumor associated macrophages (TAM) (31, 32, 151–154). However, metabolic stresses are less tolerated in quickly proliferating T effector cells, leading to more cell death as well as less proliferation and cytokine production (14, 18, 39, 155–158). To survive, expand and exert robust anti-tumor activities in the TME, T_{eff} cells must efficiently overcome the metabolic stress caused by the depletion of nutrients and accumulation of immune suppressive metabolites (**Figure 1B**). Recent research suggests that, the transfer of less differentiated T_{mem} subsets results in greater expansion, persistence and anti-tumor efficacy than terminally differentiated T_{eff} cells. In particular, T_{scm} has a robust capacity for self-renewal and functional plasticity, though further differentiation into T_{cm}, T_{em}, and T_{eff} subsets, thus providing a persistent anti-tumor immunity. Therefore, these cells could effectively compete with cancer stem cells over time to eradicate the tumor mass (159–161). Overall, less differentiated T_{mem} cells require increased mitochondrial FAO and spare respiratory capacity (SRC) for their long term survival and persistence (25, 162, 163). Clearly, the metabolic fitness of T cells is essential for successful adoptive immunotherapy.

METABOLIC OPTIMIZATION OF THE CLINICAL MANUFACTURE AND APPLICATION OF CAR T CELLS

The recent breakthroughs of CD19 CAR T cell therapies to cure hematologic malignancies provide an exciting promise of extending this approach to solid tumors (164, 165). However, a critical barrier to using CAR T therapy for treating solid tumors that express appropriate antigens is the tumor's hostile microenvironment. A plethora of immunosuppressive mechanisms imposed by tumor cells suppress T cell proliferation, survival and effector function (155–169). In addition to the cytokine-mediated and cell-surface receptor-mediated signals that are essential for suppressing T cell functions and responses, the TME represents a dramatic example of metabolic derangement. Insufficient tumor vascularization due to disorganized blood vessel networks leads to hypoxia, lack of nutrients, acidosis, and the accumulation of metabolic waste and free radicals (170, 171). In addition, the increased nutrient and oxygen demands of tumor cells imposed by heightened oncogenic signaling further aggravates the metabolic stresses that suppress effector T cells function (6, 7, 46, 74, 75, 155, 169, 172, 173). As such, a rational and effective CAR T immunotherapy for solid tumors needs to integrate novel strategies which improve T cell metabolic fitness to overcome metabolic stresses imposed by the TME.

The standard manufacturing process of CAR T cells starts by obtaining the patient's peripheral blood mononuclear cells (PBMC) through leukapheresis, followed by T cell enrichment, activation and gene modification with viral or non-viral methods. These genetically modified T cells are then expanded to the required cell numbers for therapy, and then formulated and/or cryopreserved before infusion into the patient (**Figure 2**). The production of CAR T cells requires quality control testing throughout the entire process and is subjected to Good

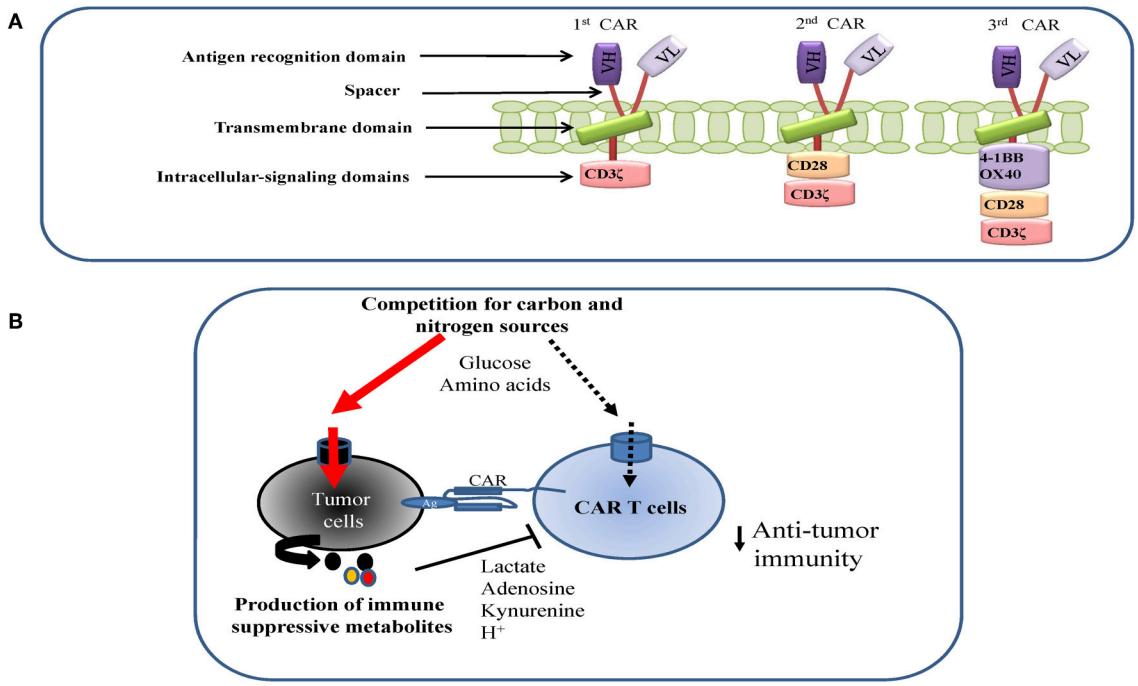

FIGURE 1 | (A) CART design and structure. **(B)** Metabolic antagonism between tumor and CAR T cells in the tumor microenvironment (TME). Tumor cells may suppress CAR T cells proliferation and function by competing for key nutrients with CAR T cells and excreting immune-suppressive metabolites into the TME.

Manufacturing Practices (GMP) guidelines (174–177). We envision using the following metabolic strategies to optimize the manufacturing process and maximize the therapeutic efficacy of CAR T cells.

Tumor Tissue Specimen Collection Before Leukapheresis

The personalized nature of CAR T therapy requires a patient-tailored strategy to ensure the robustness and reproducibility of personalized cell products. Whenever biopsy or surgery is applicable, a step-wise system of metabolic and analytic assessment is needed to determine the *in situ* immunomodulatory metabolic landscape in human tumor tissue specimens. Recently, Stable Isotope Resolved Metabolomics (SIRM) has been applied to assess the metabolic activities of thin tumor tissue slices, an adaptation of the original method of Otto Warburg's tissue slice technique (178–180). Along with conventional untargeted metabolomics, SIRM will provide complementary information, untargeted high-resolution mapping of the metabolic fate of carbon and nitrogen atoms from labeled precursors as well as quantification of nutrients and immune modulatory metabolites. In addition, the expression profile and immune modulatory impacts can be further assessed by combining RNAseq and Metabolomics-edited Transcriptomic Analysis (META) (181, 182); Using patient-derived tissue slides in studies preserves the fidelity of the original native cellular architecture and metabolic profiling on an individual patient basis. Integration of the above proposed approaches is poised to comprehensively profile the landscape of immunomodulatory metabolism in the TME, which may facilitate complementary metabolic improvements in the following steps of the manufacturing process of CAR T cells.

T Cell Activation, Genetic Modification, and Expansion

CARs are generated by combining the antigen-binding region of a monoclonal antibody with key stone intracellular-signaling domains. It consists of an extracellular targeting domain that recognizes a tumor specific antigen, which is derived from a single-chain variable fragment (scFv) of the variable heavy and variable light chains from a specific monoclonal antibody. When it is expressed on the surface of a T cell, the targeting domain allows CAR T cells to recognize and bind to the antigen that is presented by cancer cells. The intracellular signaling domain usually originates from the signal transduction subunit of co-stimulatory receptors, such as 4-1BB and CD28, which transduce extracellular ligand binding signal into CAR T cells to initiate the activation of downstream signaling cascades. CAR structure has been improved from first-generation CARs, which only had the CD3ζ signaling domain, to next generation CARs, which link the signaling endo domains of CD28, 4-1BB, and/or OX40 to provide co-stimulation signal (signal 2), which is required for optimal T cell activation (**Figure 1A**) (183–185). Optimal activation, gene transfer and culture conditions are essential to ensure the required cell number and quality are achieved for CAR T cell therapy. Due to the personalized nature of CAR T therapies, the degree of cell amplification, differentiation and functional activation

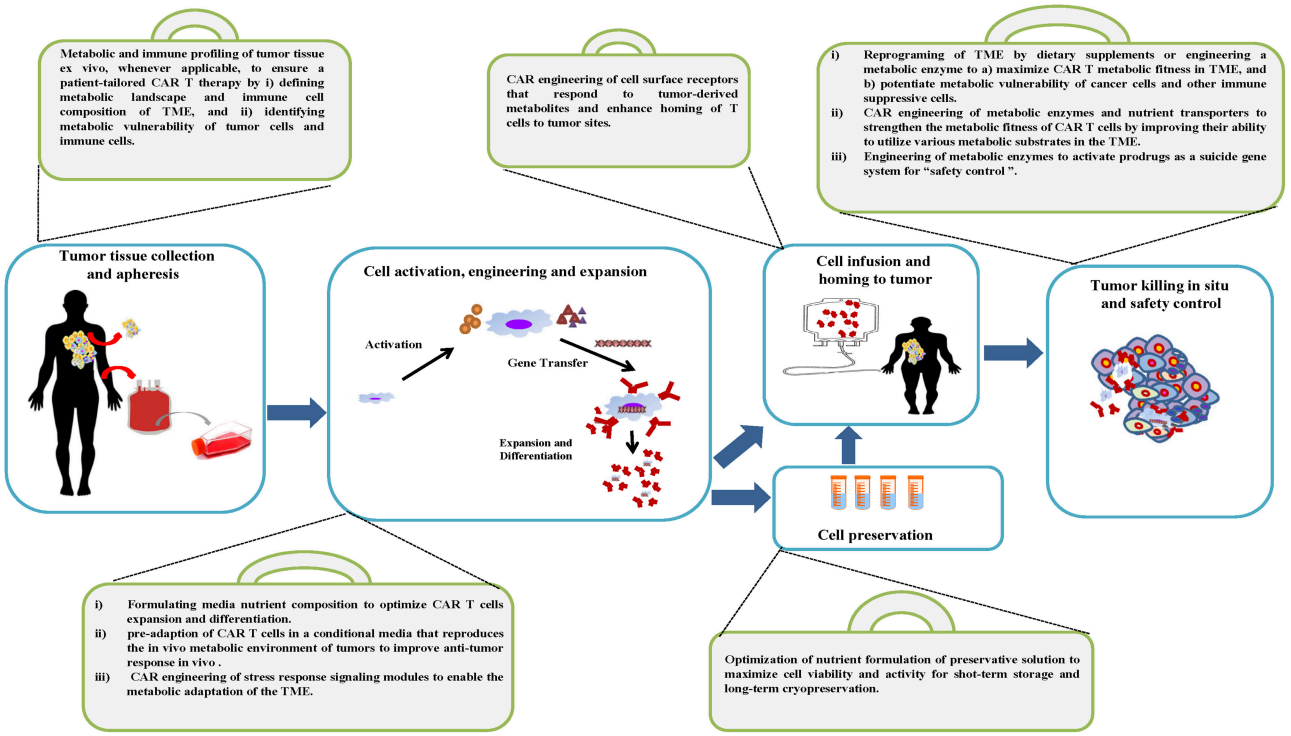

FIGURE 2 | Metabolic tool box of the CAR T cell manufacturing process and therapy. A variety of metabolic strategies are proposed to be integrated into standard work flow to optimize the manufacturing process and maximize the therapeutic efficacy of CAR T cells.

can differ significantly from patient to patient. A patient tailored nutritional formulation for cell culture media may improve the robustness and reproducibility of cell expansion. In addition, emerging evidence suggests that the formula for commercial media does not truly reflect nutrient composition in a physiological context, which is required to ensure the metabolic fitness of cells *in vivo* (186–188). CAR T cells with co-stimulatory domain 4-1BB display higher mitochondrial metabolic activity than cells with a CD28 co-stimulatory domain (30). Consistent with this finding, mitochondrial characteristics including biogenesis and membrane potential have been suggested as key indicators for metabolic fitness and effector function in T cells (163, 189). Enhancing mitochondrial biogenesis through pharmacological or genetic approaches, or by enriching for cells with low mitochondrial membrane potential through cell sorting significantly improves T cell-mediated anti-tumor activities *in vivo* (163, 189–192). Conceivably, nutritional formulations that are tailored to meet the metabolic preferences of cells with different co-stimulatory domains may further enhance the metabolic robustness of CAR T cells.

Immune effector cells have evolved to respond to fluctuations in environmental nutrient levels and thus are able to adapt to environments with either sufficient or insufficient nutrient supply (156). We reason that the pre-adaption of CAR T cells in a conditional media that reproduces the *in vivo* metabolic environment of tumors may improve anti-tumor response *in vivo*. Given that blood flow and oxygen concentration fluctuate in solid tumors, the metabolic stress imposed on CAR T cells may also fluctuate in the TME (193, 194). As such, a fine-tuned adjustment of the severity, duration and frequency of metabolic stress may better recapitulate the metabolic conditions in the TME. Interestingly, transient glutamine restriction *in vitro* via either short-term nutrient starvation or metabolic inhibitor treatment enables a robust antitumor activity of adoptively transferred T effector cells in mouse models of immunotherapy (47, 195–197).

The immune cells response to the changes in the tumor's metabolic microenvironment represents a mechanism of "metabolic checkpoint" that coordinates metabolic status with cellular signaling, and in turn, determines immune function (14). Signaling kinases and transcription factors, such as AMPK and HIF1α can mediate adaptive responses that rewire T cell metabolism to determine immune function. HIF1α-dependent glycolytic pathway is preferentially enhanced in T_H17 cells than T_{reg} cells. Ablation of HIF1α or pharmacological inhibition of glycolysis reciprocally reduces T_H17 cells and induce T_{reg} cells differentiation. However, AMPK enhances T_{reg} cells differentiation through negatively regulating OXPHOS (32, 198–205). Intrinsically edited signaling and transcriptional programs may ensure the engagement of fine-tuned metabolic programs to support T cell proliferation (associated with effector functions) or dormancy (associated with memory phenotypes) in response to the changing microenvironment (206). We reason that the engineering of the CAR by integrating a stress response signaling

module may ensure a fine-tuned metabolic adaptation of T cells in the TME.

The immunosuppressive TME is one of the critical barriers for successful CAR T treatment in the solid tumor. The solid tumor is composed of lymphatic vessels, fibroblasts, infiltrated immune cells, stroma cells, and extracellular matrix (ECM) which constitute the complex TME. During tumor progression and as a result of the heightened glycolytic metabolism of cancer cells, solid tumors are subject to depleted nutrients, acidosis, and hypoxic conditions due to aberrant vascularization. Since hypoxia is a crucial aspect of the TME, targeting hypoxia could be an important strategy for promoting CAR T cell therapy in the solid tumor. Hypoxia induces the stabilization of the HIF-1α transcription factor, which regulates T cell metabolism and function. A recent study designed a novel CAR scaffold that includes the oxygen sensitive domain of HIF1α and provided a proof of concept for engineering microenvironment responsive CAR T cells. T cells which express this engineered HIF1-CAR are cable of responding to a hypoxic environment to display robust cytolytic activity *in vitro* (**Figure 3**) (207). In addition, CAR T cells that express distinct CAR co-stimulatory domains display different metabolic characters, which in turn impact memory phenotypes. 4-1BBζ CAR T cells exhibit enhanced mitochondrial SRC and mitochondrial biogenesis, which is associated with enhanced central memory phenotypes. Inclusion of CD28ζ in the CAR structure promotes effector memory differentiation and results in increased aerobic glycolysis in CAR T cells (30). In addition to the approach of engineering CAR structure, cytokine formulation can be optimized to modulate metabolic programs and promote the T_{scm} and T_{cm} phenotype that is associated with enhanced CAR T cell persistence and anti-tumor immunity *in vivo* (208). T cells, activated with anti-CD3/CD28 antibody, followed by expansion in the presence of IL-15 and IL-7, not only mimic a more T_{scm} like phenotype, but and also exhibit increased production of IFNγ, TNFα, and IL-2 as well as cytolytic activity against target cells expressing the CAR specific antigen (209, 210). It has been suggested that IL-15 promotes SRC and FAO by upregulating carnitine palmitoyl transferase, a rate-limiting metabolic enzyme that controls FAO. Since T_{mem} cells preferentially engage OXPHOS rather than glycolysis, IL-15 may promote T_{mem} cell differentiation and bioenergetic stability partially by regulating mitochondrial metabolism (162).

CAR T Cell Preservation

The nutritional optimization of preservative solutions is an important factor for ensuring successful reperfusion and CAR T therapy. As such, a detailed understanding of the metabolic impact on cells during short-/long-term storage or during the reperfusion period is required. The nutritional formulation requires the capability to regenerate ATP, buffer ions and scavenge free radicals in CAR T cells during the period of preservation (211–215). Finally, any inconsistency between the nutrient formulations that are utilized for preservation and those used for *ex vivo* cell manipulation and expansion may potentially render the cells more susceptible to preservation damage.

CAR T Cell Infusion and Homing to Tumor

Trafficking of T cells into the site of the tumor is required for achieving an optimal CAR T therapeutic response (216, 217). Novel synthetic biology approaches are needed to engineer T cells to logically respond to metabolites that are enriched in the TME, ensuring a fine-tuned tumor recognition and homing response (218, 219). For instance, adenosine is enriched in the TME and suppresses the T cell response partially by engaging cell-surface purinergic receptors-mediated signaling in T cells (220, 221). One theoretical strategy is to engineer an adenosine-responding CAR by fusing the extracellular domain of adenosine receptor to intracellular costimulatory domains, thus artificially switching a normally suppressive signal to an activating signal. We envision that a dual CAR system, with one for a tumor antigen and one for a tumor-associated metabolite, may further enhance the specificity and responsiveness of CAR T cells that may be deployed against tumors in a wide range of sites.

Tumor Killing *in situ* and Safety Control

The capacity for cell proliferation and persistence in the TME is the best predictor of clinical efficacy in CAR T therapy. Nutritional supplements may overcome the metabolically suppressive microenvironment and may enable CAR T cells to persist and expand, ensuring the optimal "effector vs. target" ratio for the clearance of the tumor *in vivo*. L-arginine is considered a conditionally essential amino acid and supplementation with L-arginine enhances the antitumor response of adoptively transferred T cells in animal models (107). Clearly, arginine is also a metabolic vulnerability of cancer cells in the TME, since circulating arginine is essential for supporting tumor growth (222, 223). Therefore, we need to understand the impact of supplemental nutrients on both T cells and tumor cells to better stratify this approach in the future.

The genetic and enzymatic approaches that enable the conversion of immune suppressive metabolites to either inert compounds or pro-inflammatory compounds are promising strategies to reprogram metabolic TME. The concentration of lactate in vertebrate plasma ranges from 1 to 30 mM under physiological and pathological conditions (224). While lactate accumulated in the TME is generally considered as metabolic "waste," muscle cells, neurons and certain tumor cells are known to be able to take up and oxidize lactate (225–227). Consistent with these findings, emerging evidence suggests that lactate is a key carbon source *in vivo* and can be oxidized in the mitochondria to generate energy and feed into cataplerotic routes of the TCA cycle (228–236). Enforced expression of phosphoenolpyruvate carboxykinase 1 (PCK1), which presumably increases gluconeogenesis from lactate and thus alleviates the stress from glucose restriction in the TME, has been shown to enhance anti-tumor T cell responses in animal models (167). Also, we envision a rewired lactate flux, by enforcing LDHB expression, may render T cells capable of utilizing tumor-derived lactate, and thus may strengthen the metabolic fitness of CAR T cells in the TME.

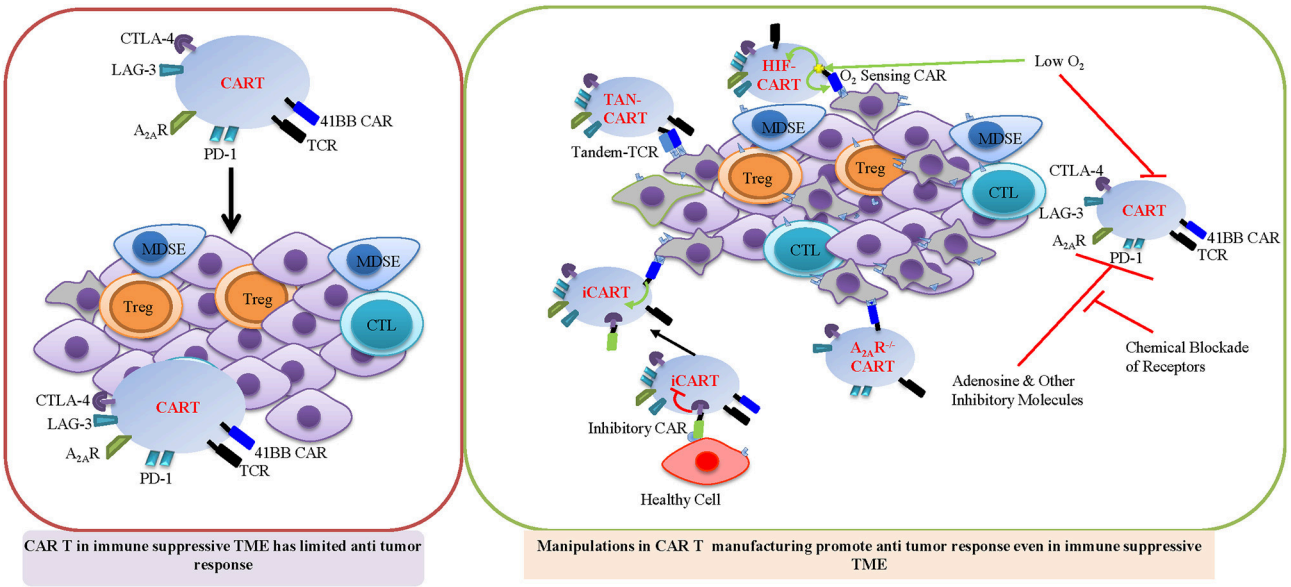

FIGURE 3 | Important strategy for promoting CAR T cell therapy in immune suppressive TME. Manipulations during CAR T manufacturing HIF-CAR, TAN-CAR, and iCAR will promote CAR T function in immune suppressive TME. HIF-CAR T cells are activated by low oxygen concentrations. Inhibitory CAR (iCAR) limits T cell activation in the presence of off-target cells. Tandem CARs (Tan CAR) requires bispecific activation which reduces off target effects. A2AR$^{-/-}$ CAR T cells are resistant to adenosine mediated INF-γ suppression.

One recent study has demonstrated that the systemic delivery of an enzyme that efficiently depletes kynurenine in the TME can enhance the T cell-mediated antitumor immunity (237). Similarly, polyethylene glycol–conjugated adenosine deaminase (PEG-ADA) and ADA gene therapies have been successfully employed to treat ADA-SCID patients (238, 239). Since only immature and transformed T cells display high ADA activity (240–242), systemic ADA treatment through PEG-ADA supplementation may be a new, complementary strategy to optimize the potency and durability of CAR T therapy. It is also conceivable that an engineered oncolytic virus or CAR T cells which express ADA may offer additional strategies to locally deliver ADA into the TME. Nucleoside transporters can rapidly remove adenosine from the extracellular nucleoside pool and direct adenosine into nucleoside salvage to support RNA/DNA synthesis. Thus, expression of high affinity nucleoside transporters may divert adenosine from eliciting an immune-suppressive purinergic signaling response into promoting T cell proliferation (243, 244). Similarly, genetic strategies which enforce the expression and affinity of transports of the key carbon and nitrogen donors (i.e., glucose and glutamine) may confer a selective advantage on T cells over tumor cells in the TME.

Another promising therapeutic strategy for remodeling the microenvironment is to modulate ion and pH balances. Reducing the potassium level through enforced expression of potassium channels in T cells can enhance their antitumor activity (122). A lactate-binding compound has been recently developed as a potent pharmaceutical approach for engineering metabolic flux of lactate and normalizing the pH *in vivo* (245).

The naturally occurring T cell mediated response is largely controlled through cell autonomous mechanisms. However, CAR T therapies often lead to a series of adverse effects that may be reduced through the fine-tuned regulation of the timing, location and amplitude of T cell activity. The principal of the recently developed "ON-switch" CARs can be theoretically extended by employing "metabolic switches" to control T cell activities in real-time (246, 247). In addition, the engineering of metabolic enzymes to activate prodrugs has been exploited as a suicide gene system (248–250). A similar strategy can be exploited as a "safety switch" to enable the selective ablation of CAR T cells at will by providing prodrugs and therefore limiting the on-target, but off-tumor toxicities of CAR T cells (251–253).

FUTURE DIRECTIONS

Cancer cachexia, which is responsible for the death of more than 20% of cancer patients, is characterized by dramatic body weight loss and disproportionate wasting of skeletal muscle (254–256). Supportive care including dietary treatment and physical exercise that can maintain energy balance, as well as increase insulin sensitivity, protein synthesis rate, and anti-oxidative enzyme activity, which is beneficial for relieving symptoms of cachexia (257–260). Similar strategies may be applied as systemic approaches to enhance anti-tumor immunity by fostering an optimized metabolic environment for immune cells.

The fast moving CAR T therapy field has generated tremendous excitement and will likely change the paradigm of therapeutic interventions for solid tumors. Manipulation of

metabolism has been demonstrated as a potential approach to the regulation of immune responses in various preclinical settings. Beyond CAR T therapy, the above potential metabolic modulations can also be considered as adjunctive therapy together with other immunotherapies, including checkpoint blockade and cancer vaccines (261–265). TME consists of an intricate, highly complex and dynamic network of immune cell subsets. Other immune cell subsets, such as natural killer (NK) cells, TAM and MDSC are universally found in the TME and are key players in anti-tumor immunity (266, 267). We will need to continue to decipher the essential metabolic pathways by analyzing metabolic flux and assessing the consequences of metabolic intervention on these pathways in all key cellular components of the TME. Understanding how control of (or by) metabolic pathways impacts anti-tumor immune responses in these cell types is required to selectively strengthen the metabolic fitness in effector T cells and potentiate the metabolic vulnerabilities of tumor cells and immune-suppressive cells. Finally, Strategies to improve immune cell metabolic fitness may be applicable across a broad panel of cancer immunotherapies including checkpoint blockade and cancer vaccines (261–265).

AUTHOR CONTRIBUTIONS

XX, JG, and JS wrote the manuscript and RW wrote and finalize the manuscript.

ACKNOWLEDGMENTS

This work was supported by 1UO1CA232488-01 and 1R01AI114581 from National Institute of Health, and 128436-RSG-15-180-01-LIB from the American Cancer Society (RW).

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

The handling editor declared a shared affiliation, though no other collaboration, with the authors at the time of review.

frontiers in Immunology

REVIEW
published: 16 May 2019
doi: 10.3389/fimmu.2019.01114

Innate Immune Cells: A Potential and Promising Cell Population for Treating Osteosarcoma

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OPEN ACCESS

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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Immunology

Received: 24 November 2018
Accepted: 01 May 2019
Published: 16 May 2019

Citation:
Wang Z, Wang Z, Li B, Wang S, Chen T and Ye Z (2019) Innate Immune Cells: A Potential and Promising Cell Population for Treating Osteosarcoma. Front. Immunol. 10:1114. doi: 10.3389/fimmu.2019.01114

Advanced, recurrent, or metastasized osteosarcomas remain challenging to cure or even alleviate. Therefore, the development of novel therapeutic strategies is urgently needed. Cancer immunotherapy has greatly improved in recent years, with options including adoptive cellular therapy, vaccination, and checkpoint inhibitors. As such, immunotherapy is becoming a potential strategy for the treatment of osteosarcoma. Innate immunocytes, the first line of defense in the immune system and the bridge to adaptive immunity, are one of the vital effector cell subpopulations in cancer immunotherapy. Innate immune cell-based therapy has shown potent antitumor activity against hematologic malignancies and some solid tumors, including osteosarcoma. Importantly, some immune checkpoints are expressed on both innate and adaptive immune cells, modulating their functions in tumor immunity. Therefore, blocking or activating immune checkpoint-mediated downstream signaling pathways can improve the therapeutic effects of innate immune cell-based therapy. In this review, we summarize the current status and future prospects of innate immune cell-based therapy for the treatment of osteosarcoma, with a focus on the potential synergistic effects of combination therapy involving innate immunotherapy and immune checkpoint inhibitors/oncolytic viruses.

Keywords: osteosarcoma, innate immune cell, adoptive cell therapy (ACT), vaccine, immmune checkpoint

INTRODUCTION

Osteosarcoma is the most common primary malignant bone tumor and it often leads to pulmonary metastasis, which is the major cause of death of osteosarcoma patients (1). Surgical resection combined with neoadjuvant and postoperative chemotherapy has increased long-term survival rates to 70% for patients with localized osteosarcomas, but <20% for patients with recurrent and/or metastasized osteosarcomas. The current standard treatment strategy has remained unchanged for decades (2). Therefore, there is urgent need to develop novel therapies to improve the overall survival rates of osteosarcoma patients, particularly those experiencing relapse and/or metastasis.

Immunotherapy is becoming an attractive therapeutic strategy for the treatment of osteosarcoma. The human immune system, which consists of innate and adaptive immunity, plays a critical role in suppressing tumor growth. The major effector cells in adaptive immunity targeting osteosarcoma are cytotoxic T cells (CTLs). A previous study demonstrated that CTLs played an important role in immune surveillance in osteosarcoma patients (3). In addition, adoptive transfer of T cells successfully resulted in tumor inhibition in mouse models of osteosarcoma (4–6).

Recently, the role of innate immune cells in the control of tumor progression has been characterized. Innate immune cells contribute to tumor suppression through direct recognition and killing, through self-activation to trigger a strong adaptive immune response, or through both mechanisms (7). The antitumor immunocompetence of innate immune cells provides a rational basis for innate immune cell-based therapy, which has shown promise for the treatment of hematopoietic malignancies and solid tumors (8). Indeed, successful treatment of osteosarcomas in preclinical studies using innate immune cells has been reported (9, 10). Our previous studies have shown that innate immune cells were effective against osteosarcoma (11–14). In this paper, we describe the anti-osteosarcoma roles of the following major classes of innate immune cells: dendritic cells (DCs), macrophages, natural killer (NK) cells, natural killer T cells (NKT) cells, and γδ T cells. We also review the current status of innate immune cell-based therapy for the treatment of osteosarcoma and potential future improvements based on the results of treatment of other types of tumors. Moreover, immune checkpoint inhibitors (ICPIs) represent a new frontier in cancer therapy and have shown a certain degree of therapeutic effects in osteosarcoma patients (15). Some immune checkpoints are not only expressed on T cells, but also on DCs, macrophages, NK cells, NKT cells, and γδ T cells; blocking these immune checkpoints reverses their anti-tumor activity in tumor immunity. Therefore, we detail the effects of immune checkpoint-inhibition on immune cells and the potential for synergy based on combining innate immune cell-based therapy with immune checkpoint manipulation for the treatment of osteosarcoma. In addition, as oncolytic virus (OV) therapy is known to induce an innate immune response, we also discuss the combinational potential of innate immune cell-based therapy and OVs.

DENDRITIC CELLS

DCs, which are professional antigen-presenting cells (APCs), take up and present antigens to naïve T cells, ultimately stimulating them to differentiate into tumor killers (16). Recently, a series of studies have shown that DCs can also activate innate immune cells with robust antitumor activity such as γδ T cells, cytokine-induced killer (CIK) cells (17–19).

However, established tumors always endeavor to reduce the availability of antigen presentation by APCs, resulting in immunosuppression, which disrupts the generation of antitumor immune responses (20, 21). In response, DC vaccines have been developed to bypass this mechanism. This procedure can be summarized as follows: DCs are isolated from peripheral blood mononuclear cells (PBMCs), matured, and loaded *ex vivo* with tumor antigens with defined cocktails, and then infused back into the patient (**Figure 1**). Theoretically, these antigen-activated DCs can successfully boost the immune response. Recent preclinical studies of osteosarcoma DC vaccines are listed in (**Table 1**). They can be classified into three major groups based on the protocols for loading various sources of antigens (33): (1) DCs co-cultured with peptides, proteins, or tumor-cell lysates; (2) DCs transfected with DNA, RNA coding for antigens, or total RNAs derived from tumor cells; and (3) fusions between DCs and devitalized tumor cells. Yu et al. (23, 24) tested the efficacy of osteosarcoma DC vaccines either fused with whole-tumor cell or transduced with total tumor RNA. Most immunized tumor-free rats acquired partial or complete protection from tumor challenge. In addition, vaccination induced tumor suppression in tumor-bearing mice (23, 24). Other studies tested the potential of combination therapy consisting of a DC vaccine and targeted drugs such as anti-transforming growth factor-β (TGF-β)/glucocorticoid-induced tumor necrosis factor receptor (GITR) antibodies (30, 32). The results of these studies showed that primary and metastatic tumor growth was inhibited. In addition, the tumor microenvironment (TME) was remodeled with reduced number of regulatory T lymphocytes (Tregs), reduced levels of immunosuppressive cytokines, and an increased number of $CD8^+$ T lymphocytes (30, 32). However, DC vaccines were less effective for the treatment of osteosarcomas in clinical trials (34–36). For instance, only two out of 12 patients exhibited a strong anti-tumor immune response, and none exhibited any clinical effects, after receiving 3 weekly DC vaccine administrations (35). However, DC vaccines were well-tolerated in all the clinical trials.

Three explanations can be proposed for the lack of clinical benefits in patients. (1) Compromised quality and quantity of the immune effector cells in patients. Osteosarcoma patients commonly receive a full course of upfront chemotherapy, which may damage the innate and adaptive immune effectors and thus limit their availability and efficacy to respond to the increased antigen presentation. (2) Poor migration of effector cells to the tumor site, probably due to down-regulation of chemokine expression. (3) Other strong immunosuppressive mechanisms, for example, immune checkpoints on immune cells. An effective cancer vaccine should be able to overcome tumor-associated immune suppression and reinstate immune surveillance (37). Therefore, increasing the ratio of active effector cells to tumor target cells, enhancing the infiltration of the effectors, or remodeling the TME in combination with administering DC vaccines may enhance antigen presentation, immune response, and clinical efficacy.

MACROPHAGES

In normal bone biology, osteoclasts, which are highly specialized macrophages, are involved in bone resorption and have central functions in bone homeostasis (1). Macrophages in the vicinity of osteosarcoma cells are identified as tumor-associated macrophages (TAMs). They consist of a large variety

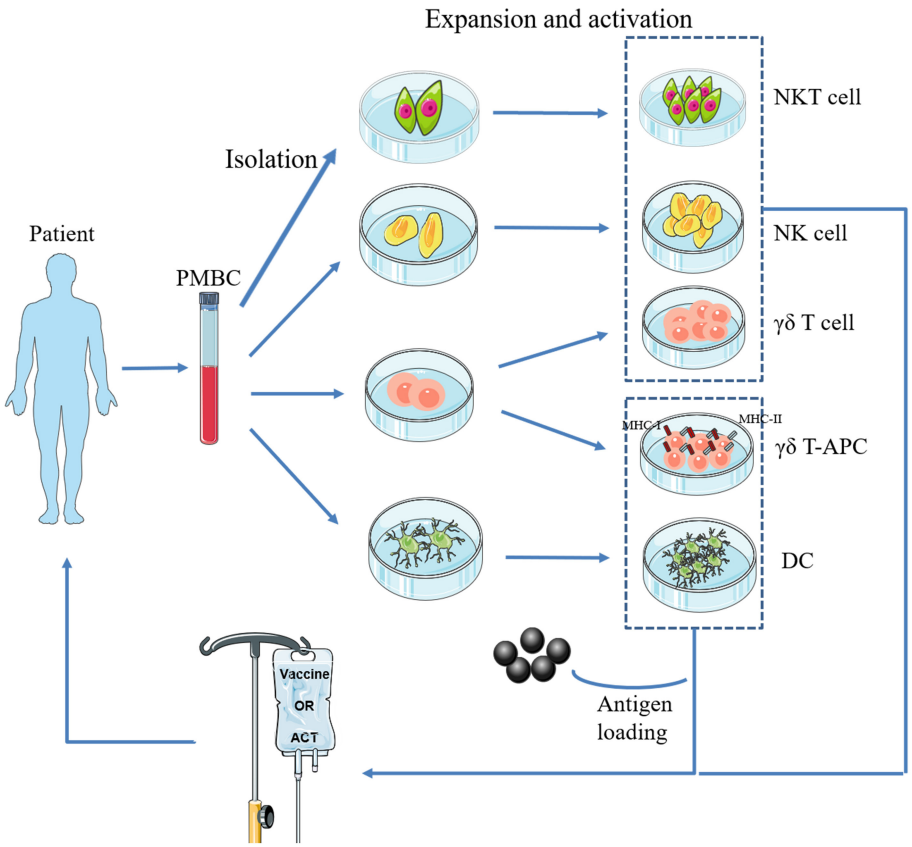

FIGURE 1 | Basic procedure of adoptive transfer of innate immune cells. NKT cells, NK cells, γδ T cells, and DCs are isolated from a patient's PBMCs, expanded and activated *ex vivo,* and then infused back into the body. In particular, γδ-APC and DCs need to be loaded with tumor antigen(s).

of subpopulations, which were initially classified as anti-tumor M1-polarized macrophages and pro-tumor M2-polarized macrophages (38). TAMs infiltrate massively into osteosarcoma tissues and contribute to tumor progression through multiple pathways. In preclinical models, macrophages recruited by interleukin (IL)-34 released by osteosarcoma cells promoted tumor progression and the metastatic process (39). Han et al. (40) found that osteosarcoma patients with detectable metastasis at diagnosis have more TAMs in the primary site. Interestingly, TAMs occurred at a higher rate in osteosarcoma lung metastases than in the corresponding primary lesions and promoted lung metastasis and induced epithelial-mesenchymal transition in osteosarcoma by activating the cyclooxygenase (COX)-2/signal transducer and activator of transcription (STAT)-3 axis (40). Additionally, Han et al. revealed that the number of M2-TAMs was correlated with the frequency of suppressive T-cell immunoglobulin and mucin-domain containing-3 (TIM-3)$^{+}$ programmed cell death 1 (PD-1)$^{+}$ T lymphocytes in osteosarcoma patients (41). TIM-3/Gal9 interactions between T cells and monocytes have been shown to resulted in an immunosuppressive response (42). These results indicate that TAMs promote tumor growth by suppressing intra-tumor T-lymphocytes. However, several studies have reached different conclusions. A study by Buddingh et al. demonstrated that TAMs were associated with metastasis inhibition in high-grade osteosarcoma patients (43). This result was recently confirmed in orthotopic osteosarcoma mouse models (44). Moreover, a biopsy study revealed that a high level of CD163 (a marker of M2-polarized macrophages) was related to longer metastasis progression-free survival (MPFS), and CD68 (a marker for macrophages) exhibited a similar association (45). The possible reason may be that the polarization/phenotype and infiltration of TAMs change dynamically during tumor growth, and the current studies do not fully represent the whole dynamic process of TAMs in the TME.

Despite the contradictory roles of TAMs in the TME, three therapeutic strategies targeting TAMs have shown potential for treating osteosarcoma. (1) Preventing polarization of M1 macrophages to M2, or directly suppressing the M2 phenotype. Pharmacological therapy for the treatment of osteosarcoma using all-trans retinoic acid (46), resveratrol (47), and dihydroxy coumarins (48) has shown favorable results involving the suppression of M2-polarized macrophages. (2) Enhancing non-TAM macrophages recruitment. A study showed that upregulation of Secreted Protein, Acidic and Rich in Cysteine-like 1 (SPARCL1) protein induced osteosarcoma

TABLE 1 | Pre-clinical studies of DC-based vaccines for osteosarcoma.

Type of DC vaccine	Study type	Ancillary therapy	Effect	References
Autologous DCs transfected with total tumor mRNA	*In vitro*	CIK cells	Effective osteosarcoma cytolysis	(19)
	In vivo	None	Induction of specific CTL responses, tumor rejection in 70% of vaccinated tumor-bearing rats, and development of long-term immunological memory to reject a subsequent tumor rechallenge	(22)
	In vivo	None	Induction of specific CTL responses, tumor rejection in 80% of vaccinated tumor-bearing rats and development of long-term immunological memory to reject a subsequent tumor rechallenge	(23)
Allogeneic DCs fused with tumor cells	*In vivo*	None	Protection from tumor challenge in 70% of pre-vaccinated rats and tumor rejection in 60% of tumor-bearing rats	(24)
	In vitro	None	Effective activation of T cells	(25)
Autologous DCs fused with tumor cells	*In vitro*	None	Effective activation of T cells	(26)
	In vivo	None	Atrophy or disappearance of tumor bodies and higher survival times and rates	(27)
Autologous DCs loaded with tumor cell lysate	*In vitro*	None	Increased induction of CTL activity	(28)
	In vivo	None	Increased number of $CD8^+$ T lymphocytes in the metastatic areas, and reduced pulmonary metastases	(29)
	In vivo	Anti-TGF-β antibody		(30)
	In vivo	Anti-CTLA-4 antibody		(31)
	In vivo	Anti-GITR antibody	Increased number of $CD8^+$ T lymphocytes in tumor tissue and serum, inhibition of primary tumor growth, and prolonged survival	(32)

cells to secrete chemokine ligand 5, resulting in macrophage recruitment. The recruited macrophages exerted anti-tumor effects and inhibited osteosarcoma metastasis (49). (3) Activating macrophages. Mifamurtide, an immunoadjuvant currently approved for osteosarcoma therapy in the European Union, can activate the tumoricidal properties of macrophages and inhibit human osteosarcoma cell growth (50, 51). A report from the international Children's Oncology Group found that the addition of mifamurtide to chemotherapy significantly improved overall survival from 70 to 78% and resulted in a trend toward improved event-free survival (EFS) among patients with no signs of metastasis (52). Similar benefits were observed in patients with metastatic osteosarcomas, although the results were not statistically significant (53).

NATURAL KILLER CELLS

NK cells express a repertoire of activating and inhibitory receptors (**Table 2**) that recognize altered expression of proteins on target cells, allowing for control of NK cell functions. After activation, they exhibit spontaneous cytolytic activity against cells undergoing malignant transformation (54). Recently, immunologists found that NK cells could stimulate DC recruitment into the TME, resulting in inhibition of tumor growth (55). Osteosarcoma patients had lower numbers of NK cells at the time of diagnosis compared to normal controls (56). After IL-2 administration and polychemotherapy, osteosarcoma patients had increased numbers, and increased activity, of NK cells in the blood, the magnitude of which strongly correlated with the clinical outcomes (57). These data indicate that NK cells have anti-tumor immune activity and play a role in immune surveillance in osteosarcoma patients. Importantly, osteosarcoma cell-surface molecules make osteosarcoma cells particularly susceptible to NK cell-mediated killing. CD54 and CD58 (both of which are adhesion molecules) are fully expressed on osteosarcoma cells, allowing for easy recognition by, and a strong association with, NK cells (58, 59). In addition, human leukocyte antigen (HLA) class I (a ligand for inhibitory receptors on NK cells) is typically downregulated (3), while

TABLE 2 | Activating and inhibitory receptors on human NK cells.

Type	Receptors	Ligands
Activating receptors	NKG2D	MICA/B, ULBP1–4
	CD94-NKG2C	HLA-E
	KIR2DL4	HLA-G
	KIR2DS1	HLA-C2
	KIR2DS2	HLA-C1
	KIR2DS3	Unknown
	KIR2DS4	HLA-A11
	KIR2DS5	Unknown
	KIR3DS1	HLA-Bw4
	NKp30	B7H6, BAT3, pp65 of HCMV, viral HA PfEMP1 of *Plasmodium falciparum*
	NKp46	Heparin, viral HA and HN
	NKp44	Viral HA and HN, PCNA, proteoglycans
	DNAM-1	CD112, CD155
Inhibitory receptors	KIR2DL1	HLA-C2
	KIR2DL2	HLA-C1
	KIR2DL3	HLA-C1
	KIR3DL1	HLA-Bw4
	KIR3DL2	HLA-A3, -A11
	NKR-P1A	LLTI
	CD94-NKG2A	HLA-E
	ILT2 (CD85j)	HLA-A, -B, -C, HLA-G1, HCMV UL18
	CD244(2B4)	CD244(2B4)

major histocompatibility complex class I chain-related protein A/B (MICA/B) and UL16-binding protein (ULBP) (ligands for activating receptors on NK cells) (60, 61) are overexpressed on osteosarcoma cells, allowing for easy activation of NK cells.

Treatment of patients with cells that have been isolated, manipulated, and expanded *ex vivo*, and then reinfused into the patient, is called adoptive cell therapy (ACT) (**Figure 1**). Infused immune cells migrate and infiltrate into the tumor site and mediate antitumor effects. There are three ancillary strategies to further improve the therapeutic effectiveness of adoptive NK cell transfer in osteosarcoma immunotherapy (**Table 3**). First, epigenetic drugs, such as histone deacetylase inhibitors (HDACi, e.g., valproic acid [VPA], entinostat) and DNA-methylation inhibitors (DNMTi, e.g., hydralazine) can increase the expression of ligands for activating receptors (MICA/B, ULBP, and CD155) or death receptors (Fas) on osteosarcoma cells, enhancing NK cell-mediated lysis (62, 63, 65). Another DNA-methylation inhibitor, decitabine, has been shown to enhance γδ T cell-mediated cytotoxicity by inducing ligands for activating receptors (natural killer group 2D, member D [NKG2D] ligands [NKG2DLs]) on osteosarcoma cells (12). Combining decitabine with the NK cells might be equally effective for treating osteosarcoma. Additionally, some traditional chemotherapeutic drugs (including doxorubicin, cisplatin, and gemcitabine) have been found to increase NK cell-activating ligand expression in tumors (71). Though similar studies in osteosarcoma are rare, chemotherapeutic drugs can modulate death receptors (DRs) on osteosarcoma cells, which may make them more sensitive to Fas-mediated NK cell cytotoxicity. For example, gemcitabine up-regulated cell-surface Fas expression and was effective in treating osteosarcoma lung metastases (72). Interestingly, treatment with cisplatin could not upregulate the cell-surface Fas antigen but it did sensitize human osteosarcoma cells to Fas-mediated apoptosis by down-regulating the expression of FLICE inhibitory protein long form (FLIP-L). Second, cytokine therapy can enhance the conjugate-forming capacity of NK cells to osteosarcoma targets by augmenting the expression of CD18 and CD2 (68) (both of which are adhesion molecules on NK cells), and intercellular adhesion molecule (ICAM)-1 (67) and fibronectin (69) (both of which are adhesion molecules on osteosarcoma cells). Interestingly, cytokine therapy can also increase the killing activity of NK cells. For instance, IL-15, the most promising NK cell-activating cytokine, can strongly enhance NK cell-mediated cytolytic activity toward chemotherapy-resistant osteosarcoma (60, 66). IL-2 can also strongly augment NK cell activity (73). It has been widely shown that, in neuroblastoma, IL-2 administration combined with immunotherapy (involving anti-GD2 antibody) enhanced NK cell proliferation and cytotoxicity (74), and showed promising results in clinical trials (75). Importantly, IL-2 aerosolization in dogs and mice with osteosarcoma lung metastasis similarly enhanced the local proliferation and cytotoxicity of NK cells and induced metastatic regression (76, 77). Third, monoclonal antibodies can target various receptors on NK cells to improve NK cell cytotoxicity. One approach is to develop a monoclonal antibody (mAb) to facilitate antibody-dependent cell-mediated cytotoxicity (ADCC) against osteosarcoma cells. Cetuximab, a mAb that targets epidermal growth factor receptor (EGFR) on target cells, with an Fc region that binds to CD16 on NK cells, increases NK-dependent lysis of EGFR-expressing osteosarcoma cell lines by enhancing ADCC (70). Another approach is to block the inhibitory NK cell receptors (such as NKG2A or KIR2DL-1,−2, and−3) using mAbs (78, 79). However, this approach has not been evaluated for treating osteosarcoma. Emerging evidence has shown promising strategies for osteosarcoma treatment, and carefully designed clinical trials may demonstrate the effectiveness of these therapies.

Genetic engineering of immune cells can endow them with additional antitumor specificity. For instance, transduction of precise and functionally active chimeric antigen receptors (CARs) into NK cells has led to stronger cytotoxicity toward osteosarcomas. A receptor designated NKG2D-DAP10-CD3ζ (comprising the NK cell- activating receptor NKG2D and two key signaling molecules, DAP10 and CD3ζ) was recently developed. Transduction with this chimeric receptor markedly increased NKG2D surface expression on NK cells and the transmission of activating signals. In a xenograft model of osteosarcoma, adoptive transfer of these CAR-NK cells significantly decreased the overall tumor burden (80). However, there are technical challenges to overcome to obtain sufficient numbers of functionally active NK cells from a patient's blood. The emergence of the human NK92 cell line consisting of activated NK cells may resolve the challenges faced by CAR-NK cell-based therapy, as NK92

TABLE 3 | Classification of immunomodulatory strategies for improving the killing effectiveness of adoptive NK cell transfer therapy against osteosarcoma.

Immunomodulatory strategy		Mechanism	Study type	Comment
Epigenetic drug	VPA	Augmented expression of MICA/B on tumor cells	*Ex vivo*	VPA sensitized human osteosarcoma cells to cytotoxicity of NK cells (62)
	Entinostat	Augmented expression of MICA/B, ULBP, and CD155 on tumor cells	*In vivo*	Entinostat failed to augment the efficacy of NK cell therapy in a nude mouse model of human osteosarcoma lung metastasis (63)
	Entinostat	Downregulation of the anti-apoptotic protein, c-FLIP, and increased levels of Fas within the membrane lipid rafts on tumor cells	*Ex vivo*	Entinostat sensitized osteosarcoma cells to NK cell-mediated apoptosis (64)
	VPA+ hydralazine	Augmented expression of MICA/B and Fas on tumor cells	*Ex vivo*	VPA combined with hydralazine enhanced the susceptibility of osteosarcoma cells to Fas- and NK cell-mediated cell death (65)
Cytokine	IL-15	Enhanced DNAM-1 and NKG2D signaling pathways	*Ex vivo*	IL-15 enhanced cytolytic activity against chemotherapy-resistant osteosarcoma cells (60)
	IL-15	Prevention of down-regulation of NKG2D on NK cells	*Ex vivo*	IL-15 reversed inhibition of NK cell-mediated cytolytic activity against osteosarcoma (66)
	IL-12+IFN-γ+IL-18	Enhanced expression of ICAM-I on HOS cells	*Ex vivo*	IL-12 enhanced NK-mediated cytolysis of HOS cells in the presence of IFN-γ and with IL-18 (67)
	IL-12+IL-2	Increased density of CD18 and CD2 molecules on NK cells	*Ex vivo*	A combination of IL-12 and IL-2 increased lytic activity against and binding to osteosarcoma cells (68)
	IL-17	Increased expression of fibronectin on U2 OS cells	*Ex vivo*	IL-17 enhanced NK cell-mediated adhesion and cell lysis activity against osteosarcoma (69)
Monoclonal antibody	Cetuximab	ADCC	*Ex vivo*	Cetuximab augmented cytolytic activity of resting NK cells, which was specifically directed toward osteosarcoma cells (70)

cell line is relative ease in *ex vivo* large-scale expansion and effective receptor transfection (81). Adoptive transfer of NK-92 cells transduced to express various CARs was shown to cause tumor regression in various tumor xenografts (82, 83). CAR-NK-92 cell-based therapy is currently being evaluated in clinical trials for $CD33^+$ acute myeloid leukemia (AML; NCT02944162) and $CD7^+$ leukemia and lymphoma (NCT02742727). Therefore, utilizing NK-92 cell line for producing sufficient CAR-NK cells (e.g., NKG2D-DAP10-CD3ζ-transduced NK92 cells) to effectively target and eliminate osteosarcoma is a promising strategy that requires further evaluation. However, NK92 cell line must be irradiated before being infused into patients (81), which limits the survival and proliferation of NK cells—two key factors that are known to influence the efficacy of NK cell-based immunotherapy (84). In contrast, large-scale differentiation of human induced pluripotent stem cells (iPSCs) into NK cells (with phenotypic and functional similarities to NK cells isolated from peripheral blood) is relatively easy (85). After CAR transduction, the efficiency of NK cell production from iPSCs is similar to the efficiency of NK cell production from non-CAR-expressing iPSCs (86). Moreover, NK cells derived from human iPSCs that express CARs (CAR-iPSC-NK cells) have a typical NK cell phenotype. In a mouse xenograft model of ovarian cancer, CAR-PSC-NK cells (with a CAR comprising the NK cell-activating receptor NKG2D, the co-stimulatory domain 2B4 and the key signaling molecule CD3ζ) showed increased *in vivo* expansion and improved activity with less toxicity (87). CAR-iPSC-NK cells mediate their activity without requiring HLA matching; therefore, theoretically, they can also be used to treat other solid tumors including osteosarcoma. Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) technology has been used to edit CAR T cells (88). For example, knocking out immune checkpoints may protect CAR T cells from being exhausted (89). Knocking out αβ T-cell receptors (TCR) (88) or β2-microglobulin (β2M) (90) minimized the risks associated with "off-the-shelf" CAR T cells. Delivering a CAR gene to a specific locus, TCR α constant (TRAC), yielded therapeutic CAR T cells that were more potent (91). To achieve a robust anti-tumor effect, applying CRISPR/Cas9 technology to edit CAR-NK cells (e.g., by knocking out immune checkpoints) should be further investigated.

NATURAL KILLER T CELLS

NKT cells express molecular markers of both NK cells (e.g., NK1.1, Ly49, NKRs, and KIRs) and T cells (e.g., αβ TCR, CD44, CD69, and CD122). In tumor immunity, activated NKT cells are able to kill tumors via different NK and T cell-associated mechanisms (92, 93). In addition, high numbers of tumor-infiltrating NKT cells correlated with good clinical outcomes in cancer patients (94, 95). However, in some tumor types, the number of NKT cells was higher compared to the number in normal tissue (94, 96). Further studies focusing on function and phenotype of tumor-infiltrating NKT cells showed that they expressed fewer activating receptors and produced lower

amounts of pro-inflammatory cytokines compared with para-carcinoma tissues (97, 98).

A similar contradictory function of NKT cells in osteosarcoma immunity was observed. One research group found that NKT cells purified from human PBMCs and expanded *ex vivo* enhanced osteosarcoma cell death induced by standard chemotherapy (doxorubicin, cisplatin, and methotrexate) (99). In contrast, other researchers found that tumor-infiltrating NKT cells had a negative regulatory role, involving suppression of CTL function (100). A hypothetical model of NKT cell functional transformation in osteosarcoma is as follows: during the early tumor stage, the NKT cell subpopulation exerts effective antitumor immune responses against tumors. However, during tumor progression, NKT cells become overstimulated and anergic, and they finally transform, contributing to tumor immune escape (101).

Two major aspects of current NKT cell therapeutic strategies should be carefully considered in light of this hypothetical model. (1) *in situ* expansion and activation of NKT cells in early tumor stages or adoptive transfer of *ex vivo* expanded and activated autologous NKT cells into patients (**Figure 1**). α-galactosylceramide (GalCer) or α-GalCer-pulsed autologous DCs is a common strategy to activate NKT cells *in vivo* or *ex vivo* (102, 103). Recent studies found that iPSCs might be more effective at amplifying the numbers of autologous NKT cells (104, 105). (2) Skewing of pro-tumor NKT cells toward anti-tumor subtypes in advanced tumor stages. The addition of IL-12 (106) or chemical modification of α-GalCer (107) skewed the conventional α-GalCer-produced TH1- and TH2-associated cytokines toward only TH1-associated cytokine production. These data indicate that pro-tumor NKT cells were transformed to anti-tumor subtypes following this intervention.

γδ T CELLS

It has been found that γδ T cells can mediate effective antitumor immune responses. In a methylcholanthrene (MCA)-induced sarcoma model, γδ T cell-deficient mice had an increased incidence of tumor development (108). Preclinical studies found that γδ T cells could directly kill malignant cells through the generation of cytokines (tumor necrosis factor [TNF]-α and interferon [IFN]-γ), upregulation of activating receptors or their ligands (Fas-L, NKG2D, TRAIL, and TNF), expression of CD16 for ADCC, and release of granzymes and perforin (109). Recent studies indicated that, in the short-term, γδ T cells possess phenotypic characteristics of DCs after activation by phosphoantigens (110). The effect of priming a strong $CD8^+$ T cell-mediated anti-tumor response using peptide-pulsed γδ T cells was even more powerful than the effect induced by DCs (111, 112).

The main advantages of adoptive γδ T cell transfer immunotherapy (**Figure 1**) are as follows: (1) γδ T cells can infiltrate the TME (113, 114); (2) they exert cytotoxic activity against cancer cells in an HLA-independent manner; and (3) they can be expanded and activated *ex vivo* by simple yet effective protocols (115). Kato et al. (116) initially reported the ability of γδ T cells to directly recognize and kill osteosarcoma cell lines NY, SAOS2, and OST. However, these cell lines were only moderately susceptible to γδ T cell cytotoxicity. Therefore, later studies have focused on adjuvant therapies to potentiate the immunosensitivity of osteosarcoma cells to γδ T cells (**Table 4**). Zoledronate (ZOL) significantly reduces skeletal complications in patients with bone metastases from solid tumors (120) and inhibits osteosarcoma growth (121). Our group and other researchers demonstrated that ZOL could also enhance the anti-osteosarcoma activity of γδ T cells (14, 117). However, the specific mechanisms have not been elucidated and a high dose of ZOL is necessary to achieve this effect, while the ZOL concentration in the blood declines rapidly (122). Recently, a study by our group found that a ZOL-related mechanism was associated with increased accumulation of mevalonate pathway intermediates (11). We also found that VPA (the HDACi) and ZOL had a synergistic effect on the enhancement of γδ T cell-mediated cytotoxicity against osteosarcoma cells by facilitating the accumulation of mevalonate pathway intermediates (11). More usefully, this combination therapy reduced the ZOL dose required in adoptive γδ T cell transfer immunotherapy, facilitating its clinical application (11). In addition, the expression of human epidermal growth factor receptor 2 (Her-2) was associated with tumor progression and poor prognosis in osteosarcoma patients (123). However, no therapeutic effectiveness was observed pre-clinically or clinically for trastuzumab (an anti-Her-2 monoclonal antibody)-driven osteosarcoma therapy (124). However, Liu et al. reported that trastuzumab aided γδ T cell-mediated lysis of osteosarcoma cells by enhancing ADCC (13), suggesting a promising novel combination regimen to treat osteosarcoma. Additionally, it was reported that bispecific antibodies could enhance the cytotoxicity of γδ T cells. For example, a research group designed a bispecific antibody, Her2/Vγ9, that binds to Vγ9 on γδ T cells and Her-2 on pancreatic tumor cells (125). Infusion of this novel bispecific antibody improved recognition and binding between adoptively transferred γδ T cells and tumor cells, significantly reducing pancreatic tumor growth in mouse models. This result suggests that Her2/Vγ9 antibody might promote the capacity of γδ T cells to lyse osteosarcoma cells to a greater extent than Her2 antibody. Furthermore, IFN-γ and decitabine (a DNA demethylation drug) increased γδ T cell cytotoxicity against osteosarcoma cells by increasing the expression of Fas and NKG2DLs on tumor cell surfaces (12, 118).

Recent achievements in cell engineering and further studies of γδ T cell physiology have provided an improved foundation for improving γδ T cell-based immunotherapies. Three potential perspectives related to potentiating the cytotoxicity of γδ T cells are as follows. (1) T cells transduced with TCRs that specifically target the NY-ESO-1 antigen on tumors are called NY-ESO-1-specific TCR-engineered T cells. These cells can be activated upon encountering NY-ESO-1 antigens presented by HLA molecules and they then specifically target and kill tumor cells. Adoptive transfer of NY-ESO-1-specific TCR-engineered T cells represents a potentially effective therapeutic approach for the treatment of osteosarcoma (126). However, introduction

TABLE 4 | Chronological summary of studies on γδ T cell therapy against osteosarcoma.

References	Ancillary therapy	Study type	Cell type and source	Mechanism	Result
Muraro et al. (117)	ZOL + IL-2	*In vitro*	γδ T cells from HD	Unknown	Potent anti-tumor activity of γδ T cells against osteosarcoma cell lines
Li et al. (118)	IFN-γ	*In vitro*	γδ T cells from HD	Up-regulated expression of Fas on osteosarcoma cell lines	Enhanced cytotoxic effect of γδ T cells against osteosarcoma cell lines
Li et al. (14)	ZOL	*In vitro*	Vγ9Vδ2 T cells from OP and HD	TCR-mediated and partly NKG2D-mediated granule exocytose and TRAIL pathways	Potent anti-tumor activity of Vγ9Vδ2 T cells
Liu et al. (13)	Trastuzumab + ZOL	*In vitro*	Vγ9Vδ2 T cells from HD	ADCC	More efficient ability of Vγ9Vδ2 T cells to recognize and lyse osteosarcoma cell lines.
Li et al. (119)	Celastrol	*In vitro*	γδ T cells from OP and HD	Up-regulation of death receptors 4/5 on osteosarcoma cell lines	Increased osteosarcoma cell lysis by γδ T cells
Wang et al. (11)	ZOL+VPA	*In vivo*	γδ T cells from OP and HD	Increased accumulation of the mevalonate pathway intermediates in osteosarcoma primary cells and cell lines	Enhanced γδ T cell migration and antitumor effect.
Wang et al. (12)	Decitabine	*In vivo*	γδ T cells from OP	Increased expression of NKG2DLs on osteosarcoma cell lines	Enhanced antitumor effect of combination therapy of γδ T cell infusion and decitabine administration

HD, healthy donors; OP, osteosarcoma patients.

of α/β chains has the potential to result in mispairing with endogenous α/β TCR chains, resulting in mixed TCR dimers with unknown specificities, which can lead to adverse complications such as autoimmune responses and toxicity. However, previous studies showed that α and β TCR chains could not form heterodimers with γ and δ TCR chains when transduced into γδ T cells (127). Meanwhile, αβ TCR-transduced γδ T cells exhibited high levels of cytokine release and cytotoxic activity (127, 128). Therefore, using NY-ESO-1-specific αβ TCR-transduced γδ T cells to treat osteosarcoma may be a safe and effective strategy. (2) γδ T cells may be ideal candidates for cell vaccine manufacturing (**Figure 1**). The advantages of γδ T cell vaccines compared to DC vaccines are as follows (129): first, obtaining and expanding γδ T cells to create an unlimited number is easy, economical, and highly selective; second, γδ T cell vaccines display excellent survival during *ex vivo* preparation, allowing for possible freezing for storage and shipment to cancer clinics in large quantities; third, the status of γδ T cells is uniform (effector-memory), while DCs remain heterogeneous (immature-mature-exhausted); finally, γδ T cells have functional uniformity with stable induction of primarily pro-inflammatory responses. (3) Mechanistic target of rapamycin (mTOR) is important for regulating T cell metabolism and function. Recent studies have demonstrated the important role of mTOR in γδ T cells. Rapamycin (the US Food and Drug Administration [FDA]-approved mTOR inhibitor) increased the yield and durability of the elicited γδ T cell response (130). Later studies demonstrated that the immune stimulatory effects of rapamycin are mediated by boosting perforin release, enhancing tumor core infiltration, and upregulating NKG2D and TNF-α (131, 132). Therefore, it is conceivable that inhibition of mTOR receptors could contribute to γδ T cell-mediated osteosarcoma cell killing.

COMBINATION THERAPY WITH IMMUNE CHECKPOINT INHIBITORS

Immune checkpoint molecules are key modulators of the anti-tumor T cell immune response by a narrow definition. Actually, multiple immune checkpoint molecules are also expressed on innate immune cells, which function as immunomodulators. Their interactions activate either inhibitory or activating immune signaling pathways. Indeed, metabolic pathways play a critical role in the functional modulation of immune cells and could, by extension, be considered as immune checkpoints. Here, we focus on the inhibitory immune checkpoints that influence adaptive and innate immune cells. Blocking inhibitory checkpoints can reverse the exhaustion state of immune cells and inhibit tumor growth. Importantly, one clinical trial demonstrated the immune response to ICPIs in osteosarcoma patients (15) and rational combinations of immunotherapies, particularly those involving ICPIs, have demonstrated increased efficacy in cancer patients (133). Therefore, ICPIs have the potential to improve efficacy of innate immune cell-based therapy for osteosarcoma.

Programmed Cell Death 1

Programmed cell death 1 (PD-1) is a receptor expressed on the surface of T lymphocytes, and innate immune cells. PD-1 binds a specific ligand, programmed cell death ligand 1 (PD-L1), which is expressed on several types of malignant cells and APCs in tumor foci. It is widely accepted that PD-1 is an exhaustion marker for CTL (134), which is the main anti-tumor effector cell during checkpoint blockade therapy. A study aiming to find predictors of DC vaccine responses showed that glioblastoma patients with tumor-infiltrating lymphocytes (TILs) with a higher $PD\text{-}1^{+}/CD8^{+}$ ratio had worse prognosis (135). These data indicated that DC vaccine-primed $CD8^{+}$ T cells

became exhausted via the PD-1-PD-L1 axis, which is one of the reasons that DC vaccines have showed unsatisfactory results in osteosarcoma patients. This obstacle might be overcome by ICPIs. On the other hand, evidence indicates that a mechanism of acquired resistance to ICPIs involved alterations in antigen presentation (136). This problem can be solved by growing DC vaccines *ex vivo*. Therefore, PD-1 inhibitors and DC vaccines have complementary roles regarding antitumor efficacy (37, 137, 138). For instance, an *ex vivo* study demonstrated that anti- PD-1 treatment enhanced T-cell responses induced by DC vaccines fused with myeloma cells (137). Furthermore, in melanoma-bearing mice, anti-PD-1 treatment increased the function and infiltration of TILs induced by DC vaccines, and augmented anti-tumor activity (138). Currently, there are ongoing phase I/II clinical trials studying the effects of different types of DC vaccines combined with nivolumab (a mAb that blocks PD-1) for the treatment of glioma (NCT02529072), glioblastoma multiforme (NCT03014804, NCT02529072), and solid tumors (NCT02775292).

Interestingly, some cancer types exhibit low MHC I expression and/or neoantigen burden, which renders them resistant to recognition by $CD8^+$ T cells, but sensitive to PD-1/PD-L1 axis blockade (139). This suggests that other immune cell types might also be suppressed by this axis. PD-1 expression on NK cells has been detected in cancer patients, including those with Kaposi sarcoma and ovarian carcinoma (140, 141). Preclinical observations showed that PD-L1 upregulation on several types of tumor cells or DCs suppressed NK cell-mediated tumor cell lysis, and blockade of PD-1 restored NK cell anti-tumor activity and inhibited tumor growth (141, 142). Importantly, a recent clinical study demonstrated that blocking PD-1 and PD-L1 elicited a strong NK cell response that was indispensable for the full therapeutic effects of immunotherapy (139). These data suggested the importance of the PD-1/PD-L1 axis in inhibiting NK cell responses *in vivo* and revealed that NK cells mediate the effect of PD-1/PD-L1 blockade immunotherapy. In addition, combination therapy consisting of NK cell transfusion and PD-1 blockade resulted in more potent cytolytic activity against tumor cells *in vitro* (142, 143). Unfortunately, a phase II clinical trial evaluating the effects of pembrolizumab, an anti-PD1 mAb, on the NK cell exhaustion phenotype in patients with unresectable stage III/IV melanoma (NCT03241927) has just been terminated because of difficult participant enrollment. Otherwise, this trial can aid in understanding how NK cell activity and exhaustion interplay with PD-1 expression and function, and it can lead to the development of more effective combination therapies.

$PD\text{-}1^+$ TAMs, which exhibited an M2-like surface profile and M2-like functional characteristics and suppressed $CD8^+$ (144) and $CD4^+$ (145) T cell function, were detected in human cancers. In a human LM7 osteosarcoma mouse model, macrophages in lung metastases highly expressed PD-1 (146). PD-1 blockade significantly decreased the number of osteosarcoma lung nodules by increasing the macrophage tumor infiltration and polarization from M2 to M1 (146). Other research showed that PD-1 levels on tumor-infiltrating DCs were increased during tumor progression, and these DCs responded poorly to tumor antigens, and suppressed T cell activity and infiltration (147). In a murine model of ovarian cancer, targeting PD-1 on DCs significantly enhanced antigen-specific T cell responses and slowed tumor growth (147).

PD-1/PD-L1 expression was increased in osteosarcoma patients and correlated with poor prognosis (148, 149). In preclinical trials, PD-1 blockade resulted in anti-metastatic effects in osteosarcoma murine models (150, 151). However, PD-1 blockade was ineffective in an orthotopic osteosarcoma model (152). In addition, data from a multicenter, two-cohort, single-arm, open-label, phase II trial revealed that the effect of pembrolizumab (a PD-1 inhibitor) on osteosarcoma patients was poor (only one [5%] of 22 patients showed a partial response) (15). Therefore, it was urgent to improve the therapeutic effects of PD1/PDL-1 inhibitors. Recently, oncologists defined tumors lacking various inflammatory immune cell infiltration as "cold tumors," and the opposite as "hot tumors" (153). Hot tumors are more susceptive to ICPIs. However, osteosarcomas are relatively "cold tumors." A potential approach for reducing acquired resistance to ICPIs is turning a cold tumor into a hot tumor, resulting in enhanced infiltration of inflammatory immune cells (both adaptive and innate immune cells) into the tumor (154, 155). Therefore, further investigation of combination therapy involving an ICPI with an innate immune cell-based therapy (such as ACT and vaccines) for the treatment of osteosarcoma may be of value.

Cytotoxic T-Lymphocyte-Associated Protein 4

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is another major immune checkpoint molecule on T cells induced by activation. CTLA-4 negatively regulates T cell function (156), and blocking CTLA-4 can reactivate T cells and enhance the efficacy of osteosarcoma vaccines. For example, in a C3H murine osteosarcoma model, tumor lysate-pulsed DCs with CTLA-4 blockade prevented lung tumor metastasis (31). Furthermore, a clinical study on the combined effects of a synthetic mRNA-electroporated DC vaccine and ipilimumab (an anti-CTLA-4 mAb) for patients with pretreated advanced melanoma showed a 6-month disease control rate of 51% and a promising overall response rate of 38% (eight complete and seven partial responses) (157). These results greatly increased interest in combination therapies involving vaccines and ICPIs. However, studies focusing on CTLA-4 expression on NK cells are scarce. CTLA-4 was detected on tumor-infiltrating NK cells in tumor-bearing mice and was closely associated with the inhibition of DC-induced IFN-γ production by NK cells (158). No studies have evaluated the expression of CTLA-4 on human NK cells. However, CTLA-4 may exist on human NK cells and may modulate their effector functions in cancer immunity.

CTLA-4 is significantly associated with carcinogenesis of osteosarcomas, which provides a potential therapeutic target (159). In a preclinical study, co-inhibition of CTLA-4 and PD-L1 resulted in complete control of metastatic osteosarcoma (151). Combined therapy involving anti-CTLA-4 antibody and a DC vaccine led to a similar outcome (31). Future studies should explore the possibility of combining anti-CTLA-4 mAb and NK cell-based therapy.

T-cell Immunoglobulin and Mucin-Domain Containing-3

T-cell immunoglobulin and mucin-domain containing-3 (TIM-3) is expressed by innate and adaptive immune cells. Importantly, all TIM-3$^+$ T cells in cancer patients co-express PD-1 (160). The current view is that CTLs with TIM-3-PD-1 co-expression are functionally more "exhausted" than those that express PD-1 alone (161, 162). Therefore, a DC vaccine combined with co-inhibition of TIM-3 and PD-1 may further prime T cells and maintain their cytotoxicity against malignant cells.

The inhibitory function of TIM-3 on innate immune cells (including NK cells, NKT cells, DCs, and macrophages) is consistent with its function on T cells (163). TIM-3 expression on peripheral NK cells correlated with their exhausted phenotype and predicted poor prognosis of patients with advanced melanoma and lung adenocarcinoma (164–166). Blockade of TIM-3 on NK cells from these patients increased NK cell-mediated cytotoxicity and IFN-γ production. Interestingly, researchers found that co-expression of TIM-3 and PD-1 is a marker of functionally exhausted NK cells in advanced tumors, as is the case for T cells (167). TIM-3 expression on macrophages is associated with inhibitory function in inflammatory diseases and cancers (168–170). For instance, in hepatocellular carcinoma, TIM-3 expression on TAMs was significantly enhanced by tumor-derived signals, which caused the macrophages to undergo alternative activation and inhibited CTL activation. Subsequent interference with TIM-3 on the TAMs successfully suppressed hepatocellular carcinoma growth (170). Recent studies showed that M1 macrophages had low expression of TIM-3, providing further evidence of its negative regulatory function in macrophages. In DCs, TIM-3 inhibits DC activation and maturation via the Btk-c-Src signaling pathway (171). In the TME, the interaction between TIM-3 and high-mobility group box 1 (HMGB1) prevented activation of tumor associated DCs by impeding sense of immunogenic nucleic acids, thereby suppressing anti-tumor responses (172). In γδ T cells, TIM-3 served as an exhaustion marker and protected the human body from inflammatory attack in different diseases (173, 174). Its role in tumor infiltrating γδ T cells has not been characterized.

Co-blocking CTLA-4 and PD-1 led to synergistic anti-tumor effects (175, 176). Interestingly, anti-CTLA-4 antibody showed a unique curative effect in anti-PD-1-resistant cancer (177). These results indicate that TIM-3 plays an essential role in tumor immunity. Therefore, TIM-3 is a candidate target for improving the effect of innate immune cell-based therapy.

CD39/CD73 and Adenosine Receptors

In the TME, ATP conversion to ADP and/or AMP occurs in the presence of CD39 (also known as NTPDase 1), while CD73 (also known as 5′-NT) dephosphorylates AMP to adenosine. Accumulated extracellular adenosine exerts regulatory functions by binding to one of four adenosine receptors (ARs), A1R, A2AR, A2BR, and A3R (**Figure 2**).

A2AR activation increased cell-surface expression of PD-1 and CTLA-4 on T cells and inhibited proliferation and pro-inflammatory cytokine production (178). Similarly, a recent study showed that tumor-infiltrating CD8$^+$ T cells expressed high levels of CD39 and exhibited an exhausted phenotype with impaired production of cytokines and high expression of inhibitory receptors (179). These observations suggested that CD39 was an immune checkpoint that could be targeted to restore the T cell immune response against tumors. In addition, genetic ablation or therapeutic inhibition of CD73 or AR improved the effector functions and infiltration of CTLs, and significantly reduced tumor growth (180–182). Importantly, these interventions augmented the efficacy of adoptive T cell anticancer therapy against ACT-resistant tumors (183, 184). These results indicated the potential to improve the efficacy of vaccines by inhibiting the adenosinergic pathway. Intravenous administration of CD73-specific small interfering RNA (siRNA)-loaded chitosan-lactate nanoparticles (ChLa NPs) potentiated the antitumor effects of a DC vaccine in 4T1 breast cancer-bearing mice, with augmented CTL effector function, improved T cell proliferations, and increased production of inflammatory cytokines (185). Similarly, another study demonstrated that co-targeting of A2AR and CD73 in conjunction with a DC vaccine successfully reduced tumor growth, prolonged survival, and enhanced specific antitumor immune responses in the same mouse model of breast cancer (186).

Notably, A2AR is abundantly expressed on NK cells (at a 5-fold higher level, at the mRNA level, compared to that in T cells), and A2AR activation inhibited NK cell cytotoxicity and proliferation in several tumors (187–189). A recent study found that co-inhibition of A2AR and PD-1 in a B16F10 lung metastasis model resulted in a therapeutic effect that was more dependent on infiltrating NK cells than T cells (190). These findings indicate an important role of A2AR regarding NK cell function in tumor immunity. In addition, antagonism of A2AR reduced the percentage of CD56^{bright} NK cells in favor of accumulation of mature CD56^{dim} NK cells with high cytotoxic activity (191). This suggested that A2AR antagonism could enhance adoptive NK cell immunotherapy. Adenosine-differentiated DCs displayed high levels of tolerogenic molecules (VEGF and indoleamine 2,3-dioxygenase [IDO]) and anti- inflammatory cytokines (IL-10), which impaired the DC antigen presenting function and subsequent T cell priming, resulting in accelerated tumor growth in mice (192, 193). Selective inhibition of A2BR improved DC activation and chemokine release, and subsequently increased T cell infiltration and adaptive responses in mice, resulting in reduced growth of carcinomas (194). Moreover, activation of the A2AR pathway in DCs increased the expression of programmed cell death 1 ligand 2 (PDL2, a ligand for the inhibitory receptor PD1), which directly inactivated effector T cells (195). Similarly, A2BR plays a prominent role in M2 polarization of macrophages (196). Macrophages differentiated in the presence of adenosine expressed arginase, IDO, and TGF-β, and had limited T cell stimulatory activity (196). Additionally, TAMs expressing CD39 and CD73 contributed to tumor growth through the production of adenosine (197, 198). Studies of the effects of adenosine-related molecules on γδ T cells are sparse. Upregulation of CD39 on human Vγ9Vδ2 T cells directly abrogated the γδ TCR agonistic activity of phosphoantigens (199). Through this

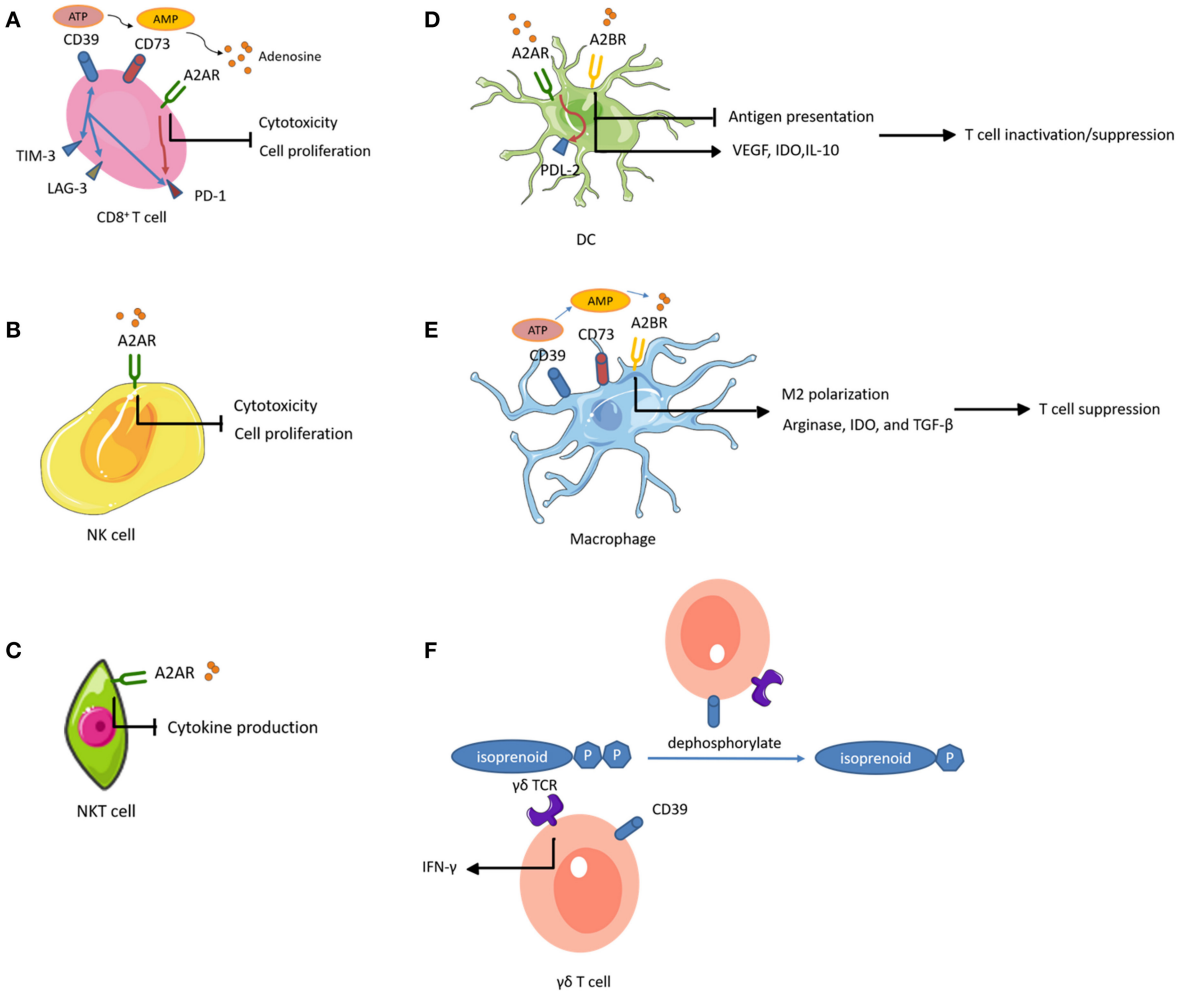

FIGURE 2 | Adenosine-mediated immunosuppression of immune cells. Expression of CD39 and CD73 generates adenosine, an immunosuppressive metabolite. Activation of adenosine receptors (ARs) suppresses the proliferation and effector functions of cytotoxic lymphocytes, and promotes polarization toward exhausted or immunosuppressive function. **(A)** $CD39^+$ $CD8^+$ T cells highly express other inhibitory immune checkpoints such as PD-1, TIM-3, and lymphocyte activating 3 (LAG-3). **(A,B)** On $CD8^+$ T cells and NK cells, A2AR activation inhibits their proliferation. **(A–C)** On $CD8^+$ T cells, NK cells, and NKT cells, A2AR activation impairs their cytotoxic potential. **(A,D)** A2AR signal path on $CD8^+$ T cells and DCs promotes the expression of other inhibitory immune checkpoints. **(A,E)** CD39 and CD73 expression on $CD8^+$ T cells and macrophages contributes to adenosine accumulation. **(D)** On DCs, A2BR stimulation impairs DC antigen presentation and subsequent T cell priming while inducing VEGF, IDO, and IL-10 secretion and subsequent T cell suppression. **(E)** Activation of A2BR on macrophages favors M2 phenotype polarization and induces arginase, IDO, and TGF-β, mediating T cell suppression. **(F)** The ecto-ATPase CD39 inactivates isoprenoid-derived Vγ9Vδ2 T cell phosphoantigens.

pathway, CD39 reduced Vγ9Vδ2 T cell activation and IFN-γ production. This study revealed a previously unrecognized immunoregulatory function of CD39, which is independent of the adenosinergic pathway. A2AR activation also increased anti-inflammatory cytokine production in NKT cells, indicating that A2AR played a negative immune regulatory role in NKT cells (200).

Recent studies showed that intratumoral hypoxia and hypoxia inducible factor-1α (HIF-1α)-dependent pathways up-regulated the tandem activities of CD39 and CD73, leading to adenosine accumulation in the TME and tumor immune escape (201, 202). Adenosinergic pathways have not been characterized in osteosarcoma. However, studies have shown that hypoxia contributed to human osteosarcoma progression (203). It is conceivable that hypoxia-mediated tumor protection is dependent on adenosinergic pathway-mediated immunosuppression. Therefore, targeting CD39, CD73, and ARs has the potential to reinstate osteosarcoma immunity and improve immunosensitivity to innate immune cell-based immunotherapy.

Clinical Studies of Innate Immune Cell-Based Immunotherapy and Immune Checkpoint Inhibitors

In this section, we mainly discuss the results of major clinical studies and ongoing clinical trials for treatment of osteosarcoma

TABLE 5 | Clinical trials of DC vaccination, cell infusion, and ICPIs for treating osteosarcoma.

Intervention	Ancillary therapy	Trial phase	Status	References
Autologous DCs loaded with tumor cell lysates	None	I	Unknown	(35)*
	None	I/II	Unknown	(36)*
	Gemcitabine	I	Recruiting	NCT01803152
Autologous DCs loaded with TAAs or TAA-derived peptides (MAGE-A1, MAGE-A3, NY-ESO-1)	Decitabine	I/II	Completed	NCT01241162
Pembrolizumab (targeting PD-1)	None	II	Active, not recruiting	NCT02301039
SHR1020 (targeting PD-1)	Apatinib	II	Active, not recruiting	NCT03359018
Nivolumab (targeting PD-1) with ipilimumab (targeting CTLA-4)	None	II	Not yet recruiting	NCT02982486
Nivolumab with or without ipilimumab	None	I/II	Recruiting	NCT02304458
	None	II	Suspended	NCT02500797
NK cell infusion	None	I/II	Unknown	NCT02409576
	Haploidentical stem cell transplantation	II	Active, not recruiting	NCT01807468
	Hematopoietic cell transplantation	II	Recruiting	NCT02100891

TAA, tumor-associated antigen.
**These studies were not found in ClinicalTrials.gov.*

on innate immune cell-based immunotherapy and ICPIs for the treatment of osteosarcoma. As discussed above, the results of the initial clinical trials of DC vaccines were unsatisfactory (34–36), possibly due to tumor-associated immune suppression. A recent clinical trial (NCT01803152) has reported some improvements. The DC vaccine used was similar to the previous study (34–36), but the vaccine was combined with gemcitabine, which inhibits myeloid-derived suppressor cells (MDSCs) that play a vital role in tumor-associated immune suppression. In the field of innate cell infusion, NK cells are at the forefront. In an early clinical study, NK92 cells were infused into a patient with advanced osteosarcoma, though no treatment response was observed (204). More trial participants are required. We found several ongoing studies of expanded, activated haploidentical NK cell infusions for the treatment of sarcomas (these studies are summarized in **Table 5**), which should provide information on the effectiveness and safety of this approach. Only one clinical study published results regarding the curative effects of ICPIs for the treatment of osteosarcoma, which showed a 5% response rate to pembrolizumab (a PD-1 inhibitor) (15). Multiple clinical trials targeting PD-1 and/or CTLA-4 are ongoing (**Table 5**), and we expect an improved curative effect, which will provide a foundation for combination regimens involving targeting PD-1 and/or CTLA-4 along with innate immune cell-based immunotherapy.

COMBINATION THERAPY WITH ONCOLYTIC VIRUSES

Oncolytic viruses (OVs) are emerging as a novel therapeutic class, which selectively replicate in and lyse cancer cells without harming normal cells. Like chemotherapy and radiotherapy, the therapeutic outcomes of OVs are determined not only by direct cancer cell lysis, but also by immune activation (205). Here, we mainly discuss the innate immune responses induced by OVs.

Virus-infected cancer cells tend to down-regulate their MHC-I molecules making themselves more sensitive to NK cells (206). In this regard, several studies have been conducted to examine the anti-tumor effect of the combination of NK cells with OVs. As expected, combination therapy showed an additive or synergistic anti-tumor effect (207, 208). In addition, OV infection can lead to increased tumor infiltration of M1 type macrophages and NK cells (209, 210). Furthermore, infected cells can trigger a Toll-like receptor response due to the expression of pathogen-associated molecular patterns (PAMPs) on the cell surface or due to detection by intracellular components of Toll-like receptors (211). Additionally, OV infection can cause the exposure of calreticulin, HMGB-1, nucleic acids, and type I IFNs (212), and the induction of immunogenic cell death (213), which are essential ligands and innate immune sensing pathways for activation of DCs and macrophages (7). Oncolysis by OVs could also cause the release of tumor associated/specific antigens that are then cross-presented by DCs, ultimately eliciting an adaptive immune response against the tumor (214, 215). Some OVs, such as reovirus (216) and M protein mutant vesicular stomatitis virus (DeltaM51-VSV) (217), can directly activate DCs and facilitate their antigen presentation function.

CONCLUSION

In view of the recent insights into the biology and immunology of osteosarcoma, immunotherapy is becoming an increasingly attractive treatment strategy. It is generally assumed that

adaptive immune cells, especially CTLs, have the greatest potential to eliminate tumors, due to their professional antigen recognition activity and specific killing of tumors (218). However, the characteristics of osteosarcomas (e.g., low expression of MHC-I molecules, absence of specific tumor antigens, and impaired antigen presentation) impede the anti-tumor capacity of CTLs (3, 20). Innate immune cells have unique advantages related to eliminating osteosarcoma due to their roles in antigen presentation, antigen-specific T cell priming, and MHC-independent direct cell killing. Efficacy can be further improved by using auxiliary strategies such as epigenetic modification, gene engineering, and mAb therapy. However, existing immunosuppressive mechanisms, especially the immune checkpoints imposed on immune cells, act as major obstacles to efficacy of innate immune cell-based therapy. Considering the role of OVs in induction of innate immune response, it is reasonable to combine innate immune cell-based therapy with ICPIs or OVs to treat osteosarcoma.

AUTHOR CONTRIBUTIONS

This review paper was written by ZeW, revised by ZhW, and BL, suggested by SW and TC, and edited and guided by ZY. All authors read and approved the final version of the manuscript.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (grant no. 81872173).

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

The reviewer BT and handling Editor declared their shared affiliation.

frontiers in Oncology

REVIEW
published: 31 January 2020
doi: 10.3389/fonc.2019.01529

Controlling Cytokine Release Syndrome to Harness the Full Potential of CAR-Based Cellular Therapy

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Chimeric Antigen Receptor (CAR)-based therapies offer a promising, targeted approach to effectively treat relapsed or refractory B cell malignancies. However, the treatment-related toxicity defined as cytokine-release syndrome (CRS) often develops in patients, and if uncontrolled, can be fatal. Grading systems have now been developed to further characterize and objectify clinical findings in order to provide algorithm-based guidance on CRS-related treatment decisions. The pharmacological treatments associated with these algorithms suppress inflammation through a variety of mechanisms and are paramount to improving the safety profile of CAR-based therapies. However, fatalities are still occurring, and there are downsides to these treatments, including the possibility of disrupting CAR-T cell persistence. This review article will describe the clinical presentation and current management of CRS, and through our now deeper understanding of downstream signaling pathways, will provide a molecular framework to formulate new hypotheses regarding clinical applications to contain proinflammatory cytokines responsible for CRS.

Keywords: cytokine release syndrome, chimeric antigen receptor, T cells, NK cells, Fyn-ADAP

OPEN ACCESS

Edited by:
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University of Texas MD Anderson Cancer Center, United States

Reviewed by:
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Specialty section:
This article was submitted to Cancer Immunity and Immunotherapy, a section of the journal Frontiers in Oncology

Received: *02 May 2019*
Accepted: *18 December 2019*
Published: *31 January 2020*

Citation:
Thakar MS, Kearl TJ and Malarkannan S (2020) Controlling Cytokine Release Syndrome to Harness the Full Potential of CAR-Based Cellular Therapy. Front. Oncol. 9:1529. doi: 10.3389/fonc.2019.01529

INTRODUCTION

Although great progress has been made in treating hematologic malignancies, patients having relapsed or refractory disease often have poor outcomes. In adults, one of the most common hematologic malignancies is diffuse large B-cell lymphoma (DLBCL), which affects an estimated 28,000 patients in the United States each year (1). Up to 50% of patients with DLBCL have refractory disease or experience relapse after initial treatment. For these patients, outcomes are very poor with long term survival rates of 20–53% (2, 3). In the pediatric population, precursor B-cell acute lymphoblastic leukemia (B-ALL) is the most common malignancy. Although >90% of children enter remission and have excellent rates of long-term survival, relapse can occur in approximately 20% of all cases. Those who relapse often succumb to their disease (4–7).

Chimeric antigen receptor (CAR) T-cell based therapies are an exciting, targeted approach to effectively treat relapsed or refractory hematologic malignancies and have helped bring immunotherapy to the forefront of cancer treatment. CAR-T cells are a biological drug that targets

cancer-associated antigens using genetically-modified autologous T cells transduced with the CAR (8). Successful outcomes from early clinical trials using CD19-targeted CAR T cells have led to the FDA approval of tisagenlecleucel in 2017 and 2018 for relapsed or refractory pediatric B-ALL and large B cell lymphomas, respectively, and axicabtagene ciloleucel in 2017 for relapsed or refractory large B cell lymphomas. Building off these recent successes, new clinical trials are exploring a variety of ways to expand this therapy, including the development of CAR-based natural killer (NK) cell immunotherapy to work in diseases and circumstances where CAR-T cells may falter (9). Despite high rates of complete and partial remissions, allowing for long-term durable survivals and/or providing bridging therapy until hematopoietic cell transplantation, the trials leading to approval of CAR-T products also demonstrated significant therapy-related toxicities, specifically cytokine release syndrome (CRS) and its accompanying neurological effects (10–13).

Cytokine storm was initially used to describe the systemic inflammatory response that occurred following any antibody-based immune therapy; however, since the incidence of what is now coined CRS following CAR T-cell based therapies is high, the use of this term is now fairly synonymous with the major adverse effect of this therapy (13). CRS can begin with mild symptoms such as fever, tachycardia, tachypnea, nausea, and diarrhea. Concurrently, a spectrum of neurological toxicities known as CRS-related encephalopathy syndrome (CRES) [or immune effector cell-associated neurotoxicity syndrome (ICANS)] can develop, consisting of headaches, confusion, aphasia, and seizures. In its more severe forms, the inflammation related from CRS and/or CRES/ICANS can result in hypotension, coagulopathies, cerebral edema, and multi-organ failure (14–16). If uncontrolled, death can result. Across nine separate studies of CD19 CAR T cells including 387 patients, 83% developed CRS and 51% developed severe (>grade II) CRS (13, 17–24). The ELIANA trial (a phase III trial of tisagenlecleucel for pediatric and young adult patients) illustrates the potential morbidity of CRS. After receiving tisagenlecleucel, 77% of patients developed CRS, 25% required high-dose vasopressors for cardiac support, 13% were intubated, and 9% required dialysis (17).

Numerous CAR-based clinical trials are underway to treat hematological malignancies and solid tumors and have been life-saving for many patients (13, 25–35). However, CRS caused by CAR T cells is a major limiting factor in the successful utilization of cellular immunotherapy due to the competing risk of morbidity and mortality from the treatment itself (14). In order to improve CAR-based therapies, it is important to understand the limitations of the current CRS treatment approach and explore new strategies of CRS treatment and prevention.

CAR Therapy—Structure Impacts Cytokine Production

Worldwide, well-over 500 active clinical trials are registered for CAR-based therapies, and the list continues to grow[1]. In general, autologous (or more rarely, allogeneic) T cells are transduced with retroviral, lentiviral, or transposon-based systems with the CAR construct. NK cells are also being investigated as viable alternatives to T cells (9, 36). While CD19 is the most utilized antigen in CAR trials to date, a variety of other antigens are now being investigated (37–46). Lymphodepletion with agents such as fludarabine and/or cyclophosphamide chemotherapy prior to CAR-T infusions provides reduction of T regulatory cells which can inhibit CAR-T cell activity and promotes expansion of these CAR-T cells. The use of lymphodepleting chemotherapy is now standard in clinical trials. This chemotherapy-enhanced expansion of engineered CAR-T cells can provoke initial cytokine secretion. The secondary effects of these activated T cells can later produce another wave of cytokine production by activating other surrounding immune and antigen-presenting cells, which will be discussed later (47, 48).

CARs are molecules synthetically designed to comprise an extracellular single-chain variable fragment, scFv (variable light chain, V_L, and variable heavy chain, V_H), from an antigen-specific antibody. This antigen-specific antibody allows CAR T cells to recognize tumor cells in a T-cell receptor (TCR) and human leukocyte antigen (HLA)-independent manner. In regard to CD19 as an extracellular receptor on CARs, since it is expressed throughout B cell maturation starting from the pro-B cell stage, targeting CD19 facilitates its use against different B cell tumors that have originated at distinct developmental stages. The cytoplasmic tail of CD3ζ is added as the primary signaling module, and in some cases, two out of four tyrosines within the immunoreceptor tyrosine-based inhibition motifs are mutated to optimize the signaling threshold. The extracellular domains are joined to intracellular signaling modules via hinge and transmembrane regions. The intracellular domains contain both the obligatory signaling module (CD3ζ) and the cytoplasmic tail of co-stimulatory molecules such as CD28 or CD137 (4-1BB). At a cellular level, combining the cytoplasmic tails of CD3ζ with CD28 or CD137 provides strong activating signals along with robust survival and proliferation signals. Early phase studies with CD19 CARs have demonstrated the utility of including these co-stimulatory signaling molecules in the CAR product (49–51).

CD3ζ uses ZAP-70/LAT/PLC-γ1 to activate NFAT or NF-κB pathways via augmenting Ca^{2+} and diacylglycerol (DAG) (52). Also, CD3ζ activates AP-1 through the Ras/MAPK pathway. CD28 and CD137 are also known to regulate overlapping and distinct signaling pathways (53, 54). Both CD28 and CD137 can regulate proliferation, survival, differentiation, and effector functions in CAR T cells by activating the PI(3)K/AKT/Bcl-X_L cascade (55). CD28 can also affect T cell proliferation and function through Grb2, FLNa, and Lck pathways (56, 57). Similarly, 4-1BB signaling is dependent on TNF-receptor Associated Factor, or TRAF pathway. Thus, the interactions of these multiple downstream signaling pathways from upstream inducible costimulatory molecules could be important keys to understanding how CAR-T cells produce cytokines at supraphysiologic levels.

Ultimately, the transduced CAR helps these engineered T cells to mediate a redirected response toward antigen positive tumor cells, which involves both granzyme-B and perforin for tumor lysis as well as generation of inflammatory cytokines and chemokines. The outcome of these signaling pathways in CAR T

[1]https://www.clinicaltrials.gov (cited July 10, 2019).

cells can provide effective anti-tumor cytotoxicity and enhance the production of inflammatory cytokines. Unique signaling pathways that exclusively regulate each of these functions are being defined.

Cytokine-Release Syndrome: Pathogenesis of Cytokine Production

T cells play a pivotal role in tumor immunosurveillance (58). Effector functions of T cells correlate with beneficial graft-vs.-tumor responses (59). However, they are also the primary producers of pro-inflammatory cytokines and chemokines (60). Inflammation is an essential component of effector T cell mediated immune responses. However, acute inflammation at enhanced levels is highly destructive.

Antibody-based CAR receptors possess 10 to 60-fold higher affinity than the average affinity of TCR. An intrinsic dissociation constant of an affinity matured antibody can exhibit a K_d 5 × 10^{-14} compared to a $CD8^+$ T cell-derived TCR that has a K_d 10^{-7}. While TCR-engineered T cells can also potently engage cancer targets and have been shown to cause significant toxicity, there appear to be interactive antigen differences between a CAR and a TCR (61–63). Accordingly, the potency of CAR T cells is increased significantly compared to native T cells. While the extraordinary affinity of the CAR to its cognate antigen is the basis for augmented tumor killing, it also causes significant toxicity through supraphysiologic stimulation and cytokine production (49, 64, 65).

While cytokine production can occur physiologically during severe infections and graft-vs.-host disease, CRS is a complex clinical phenomenon characterized by the high activation of immune cells and immense production of proinflammatory cytokines (11, 14–16, 65–68). Acute CRS generally begins hours to days after CAR T cell infusion. In some cases, late CRS has appeared in patients 1–4 weeks after infusion, at a time when there is a significant CAR T cell expansion (47). Patients with CRS experience symptoms associated with this elevated amount of proinflammatory cytokines, including IFN-γ, IL-2, TNF-α, MIP-1, and GM-CSF (10, 50, 69). In addition to cytokines that originate from the CAR T cells themselves, additional cytokines (including IL-6, IL-8, and IL-10) primarily generated by bystander cells and professional antigen presenting cells, are also significantly elevated during CRS (32, 50, 70). This accounts for a secondary wave of cytokine production, which can often be higher than what is directly produced from CAR-T cells (32, 47, 48). Notably, this secondary burst of cytokines is associated with other hyperactive immune disorders such as hemophagocytic lymphohistiocytosis (HLH) and macrophage activation syndrome (MAS), with inflammatory markers such as C Reactive protein and ferritin becoming elevated (32).

The role of macrophages and other myeloid cells in the development of CRS has been confirmed through not only clinical assays and patient samples, but also in animal models. Giavridis et al. established that activated CAR T cells can recruit and activate macrophages and other myeloid cells and are the main source of IL-6. Authors also confirmed the important role of IL-1 blockade in improving symptoms and suggested that its ability to cross the blood brain barrier made it an ideal pharmacological candidate compared to tocilizumab (71). Similarly, Norelli et al. also confirmed though their preclinical animal studies that IL-1 was critically important in the pathogenesis of CRS, and also suggested that Anakira be considered as part of CRS therapy (72).

Clinical Manifestations of CRS

CRS begins with mild symptoms, but it can quickly progress to life-threatening complications (13, 14, 26, 31, 35, 73–76). Multiple organ systems are involved in all phases. Fevers are an obligatory sign of CRS and typically precedes any other manifestation. Constitutionally, in addition to fevers, patients can develop rigors, nausea, and arthralgias. Hematologically, a picture consistent with disseminated intravascular coagulation can be seen, consisting of coagulopathy and hemorrhage. B cell aplasia is very common and a result of on-target-off-tumor effects. Gastrointestinal toxicities include colitis with diarrhea and abdominal pain and hepatitis. Cardiovascular side effects often prompt transfer to critical care settings, where tachycardia and hypotension can be supported with fluids and pressors. Other organ systems, including renal and pulmonary, can be affected by edema and third spacing, causing hypoxia and respiratory decompensation requiring intubation and ventilatory support. The neurological toxicities (CRES/ICANS) are some of the most puzzling and disturbing complications seen (77). While some patients can develop mild symptoms of headache and confusion, others have progressed (and in some cases rapidly) to seizures, cerebral edema, and death. Aphasia has also been identified as part of the neurotoxicity profile identified with CRES/ICANS. While some symptoms can occur during the CRS period, this has not been consistently seen, and CRES/ICANS can even occur days or weeks later (78). **Table 1** highlights a summary of CAR-T trials with focus on CRS grade results (14, 17, 19, 20, 22, 42, 46, 79–82). The degree of CRS and CRES/ICANS have appeared to correlate in most cases with the use of preceding lymphodepletion, leukemia burden, and CAR-T cell dose, although it is still not completely clear why some patients do not develop any CRS and/or CRES/ICANS. Identifying biomarkers for use in CRS prediction algorithms is an emerging area of investigation to help guide patient risk, and if combined with patient characteristics, may become a helpful tool to determine CRS and CRES/ICANS risk (32).

Treatment for CRS Includes Pharmacological Interventions and Supportive Care: The Pros and Cons of This Approach

Currently, immunosuppression is the primary therapeutic approach to treat life-threatening complications of CRS. With the concurrent development of distinct CAR-T constructs and clinical trials across different research groups, numerous CRS grading scales have developed over the years [**Table 2**; (31, 78, 83–86)]. While these scales are similar in the fact that they begin with mild CRS symptoms (grade I) and end in death (grade V), they differ in how they progress between the different grades.

TABLE 1 | Summary of published CAR-T trials with focus on CRS outcomes.

References	Target	Lymphodepletion	CRS Scale	Total patients treated	CRS Grade 1 or 2	CRS Grade 3	CRS Grade 4	CRS Grade 5	Additional comments
Lee et al. (14)	CD19	Fludarabine/ Cyclophosphamide (Flu/Cy)	Trial specific	21	10	3	3	0	4 patients received tocilizumab and/or steroids
Gardner et al. (22)	CD19	Flu/Cy ($n = 14$) No lymphodepletion ($n = 31$)	Trial specific	45	33	10			16 patients received tocilizumab; 10 received steroids
Neelapu et al. (20)	CD19	Flu/Cy	Lee	101	82	9	3	1	Grade 5 event was due to hemophagocytic lymphohistiocytosis. 43 patients received tocilizumab and 27 received steroids
O'Rourke et al. (79)	EGFRvIII	None	Not described	10		0			
Ramos et al. (46)	CD30	None	Not described	9		0			
Schuster et al. (19)	CD19	Cy-only ($n = 10$) Bendamustine ($n = 8$) RT + Cy ($n = 4$) Other ($n = 6$)	Penn	28	11	4	1	0	1 patient received tocilizumab; none received steroids
Fry et al. (42)	CD22	Flu/Cy	Lee	21	16	0	0	0	
Maude et al. (17)	CD19	Flu/Cy ($n = 71$) Cytarabine/Etoposide ($n = 1$)	Penn	75	23	16	19	0	28 patients received tocilizumab
Park et al. (18)	CD19	Cy ($n = 43$) Flu/Cy ($n = 10$)	MSKCC	53	31	13		1	23 patients received tocilizumab and/or steroids
Bishop et al. (80)	CD19	Flu/Cy or bendamustine	Penn	7	2	2	0	0	
Cao et al. (81)	CD19	Flu/Cy	Lee	11	3	6	0	0	All patients received anti-PD-1 ab therapy 3 days after CAR T cells. None required tocilizumab or steroids
Zhao et al. (82)	BCMA	Cy	Lee	57	47	4	0	0	24 patients received tocilizumab.

This makes it challenging to compare CRS results across multiple trials. New consensus workshops have identified a uniform method that will likely be adopted when considering future CRS reporting, although in the interim, it is important to look closely at the grading scale used when comparing the safety results across trials (85).

When identified early, CRS can be managed with supportive care ± anti-IL-6R mAb (tocilizumab) (13, 14, 87). Patients who respond to tocilizumab resolve CRS within a few hours to days; however, there are others who do not respond to this monoclonal antibody and require the administration of corticosteroids (31). With experience gained in identifying, classifying, and treating CRS early, outcomes have improved greatly with proactive supportive care and pre-emptive pharmacological support. Furthermore, tocilizumab was approved for treatment of CRS in August 2017, easing its ability to be accepted more uniformly as a standard of care treatment for CRS (88).

However, there are several concerns with using pharmacological agents to treat CRS. First, there is some concern that systemic immunosuppression caused by these drugs may diminish the efficacy of CAR-T cells. Furthermore, dampening the immune system may make patients who are already sick and compromised from lymphodepleting chemotherapy more prone to infections. Furthermore, with more studies focused on decreased persistence of CAR-T, it is unclear if dampening their response with immune suppressive therapies will ultimately affect their ability to persist in order to be a true "living" biological drug in patients (89). Finally, understanding the biochemistry of CRS brings to light that targeting the IL-6 receptor with tocilizumab does not always work for CRES, and by the time a direct Il-6 antagonist such as

TABLE 2 | CRS definitions across different scales.

Scale	Grade I	Grade 2	Grade 3	Grade 4	Grade 5
MSKCC (83)	Mild symptoms requiring observation or supportive care only	• Hypotension requiring any vasopressors <24 h • Hypoxia/dyspnea requiring O_2 <40%	• Hypotension requiring any vasopressors ≥24 h • Hypoxia/dyspnea requiring O_2 ≥40%	• Hypotension refractory to high-dose vasopressors • Hypoxia/dyspnea requiring mechanical ventilation	Death
Lee et al. (31)	Fever, constitutional symptoms	• Hypotension responsive to fluids or one low dose pressor • Hypoxia responsive to <40% O_2 • Organ toxicity: grade 2	• Hypotension requiring multiple pressors or high dose pressors • Hypoxia requiring ≥40% O_2 • Organ toxicity: grade 3, grade 4 transaminitis	• Hypoxia requiring mechanical ventilation • Organ toxicity: grade 4, excluding transaminitis	Death
Penn (84)	Mild reaction	• Organ toxicity: grade 2 creatinine, grade 3 transaminitis • Hospitalization for management of CRS-related symptoms	• Organ toxicity: organ dysfunction requiring hospitalization, including grade 4 transaminitis or grade 3 creatinine • Hypotension requiring IV fluids or low-dose vasopressors • Coagulopathy requiring blood product transfusions • Hypoxia requiring supplemental oxygen	• Hypoxia requiring mechanical ventilation • Hypotension requiring high-dose vasopressors	Death
CARTOX (78)	• Temperature ≥38°C • Grade 1 organ toxicity	• Hypotension responsive to IV fluids or low-dose vasopressors • Hypoxia requiring FiO_2 <40% • Grade 2 organ toxicity	• Hypotension needing high-dose or multiple vasopressors • Hypoxia requiring FiO_2 ≥40% • Grade 3 organ toxicity or grade 4 transaminitis	• Life-threatening hypotension needing ventilator support • Grade 4 organ toxicity except grade 4 transaminitis	Death
ASTCT (85)	Fever without hypotension or hypoxia	Fever with either: • Hypotension not requiring vasopressors • Hypoxia requiring low-flow nasal cannula or blow-by	Fever with either: • Hypotension requiring a vasopressor • Hypoxia requiring high-flow nasal cannula, facemask, non-rebreather mask, or Venturi mask	Fever with either: • Hypotension requiring multiple vasopressors (excluding vasopressin) • Hypoxia requiring positive pressure ventilation	Death
CTCAE5.0 (86)	Fever with or without constitutional symptoms	Hypotension responding to fluids; hypoxia responding to <40% O_2	Hypotension managed with one pressor; hypoxia requiring ≥ 40% O_2	Life-threatening consequences; urgent intervention indicated	Death

siltuximab is used, it may be too late in the process to have a substantial impact. Many have speculated that use of many of these agents, including anti IL-1, may be targeting cytokines that are further downstream from the instigating events that begin this cascade of inflammation. Thus, waiting for CRS/CRES to occur and using agents to neutralize downstream cytokines may not be the best strategy to consider. While some have advocated for pre-emptive treatment with agents such as tocilizumab to prevent severe CRS, this is still under investigation (90).

Perhaps most importantly, the outcome of CRS can be life-threatening, and there have been a number of patient deaths following treatment with CAR-T cells (16). Because current algorithms are waiting until CRS symptoms occur before treating, patients are being put at risk. Therefore, novel approaches are needed to manage and prevent CRS. Because CAR's are inherently engineered cellular products, designing a safer CAR could be one approach to consider.

Engineering Safer CAR Products: Is It Possible?

With the advent of clustered regularly interspaced short palindromic repeats (CRISPR) technology, it is now possible to engineer a CAR-T that may have different and more specifically-controlled properties than virally-transduced cells. Specifically, it has been shown that targeting a CAR-coding sequence to the T cell receptor (TCR) locus may prevent accelerated T-cell exhaustion by decreasing tonic activation and TCR-induced autoimmunity. This decreased tonic activation may also decrease CRS (91). Another method that directs the engineered CAR to operate through the native TCR is a T-cell antigen coupler, or TAC (92). The TAC has three components, including an

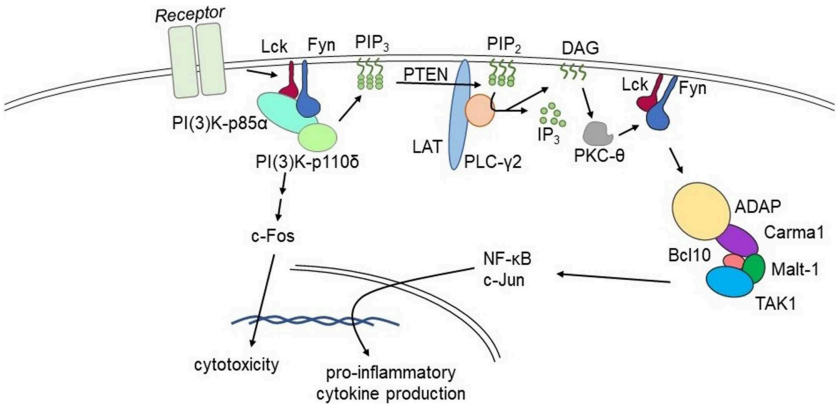

FIGURE 1 | Fyn activates divergent signaling cascades in effector lymphocytes. Signaling via the Lck→Fyn→ADAP→CARMA1→Bcl10 pathway that is obligatory for the production of inflammatory cytokines. Divergent signaling via the Lck→Fyn→PI(3)K pathway primarily facilitates cell-mediated cytotoxicity. Lack of ADAP significantly reduces pro-inflammatory cytokine production without affecting cell-mediated cytotoxicity in pre-clinical models.

antigen-binding domain, a TCR-recruitment domain, and a co-receptor domain, for a more controlled design that can also decrease off-target toxicity. Another method that has been explored is the use of synthetic Notch receptors to induce T cell response in a customizable manner. Specifically, these receptors, when engaged to its cognate antigen, induce a transmembrane cleavage that releases the intracellular transcriptional domain to penetrate the nucleus and activate a synthetic response (93). While these constructs are using the newest technologies for cellular engineering, they are still in the early phases of development. Finally, another method to consider is to decipher the downstream signaling pathways that become activated once a CAR is engaged with its cognate ligand. By targeting the activation of these pathways that produce excessive cytokines but maintain cytotoxic potential, there is strong potential to regulate CRS (54, 66).

The FynADAP Pathway: Can It Be Targeted to Develop a Safer CAR?

Our lab is focused on NK cell immunobiology, and NK cell intracellular signaling pathways are directly applicable to understanding T cell functionality (66). Our work has shown that adhesion and degranulation-promoting adapter protein (ADAP) serves as a positive regulator of proinflammatory cytokine production (54). The FynADAP complex exclusively regulates the production of inflammatory cytokines (94). Most importantly, lack of ADAP does not affect the NK cell-mediated anti-tumor cytotoxicity. These findings establish ADAP as a potential molecular target to reduce the production of inflammatory cytokine and chemokine production. This 130 kDa protein is expressed in multiple cell types including NK and T cells. It functions as a connecting link between the upstream Fyn and downstream signaling proteins Carma1 and TAK1. ADAP also interacts with SLP76 and SKAP55. The potential interaction sites of these signaling proteins in ADAP have been largely defined. Recently, as shown in **Figure 1**, we defined a Lck→Fyn→ADAP→CARMA1→Bcl10 pathway that is obligatory for the production of inflammatory cytokines (54). Lack of ADAP in T or NK cells significantly reduces the production of IFN-γ, GM-CSF, TNF-α, MIP-1α, MIP-1β, and RANTES; however, the anti-tumor cytotoxicity was intact. In both T and NK cells, ADAP plays an essential role in immunoreceptor tyrosine-based activation motif-dependent receptor activation and is involved in activation integrins including LFA1. Loss of ADAP in T cells decreases their proliferation and cytokine production efficiency in response to limiting antigen doses (95).

These findings provide a feasible clinical approach to reduce the production of inflammatory cytokines and chemokines, and thereby the severity of CRS. Stimulation through TCR and CD28 utilizes ADAP to facilitate signaling downstream of the Carma1-Bcl10-Malt1 (CBM) complex, which leads to phosphorylation and degradation of IκBα and nuclear translocation of NF-κB (96). While the molecular mechanism whereby ADAP regulates the formation of the CBM has not been fully elucidated, the essential function of ADAP in linking CBM via Carma1 to PKC-θ is well-documented (97). NF-κB, which is sequestered in the cytosol through binding to IκBα, translocates into the nucleus (97). Carma1 plays an obligatory role in the nuclear translocation of NF-κB following activation of T or NK cells (98, 99). In addition to interactions with Carma1, ADAP also recruits TAK1, which facilitates the phosphorylation of IKKα and IKKβ, components of NF-κB signaling pathway. In T cells, ADAP contributes to CBM complex formation in response to ITAM-containing receptors (96, 97, 99–101). Thus, targeting ADAP in T cells could help to selectively attenuate cytokine production, without reducing cytotoxicity. The feasible approaches to target ADAP include CRISPR-CAS9-based deletion of ADAP in CAR-transduced T and NK cells, small molecule-based interference of interactions in the Fyn-ADAP-CBM pathway, and utilization of small hairpin interfering RNAs.

There are three major concerns with this approach that need to be addressed prior to blocking the interaction of Fyn and ADAP in clinical trials. (1) Cytokines such as IFN-γ are obligatory to clear certain types of malignancies. In

general, cytokines and chemokines play a central role in orchestrating a productive anti-cancer response. Therefore, strategies to reduce the production of inflammatory cytokines should not completely curtail overall cytokine production, which would likely negatively impact anti-tumor cytotoxicity. (2) Cytokines are required for CAR-T homeostasis and likely survival and persistence. While curtailing cytokine production will not eliminate their production entirely, it is unclear to what degree of innate cytokine needs are necessary to maintain CAR-T perseverance. (3) As discussed earlier, cytokines responsible for CRS likely come from two sources. The primary source is the CAR T cells themselves that initiate the first wave of proinflammatory cytokine production, which this approach should help control. However, the secondary wave of cytokines, such as increased IL-6 production, originate from myeloid cells in response to augmented cytokine signaling from CAR T cells and native effector lymphocytes. In theory, reducing the levels of proinflammatory cytokines generated by CAR T cells should help to contain both the primary as well as indirectly, the secondary waves of cytokine productions.

SUMMARY AND FUTURE DIRECTIONS

CAR-T immunotherapy has changed the landscape of cancer treatment. However, CRS occurs in two-thirds of patients, and in its worst form can lead to death. While tocilizumab can be effective in treating CRS, once it occurs, CRS development may lead to increased morbidity, hospitalization, and cost, and may limit the dose of CAR T cells that can be used clinically. And while current algorithms have enhanced identification and treatment for CRS to decrease mortality, using these pharmacological interventions pre-emptively has not yet been established as a standard of care. Therefore, knowledge of the signaling pathways that uniquely regulate anti-tumor cytotoxicity and inflammation is critical in identifying potential novel targets for containing CRS. Our recent work evaluating the FynADAP pathway provides an archetypical model to validate blocking these unique signaling pathways to contain cytokine production as one method to engineer a safer CAR-T cells. Successful translation of this and other engineered strategies to reduce CRS in this intrinsic manner is a compelling approach to this important problem.

AUTHOR CONTRIBUTIONS

MT, TK, and SM wrote and edited this manuscript.

FUNDING

This work was supported in part by NIH R01 AI102893 (SM) and NCI R01 CA179363 (SM and MT); HRHM Program of MACC Fund/Children's Hospital of Wisconsin (SM), Nicholas Family Foundation (SM); Gardetto Family (SM); MCW-Cancer Center-Large Seed Grant (SM and MT); MACC Fund/Children's Hospital of Wisconsin (MT and SM); Ann's Hope Melanoma Foundation (SM and MT); and Advancing a Healthier Wisconsin (SM).

ACKNOWLEDGMENTS

We thank Lucia Sammarco and her Lulu's Lemonade Stand for inspiration, motivation, and support. We thank Cassandra Longsine for help with manuscript preparation.

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