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Enhancing CAR-T cell efficacy in solid tumors by targeting the tumor microenvironment

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Chimeric antigen receptor (CAR)-T cell therapy has achieved successful outcomes against hematological malignancies and provided a new impetus for treating solid tumors. However, the efficacy of CAR-T cells for solid tumors remains unsatisfactory. The tumor microenvironment has an important role in interfering with and inhibiting the effector function of immune cells, among which upregulated inhibitory checkpoint receptors, soluble suppressive cytokines, altered chemokine expression profiles, aberrant vasculature, complicated stromal composition, hypoxia and abnormal tumor metabolism are major immunosuppressive mechanisms. In this review, we summarize the inhibitory factors that affect the function of CAR-T cells in tumor microenvironment and discuss approaches to improve CAR-T cell efficacy for solid tumor treatment by targeting those barriers.

Keywords: CAR-T; Tumor; Microenvironment

INTRODUCTION

Chimeric antigen receptor (CAR)-T cell therapy is a type of immunotherapy in which T cells are endowed with specific tumor-killing capability through genetic engineering via CARs. In recent years, CAR-T cells against CD19 have shown great clinical responses in treating B-cell hematologic malignancies.[1–4] In 2017, two CAR-T products targeting CD19, tisagenlecleucel (KYMRIAH) and axicabta-gene ciloleucel (YESCARTA), were approved by the FDA[5,6] In addition, CAR-T cells for a variety of solid malignancies have been developed and tested in recent years. To date, approximately 200 clinical trials of CAR-T cells targeting solid tumors have been launched worldwide, with most studies on antigens involving mesothelin (MSLN), ganglioside (GD2), glypican-3 (GPC3), human epidermis growth factor receptor 2 (HER2), carcinoembryonic antigen (CEA), epidermal growth factor receptor and its variants (epidermal growth factor receptor, EGFR/EGFRvIII), prostate-specific membrane antigen (PSMA), prostate stem cell antigen (PSCA) and claudin 18.2.[7] Despite benefits for anti-claudin 18.2 CAR-T cells (33% ORR)[8] in gastric and pancreatic cancers, results in other clinical trials were disappointing, with only a few patients in studies targeting MSLN,[9–11] GPC3[12] and HER2[13–15] achieving a partial response and no significant effects for GD2,[16] EGFRvIII,[17] CEA,[18–21] and PSMA[22] CAR-T therapies.

Solid tumors and blood tumors possess different physical and physiological characteristics, which render them susceptible to CAR-T cell therapy. Tumor cells are usually present in the blood and are thus easily captured by CAR-T cells; in contrast, solid tumors exist deep in the body, and it is difficult for T cells to access them.[23] Such a relatively concealed and concentrated location endows solid tumors with a microenvironment that is distinct from that of blood tumors and has an essential role in immune-resistance and tumor progression. The solid tumor microenvironment consists of the extracellular matrix (ECM), tumor vasculature, tumor cells, stromal cells (such as endothelial cells and fibroblasts) and immune cells, multiple molecules such as chemokines and cytokines, and an abnormal metabolic environment such as hypoxia (Fig. 1). Extracellular matrix stromal cells and the extracellular matrix form physical barriers against the entry of T cells, and the aberrant tumor-related vasculature, dysregulation of adhesion molecules, and mismatching of tumor-secreted chemokines and immune cell-expressed chemokine receptors cause more challenges for T cell migration to tumor lesions[24]. Tumor cells express ligands of inhibitory immune checkpoints such as programmed death-1 ligand (PD-L1/L2)[25] and recruit inhibitory immune cells such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs)[26,27] to interfere with the cytotoxic function of effector T cells. In addition, the microenvironmental conditions resulting from abnormal metabolism of tumors, which are usually characterized by elevated interstitial fluid pressure, a low pH, hypoxia, and a reduced bioenergetic status, also have important roles in influencing T cell activity.[28,29]

Based on these properties and immune-resistance mechanisms of the tumor microenvironment, strategies targeting the tumor microenvironment have been developed or are under investigation to improve the survival and efficacy of CAR-T cells for solid tumors. In this review, we comprehensively summarize and highlight the main challenges existing in the tumor microenvironment that impact CAR-T cell efficacy and how to enhance the efficacy of CAR-T cells for solid tumors by targeting the tumor microenvironment. Major aspects, including targeting immune checkpoints, targeting the chemokine-receptor network, targeting the tumor vasculature, targeting stromal cells and the ECM, targeting metabolism and hypoxia, and targeting immune

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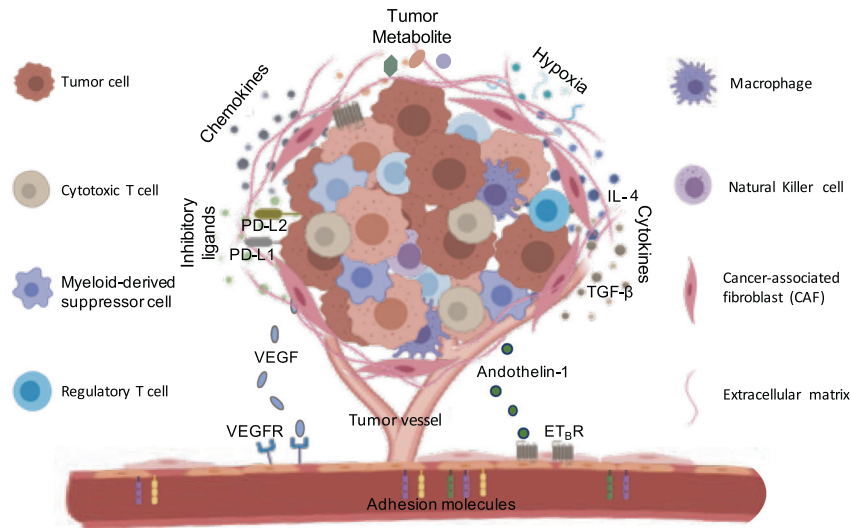
Fig. 1 Schematic diagram of the solid tumor microenvironment. The solid tumor microenvironment consists of the extracellular matrix, abnormal tumor vasculature, various cells, soluble molecules, and physiological and biochemical components produced by special tumor metabolism. The extracellular matrix together with the abnormal tumor vasculature form a physical barrier to T cell entry. Cells include tumor cells, effector immune cells, inhibitory immune cells, and stromal cells, such as cancer-associated fibroblasts (CAFs). Tumor cells usually express some inhibitory ligands or receptors, such as PD-L1/L2, to interfere with the function of T cells; other inhibitory immune cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory cells (Tregs), further inhibit the function of effector T cells. Soluble molecules such as chemokines, cytokines, and other factors act to regulate tumor vessels, such as vascular endothelial cell growth factor (VEGF) and andothelin-1. VEGF and andothelin-1 interact with VEGFR and endothelin B receptor (ET_BR) on tumor vessels, respectively, to promote angiogenesis or downregulate the expression of adhesion molecules. While some chemokines contribute to recruiting effector immune cells, others recruit inhibitory immune cells such as MDSCs and Tregs. Cytokines include effector cytokines (not indicated) and inhibitory cytokines such as IL-4 and TGF-β. Anomalous tumor metabolism produces a series of metabolites and induces hypoxia, which severely affects the survival and proliferation of T cells

suppressive cells and cytokines, are reviewed (Fig. 2). Due to the specific characteristics of the tumor microenvironment, the combination of these strategies and CAR-T cell therapy will be the major directions of future CAR-T cell therapy against solid tumors.

TARGETING IMMUNE CHECKPOINTS
Immune checkpoints refer to a series of costimulatory and coinhibitory receptors that are involved in the T cell response process. Under normal physiological conditions, immune checkpoints are crucial for the regulation and balance of the amplitude and quality of the T cell response, which contribute to the maintenance of self-tolerance and avoid overreaction-induced tissue damage. Nevertheless, it is clear that certain inhibitory immune checkpoints, such as PD-1 and CTLA-4 in T cells, can dampen T cell function.[30] The binding of PD-1 to its ligands (PD-L1 and PD-L2) results in suppression of CD28-mediated activation of the PI3K-Akt signaling pathway, thereby reducing T-cell activation, proliferation, and survival.[31] PD-L1 and PD-L2 are expressed on a variety of tumor and stromal cells.[32,33] In addition to binding PD-1, PD-L1 can interact with CD80 expressed on T cells as a second mechanism of T-cell suppression.[34] CTLA-4 is responsible for downregulating T cell proliferation and abrogating T cell activation by competitively interacting with the same ligands of CD28, CD80 (also known as B7.1) and CD86 (also known as B7.2) expressed on antigen-presenting cells.[31,35]

Such immune checkpoint-related resistance has also been observed in CAR-T cell therapy. Therefore, to improve the therapeutic efficacy of CAR-T cells in solid tumors, checkpoint blockades, such as antibodies targeting PD-1, PD-L1, and CTLA-4, have been concomitantly used with CAR-T cells in preclinical studies, increasing efficacy. In a breast cancer model, John and colleagues showed that the expression level of PD-1 on anti-Her-2

T cells increased following stimulation with Her-2 and PD-L1 double-positive tumor cells and that PD-1 blockade enhanced the in vitro and in vivo tumor-control efficacy of CAR-T cells.[36] Moon and colleagues demonstrated that the hypofunction of CAR TILs from mesothelin-positive tumors is associated with the expression of PD-1, LAG-3, TIM3, and 2B4. Furthermore, the cytotoxicity of CAR TILs was restored in vitro by an anti-PD-1 antibody.[37] The study by Gargett and colleagues demonstrated that PD-1 blockade improved both the survival and tumor-killing function of GD2 CAR-T cells following repeated antigen stimulation.[38] Cherkassky et al.[39] used an orthotopic mouse model of pleural mesothelioma and showed that CD28 CAR-T cells were more sensitive to PD-1-mediated exhaustion than 4-1BB CAR-T cells at low doses; PD-1 antibody treatment also restored the effector function of CD28 CAR-T cells. In addition to checkpoint blockades targeting PD-1, anti-PD-L1 antibodies have been evaluated. Using a liver metastasis mouse model, Burga et al.[40] found that liver myeloid-derived suppressor cells (L-MDSCs) express PD-L1 to suppress antitumor responses through the engagement of PD-1 on CAR-T cells and combined anti-CEA CAR-T cells with a PD-L1 antibody or with MDSC-depleting antibodies; both combination therapy strategies increased the efficacy of CAR-T cells. Tanoue and colleagues combined anti-HER2 CAR-T cell therapy with an oncolytic adenovirus expressing PD-L1 miniantibodies specifically secreted in the tumor microenvironment, enhancing the antitumor effects of CAR-T cells.[41] Moreover, the efficacy of combinational therapy of CAR-T cells with checkpoint blockades has been verified in the clinic for the treatment of refractory diffuse large B-cell lymphoma (DLBCL) and progressive lymphoma and neuroblastoma.[16,42]

In addition to the combination of checkpoint blockades with CAR-T cells through cell-extrinsic strategies, other studies have focused on investigating cell-intrinsic strategies, that is, directly introducing elements that block checkpoints in CAR-T

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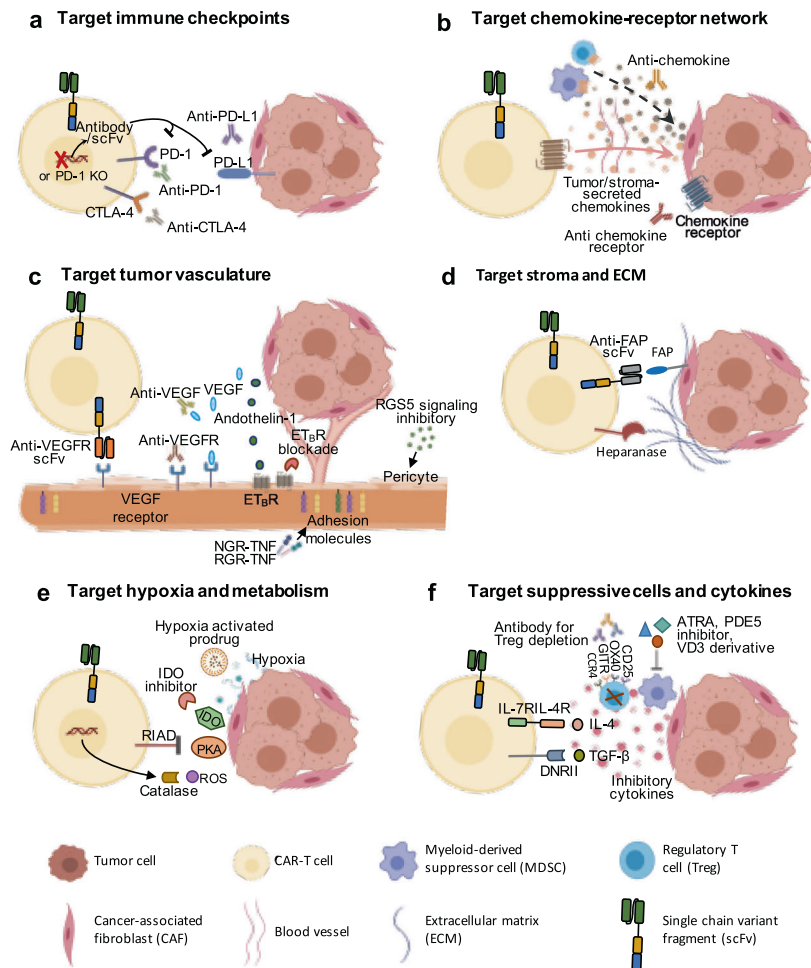
Fig. 2 Strategies to overcome barriers of CAR-T cells in the solid tumor microenvironment. **a** Combining CAR-T cells with PD-1, PD-L1, and CTLA-4 blockades, transducing genes of anti-PD-1/PD-L1/CTLA-4 antibodies/secreting scFvs into CAR-T cells, or knocking out checkpoint genes in CAR-T cells to block the inhibitory signal of CAR-T cells. **b** Expressing chemokine receptors in CAR-T cells to improve their tumor-directed trafficking or blocking chemokines and receptors expressed by tumor cells or stromal cells to inhibit the recruitment of inhibitory immune cells. **c** Disrupting the tumor vasculature with anti-VEGFR CAR or inhibiting tumor angiogenesis by blocking VEGF or promoting the infiltration of CAR-T cells by blocking ET_BR to inhibit its function on downregulation of adhesion molecules, inhibiting RGS5 signaling to normalize pericytes and tumor vasculature, and upregulating expression of adhesion molecules on a blood vessel by NGR-TNF and RGR-TNF. **d** Simultaneously targeting stromal cells and tumor cells by FAP and tumor antigen dual-targeting CAR or engineering CAR-T cells to secrete heparanase to degrade tumor extracellular matrix. **e** Inhibiting IDO, PKA, and ROS by inhibitors or RIAD-CAR-T cells (RIAD is a blocking peptide that inhibits the function of PKA) to eliminate their negative effects on CAR-T cells or engineering CAR-T cells to express enzymes such as catalase that clear ROS. **f** Depleting immune-inhibitory cells such as MDSCs and Tregs by antibodies or inhibitors and blocking inhibitory cytokines such as IL-4 and TGF-β with CAR-T cells expressing modified receptors. PD-1 Programmed death-1, PD-L1 Programmed death-ligand 1, CTLA-4 Cytotoxic T-lymphocyte-associate protein-4, VEGF Vascular endothelial cell growth factor, VEGFR Vascular endothelial cell growth factor receptor, ET_BR Endothelin B receptor, RGS5 regulator of G-protein signaling 5, NGR-TNF/RGR-TNF Vascular-targeting agents, coupling the angiogenic vessel-homing peptide with tumor necrosis factor-α, FAP Fibroblast activation protein, IDO Indoleamine 2,3 dioxygenase, PKA Protein kinase A, ROS Reactive oxygen species, RIAD Regulatory subunit I anchoring disruptor, a PKA-inhibiting peptide, DNRII Dominant-negative mutation of the TGF-β type II receptor, ATRA all-trans retinoic acid, PDE5 Phosphodiesterase-5, VD3 Vitamin D3

cells. Suarez et al.[43] developed human anti-carbonic anhydrase IX (CAIX) CAR-T cells engineered to secrete human anti-PD-L1 antibodies; carcinoma compared with anti-CAIX CAR-T cells alone, the armored CAR-T cells showed decreased expression of inhibitory receptors and increased tumor-control ability in a humanized mouse model of clear cell renal cell. Li et al.[44] and Rafiq et al.[45] developed anti-CD19 CAR-T cells able to secrete anti-PD-1-blocking scFv locally in the tumor site, which improved the capacity of CAR-T cells to eradicate human xenograft tumors or mouse tumors. As another example, Cherkassky et al.[39] engineered mesothelin-CAR-T cells with a truncated, dominant-negative PD-1 receptor without intracellular signaling domains; this receptor blocked PD-L1 or PD-L2 on tumor cells and significantly enhanced the survival and antitumor activity of

CAR-T cells in mesothelioma and lung cancer mouse models. Liu et al.[46] investigated a "switch receptor" strategy in which they fused the extracellular domain of PD-1 to the transmembrane and intracellular domains of CD28, and this PD-1:CD28 receptor enhanced CAR-T cell persistence and led to durable antitumor efficacy in mesothelioma and prostate cancer xenograft models. Additionally, Dozier et al.[47] compared the intrinsic and extrinsic strategies of checkpoint-targeted CAR-T cell therapies and found similar efficacy between the dominant-negative receptor and multiple doses of the anti-PD-1 antibody to overcome PD-L1-mediated T cell inhibition.

Genome-editing approaches are also being explored to reverse checkpoint-induced inhibitory signaling. Several groups have shown that knocking out PD-1 through CRISPR/Cas9 or TALEN

technology improves the cytotoxic activity and tumor clearance of CAR-T cells.[48–50] Ren et al. generated universal CAR-T cells by knocking out three genes, TCR, β-2 microglobulin, and PD-1, and demonstrated enhanced antitumor effector functions.[51,52]

Although several combination therapies with CAR-T cell checkpoint blockades are under clinical investigation, most of them target blood tumors,[53] whereas few clinical results for solid tumor treatment have been reported. Heczey et al.[16] used anti-GD2 CAR-T cells in combination with pembrolizumab-treated neuroblastoma but failed to reach a definitive conclusion because of the small number of clinical patients.[16] Adusumilli et al. investigated the efficacy of anti-mesothelin-CAR-T cells with PD-1 in patients with malignant pleural mesothelioma. After receiving cyclophosphamide preconditioning followed by a single dose of CAR-T cells and a subsequent anti-PD-1 agent (at least 3 doses), 8/11 mesothelioma patients showed a response, including complete metabolic responses in 2.[53] Nonetheless, the efficacy of combination therapy still needs to be explored in more clinical trials.

In addition to PD-1 and CTLA-4, other inhibitory receptors, such as TIM-3 and LAG-3, which are upregulated in T cells after chronic antigen stimulation,[54] have become popular targets for immune checkpoint blockade discovery. Some newly described inhibitory receptors, such as B7-H3, VISTA, and B7S1, which provide alternative pathways to modulate T cell responses,[55] may have great potential as targets and induce more robust antitumor responses. However, the safety of combination therapy of CAR-T cells and checkpoint blockades (CPB) needs to be considered, as combination CPB may have increasing on-target off-tumor toxicity, as most CAR-T cells targeting tumor-associated antigens are shared by normal tissues. Thus, strategies controlling the safety of CAR-T cells, such as reducing the dose, incorporating suicide genes, and finding optimal solid tumor antigen targets, need to be taken into consideration in future studies.

TARGETING CHEMOKINE-RECEPTOR NETWORK
The chemokine-receptor network has an important role in mediating cell migration, especially immune cells.[56] Previous studies have found that chemokines and their receptors can be dysregulated in the tumor microenvironment in multiple tumors, and the aberrant expression profile contributes to tumor proliferation, metastasis, and recruitment of inhibitory immune cells.[57–59] The mechanisms involved in chemokine-related tumor resistance to immune cells are comprehensive, including promoting angiogenesis and neovascularization,[60,61] recruiting inhibitory immune cells such as regulatory T cells and myeloid-derived suppressor cells,[62] preventing recruitment of effector lymphocytes[63] and inducing the generation of a hypoxic microenvironment.[64]

Given that chemokines and their receptors have crucial roles in tumor development and recruitment of immune cells, efforts have been made to target chemokine networks to promote antitumor immune responses. Peng and Nagarsheth et al. demonstrated that loss of Th1-type chemokines (such as CXCL9 and CXCL10) suppresses effector T cell and NK cell trafficking into tumors; cancer epigenetic reprogramming drugs such as EZH2 inhibitors and DNMT inhibitors also increase tumor Th1-type chemokine production and T cell trafficking into tumors and augment the therapeutic effects of checkpoint blockades and T cell therapy in preclinical models.[65–67] Enrichment of CXCL12 in the tumor microenvironment contributes to the migration of plasmacytoid DCs into tumors and tumor proliferation and metastasis.[57,68,69] Thus, antagonists of CXCL12 or its receptor CXCR4 prevent tumor metastasis and development in mouse models.[70–72] Combined immunotherapy with a CXCR4 antagonist (LY2510924) and an anti-PD-L1 antibody (durvalumab) has been evaluated in a phase 1a clinical study, showing acceptable safety and tolerability in patients with advanced refractory tumors.[73] As CCL2, CCL3, and CCL5 are involved in macrophage and neutrophil recruitment into

the tumor microenvironment and promote tumor metastasis, targeting these chemokines or their receptors prevent the accumulation of immunosuppressive myeloid cells in tumors and inhibit tumor metastasis and angiogenesis in mouse models of breast cancer, lung cancer, and ovarian cancer.[74–77] Other studies have demonstrated that CCR2 and CXCR2 blockades improve antitumor immunity and the chemotherapeutic response in pancreatic adenocarcinoma.[78] Clinical trials have been conducted to evaluate the efficacy of CCR2 blockade in treating patients with pancreatic cancer.[79] Although these strategies have not been applied in CAR-T cell therapy, combination therapy with chemokine-receptor-targeting drugs may be able to improve CAR-T cell efficacy in treating solid tumors. Further studies in preclinical models and patients are required to bring this combination approach into clinical applications.

Mismatching of tumor-secreted chemokines and T cell-expressed chemokine receptors is a major barrier for CAR-T cell trafficking to tumors. Thus, many strategies have been developed to improve the tumor-trafficking ability of CAR-T cells by engineering chemokine receptors into CAR-T cells. The first attempt was made by Kershaw and colleagues, who engineered T cells with chemokine-receptor CXCR2 and found that it enables T cells to effectively migrate toward tumor cells in vitro.[80] The same group also expressed murine CXCR2 in pmel-1 T cells and observed enhanced migration and antitumor ability in a mouse MC38 tumor model. In recent years, there have been many efforts to enhance the trafficking of T cells by modifying CAR-T cells with chemokine receptors. Di Stasi et al.[81] expressed CCR4 in CD30-targeted CAR-T cells, and these CCR4-modified CAR-T cells showed improved in vitro migration toward CCL17-secreted Hodgkin lymphoma and enhanced antitumor activity in a xenograft tumor model. Craddock and colleagues[82] showed that CCR2b-expressing GD2 CAR-T cells exhibit increased migration efficiency toward CCL2 produced by neuroblastoma cells. A similar observation was also made for CCR2b-expressing mesothelin-CAR T-cells in a malignant pleural mesothelioma mouse model.[10] Jin et al.[83] found that ionizing radiation enhances CXCL8 secretion by tumors and that transduction of CXCR1 or CXCR2 into CAR-T cells enhances T-cell trafficking and efficacy in glioblastoma, ovarian and pancreatic tumor models. Our group showed that several CXCR2 ligands are highly expressed in hepatocellular carcinoma tumors and cells and that CXCR2 transduction enhances the in vitro and in vivo migration of Glypican-3-directed CAR-T cells, improving antitumor activity in a xenograft tumor model.[84] In addition to chemokine receptors, chemokines that are related to T-cell zone formation and maintenance in lymphoid organs, such as IL-7 and CCL19, are capable of enhancing CAR-T cell trafficking and survival.[85]

Collectively, these preclinical findings indicate that the tumor-trafficking ability of CAR-T cells can be enhanced through an engineered expression of tumor-associated chemokine receptors; regardless, the efficacy in patients requires testing in clinical trials. A possible challenge lies in the heterogeneity of tumor-enriched chemokines in different tumor types and different individuals. Thus, further insight into the chemokine expression profile of different tumor types and understanding of the mechanisms that restrict the trafficking of T cells to the tumor microenvironment may help in the development of more effective strategies to further increase the localization and persistence of T cells.[24] Therefore, targeting other molecules in the tumor microenvironment, such as normalizing tumor vessels, destroying the extracellular matrix, or eliminating stromal cells, are potential methods to optimize the efficacy of CAR-T cells against solid tumors.

TARGETING TUMOR VASCULATURE
Aberrant vasculature has an important role in blocking the infiltration of T cells into solid tumors, and several major characteristics of tumor vasculature have been well studied. First,

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one important vascular cell type, the pericyte, can be absent or loosely attached, leading to leaky vessels and irregular blood flow, which interfere with the trafficking of T cells within the tumor mass.[86] Second, endothelial cells (ECs) may acquire aberrant morphology, such as upregulated endothelin B receptor (ET_BR), which can interact with tumor-secreted endothelin-1 and inhibit the expression and clustering of ICAM-1 on ECs, resulting in inefficient T-cell adhesion.[87] Third, "EC anergy", whereby over-expressed angiogenic factors such as vascular EC growth factors (VEGFs) and fibroblast growth factors (FGFs) cause downregulation of adhesion molecules such as ICAM-1 and 2, VCAM-1 and CD34 on ECs, impedes T cell infiltration into tumors.

Based on these findings, a number of studies have focused on reversing poor T cell infiltration by overcoming these barriers. Several groups have reported that hypoxia-induced expression of the regulator of G-protein signaling 5 (RGS5) is involved in abnormal pericyte maturation and tumor vasculature development.[88,89] Thus, RGS5 and related signaling pathways[90] may serve as promising targets for promoting tumor infiltration and eradiation by CAR-T cells. By targeting abnormally expressed ET_BR, Buckanovich et al.[87] found that ET_BR blockade of BQ-788 was able to enhance T-cell infiltration into tumors, promoting a tumor response to ineffective immunotherapies, such as vaccines. Hence, ET_BR may serve as another target for enhancing CAR-T cell efficacy in treating solid tumors. Another approach is to target the VEGF/VEGFR pathway. Li and colleagues showed that VEGF blockade enhanced the efficacy of GM-CSF-secreting tumor immunotherapy and prolonged the survival of tumor-bearing mice, which correlated with an increased number of tumor-infiltrating T cells.[91] Shrimali et al.[92] also found that anti-VEGF antibodies significantly increased the infiltration of adoptively transferred cells (ATCs) into tumors and improved the efficacy of ATC-based immunotherapy in a murine cancer model. Huang et al.[93] demonstrated that low doses of an anti-VEGF receptor 2 antibody caused tumor vessel normalization and enhanced the effect of vaccine therapy. Studies have also developed CAR-T cells targeting VEGFR1 and VEGFR-2,[94–96] and these CAR-T cells exhibited antitumor and antiangiogenic abilities in several mouse models. CAR-T cells targeting VEGFR-2 have been tested clinically, and of 24 patients treated, only 1 had a partial response (NCT01218867), which indicates that the efficacy of VEGFR-targeted CAR is still limited.

In other studies, prostate-specific membrane antigen (PSMA),[97] αvβ3 integrin,[98] TEM8,[99] EIIIB, a splice variant of fibronectin,[100] or CLEC14A[101] was targeted. All these markers are reported to be overexpressed in the vasculature of many solid human cancers. Although all CAR-T cells targeting these markers showed tumor inhibition in mouse models, toxicity has also been reported when targeting some markers.[94,102] Thus, the toxicity of the recognition of low levels of VEGFR-2 on normal vessels may be an important issue with these CAR-T cells.

Other molecules or drugs, such as NGR-TNF[103,104] and RGR-TNF,[105] also have the potential to be applied in combination therapy with CAR-T cells. NGR-TNF and RGR-TNF are vascular-targeting agents that couple an angiogenic vessel-homing peptide with TNF-α and appear to contribute to upregulation of T cell trafficking-related adhesion molecules and chemokines in the tumor microenvironment,[106] activation of tumor-associated ECs, and tumor vessel stabilization, favoring T cell extravasation and proliferation,[105,107,108] increasing the therapeutic efficacy of tumor vaccines and adoptive immunotherapies.[106,109,110] More-over, a phase 2 study of NGR-TNF combined with doxorubicin reported improved progression-free survival and overall survival in relapsed ovarian cancer patients.[111]

Taken together, directly targeting overexpressed markers on the tumor vasculature by CAR or a combination of drugs or reagents that induce tumor vessel normalization has the potential to enhance tumor infiltration of CAR-T cells and improve their antitumor efficacy in solid tumors. Nonetheless, targeting a single antigen may not achieve significant improvement, as indicated by limited clinical studies targeting VEGFR-2. Instead, dual-targeted CAR-T cells with the specificity of both tumor antigens and tumor vasculature markers may be a more promising strategy. It is also necessary to identify antigens that are specifically expressed in the tumor vasculature to exclude antigen-related toxicity.

TARGETING STROMAL CELLS AND THE ECM
Cancer-associated stromal cells (CASCs) promote tumor development through multiple mechanisms, including secreting growth factors, cytokines, and chemokines that promote tumor growth, metastasis, and angiogenesis.[112] In addition to blocking these cytokines or targeting these cytokines and chemokines, another strategy for enhancing CAR-T cell efficacy is directly targeting CASCs in the tumor microenvironment.

Cancer-associated fibroblasts (CAFs) are major components of the tumor-associated stroma, and CXCL12 secreted by CAFs has an important role in masking tumor cells from immune attack and excluding T cells from the tumor microenvironment. Thus, strategies have been developed to eradicate CAFs. Fibroblast activation protein (FAP) is a type 2 dipeptidyl protease that is highly expressed on CAFs in over 90% of solid tumors,[113,114] and it is therefore considered a potential target for CAR-T cell therapy.[115] Tran et al. reported that anti-FAP-CAR-T cells were specifically activated by FAP protein- or FAP-expressing cells and produced effector cytokines in vitro. However, FAP-CAR-T cells mediated limited antitumor effects in tumor-bearing mice and induced severe cachexia and lethal bone toxicities in two mouse strains, which resulted from the expression of FAP on multipotent bone marrow stromal cells (BMSCs).[116] FAP-related cachexia and anemia toxicity have also been reported by Roberts and colleagues.[117] Two other groups evaluated the efficacy of FAP-specific CAR-T cells in xenograft human tumor models. Kakarla and colleagues used a different scFv in the FAP-CAR construct, and these CAR-T cells recognized and lysed FAP-positive target cells efficiently in vitro. FAP-positive stromal cells were also significantly reduced and tumor growth decreased in an established A549 lung cancer model. In addition, combining treatment of FAP-CAR-T cells with T cells targeting EphA2 on A549 cells further enhanced overall antitumor activity and mouse survival.[118] Similar results were reported by Schuberth and colleagues: anti-FAP-CAR-T cells showed a cytokine response and tumor-killing activity in vitro and inhibited the tumor growth in the FAP-positive malignant pleural mesothelioma mouse model.[119] The limitation of these two studies is that they used mouse models of human cancer, and the on-target/off-tumor toxicity of FAP-CAR-T cells could not be addressed. However, another study by Wang and colleagues showed that by using scFv from anti-FAP antibody 73.3, anti-mouse FAP-CAR-T cells showed minimal off-tumor toxicity while exhibiting tumor-control activity in multiple types of subcuta-neously transplanted tumors. The antitumor effects of FAP-CAR-T cells were also augmented by multiple injections of CAR-T cells or by combination with other therapies.[120] The same group then demonstrated that FAP-CAR-T cells eradicated tumors through immune-independent mechanisms, which included decreasing tumor vascular density, inhibiting tumor stromagenesis, and disrupting tumor cell spatial orientation.[121] Based on these results, the efficacy of CAR-T cells targeting FAP is reassuring, and the toxicity may be related to the specificity and affinity of the scFv. In addition to CAR-T cell therapy, other approaches have been developed to target FAP, such as low molecular weight inhibitors, antibodies, immunoliposomes, and vaccines.[115]

Beyond targeting FAP on CAFs, Feig and colleagues developed another strategy targeting CAF-secreted CXCL12 and showed that combining a CXCR4 (receptor of CXCL12) inhibitor with an anti-PD-L1 antibody-induced rapid T-cell accumulation within tumors and greatly diminished cancer cells in a checkpoint

blockade-resistant pancreatic ductal adenocarcinoma (PDA) mouse model.[122]

Heparanase (HPSE), which degrades heparan sulfate proteoglycan, a component of the extracellular matrix (ECM), is another potential target. Caruana and colleagues reported that engineered HPSE-expressing CAR-T cells acquired an improved capacity to degrade the ECM, which promoted tumor T cell infiltration and antitumor activity.[123] Given the potential for the antitumor effects of stroma-targeting CAR-T cells, future rational immunotherapies might combine antistromal approaches with antitumor CAR-T cells. For example, dual-target CAR-T cells for FAP and tumor antigen or CAR-T cells carrying enzymes that degrade the ECM may result in enhanced killing efficacy in solid tumors, though potential toxicity in normal tissues must be excluded. There are two clinical trials to date that investigated the efficacy and safety of anti-FAP-CAR-T cells (NCT01722149 and NCT03932565). One of them (NCT01722149) reported that intrapleural administration of anti-FAP-CAR T-cells was well tolerated in three patients, without any evidence of treatment-related toxicity, indicating that FAP may be a safe target for CAR-T treatment; however, efficacy still needs to be explored in more patients.

TARGETING METABOLISM AND HYPOXIA

Tumor cells within the tumor microenvironment can inhibit effector lymphocytes by depriving them of nutrients, such as glucose, lipids, and amino acids, and creating a challenging environment characterized by hypoxia, acidic pH, and high levels of immunosuppressive metabolites.[28,124] Tryptophan is an essential amino acid for T cell metabolism, but tumor cells can upregulate expression of indoleamine 2,3 dioxygenase (IDO), which catalyzes the degradation of tryptophan to 3-hydroxyanthranilic acid (3-HAA) and kynurenine, contributing to T cell suppression and a poor clinical prognosis.[125–128] Liu and colleagues demonstrated that small-molecule IDO inhibitors promote T cell and NK cell growth, increase IFN-gamma production, and reduce conversion to Treg-like cells in vitro as well as inhibit tumor growth in a lymphocyte-dependent manner in vivo.[129] Several other groups have reported that IDO has an important role in impairing the antitumor efficacy of checkpoint blockades, such as CTLA-4 and PD-1/PD-L1 strategies. Indeed, combination therapy of checkpoint blockades with IDO inhibitors or knockout of IDO led to enhanced infiltration of tumor-specific effector T cells and increased the tumor-control effect and overall survival of mice in murine melanoma models or in a xenograft human glioblastoma model.[130–132]

Ninomiya and colleagues have reported that the response to CD19 CAR-T cell therapy correlates with the IDO expression level in tumor cells; they also found that CD19 CAR-T cells did not respond to IDO-positive tumors but did respond to IDO-negative tumors. The underlying mechanisms may include tryptophan accumulation-induced inhibition of interleukin expansion, cytokine secretion, proliferation, and persistence of CAR-T cells. These authors also showed that pretreating tumor-bearing mice with fludarabine and cyclophosphamide decreased the expression of IDO and enhanced the efficacy of CAR-T cells in a xenograft lymphoma model.[133]

Hypoxia, which results from excessive oxygen consumption due to rapid tumor cell proliferation and inadequate oxygen supply due to the chaotic tumor microvasculature, is another factor of the tumor microenvironment and reduces the efficacy of immunotherapies. Hypoxia has been proven to impair the antitumor immune response through different mechanisms, mainly via hypoxia-inducible factor (HIF) proteins, including HIF-1α, HIF-2α, and HIF-3α. The mechanisms include upregulating expression of PD-L1 on tumor cells and myeloid-derived suppressor cells (MDSCs) to inhibit T cell function,[134–136] inducing expression of the immune checkpoint V-domain Ig suppressor of T cell activation (VISTA) on tumoral MDSCs to suppress T cell activity,[137] upregulating expression of CD47 on tumor cells to block macrophage-mediated phagocytosis,[138,139] activating autophagy to reduce the susceptibility of tumor cells to CTL- and NK-mediated killing,[140,141] promoting the expression of nonclassical MHC-I molecules, HLA-G and HLA-E, to block the function of various immune cells,[142–144] and inducing the production of immunosuppressive metabolites.[145]

Based on these findings, many drugs targeting hypoxia have been developed, including hypoxia-activated prodrugs such as TH-302 and EO9, which are HIF-modulating drugs that can affect the expression and translation of HIF, induce its degradation, or inhibit their transcriptional and DNA-binding activities[146,147] Although these drugs have not been tested in combination therapies with CAR-T cells, it is worthwhile to attempt to modify CAR-T cells with hypoxia-targeting molecules or combine CAR-T cells with hypoxia-targeting molecules for solid tumor treatment.

In addition, two other immunosuppressive metabolites in the tumor microenvironment, prostaglandin E2 (PGE2) and adenosine, can inhibit the proliferation and functional activity of T cells. These two molecules activate downstream protein kinase A (PKA) and further inhibit T cell proliferation.[148,149] Thus, Newick and colleagues developed a type of novel CAR-T cell that coexpresses mesothelin-directed CAR with a PKA-inhibiting peptide, regulatory subunit I anchoring disruptor (RIAD). RIAD interferes with the association between PKA and the ezrin protein, disrupting PKA anchorage to lipid rafts. The results showed that CAR-RIAD T-cells exhibit high infiltration and good persistence, cytokine release, and antitumor activity in vivo.[150]

Tumor cells can upregulate ROS production in the tumor microenvironment to promote their proliferation by inhibiting immune responses. Therefore, to enhance the persistence and antitumor effect of CAR-T cells, Ligtenberg and colleagues introduced catalase (CAT) into CAR-T cells to increase their antioxidative capacity via H_2O_2 metabolism. This CAT modification reduced oxidative stress in the tumor milieu and enhanced the antitumor effect of CAR-T cells, and the CAT-CAR-T cells exerted substantial bystander protection against bystander NK cells.[151]

These studies provide clear evidence that targeting metabolic pathways or certain metabolites in the tumor microenvironment, such as IDO, PKA, ROS, and hypoxia, is a feasible approach to improve the therapeutic efficacy of immunotherapies such as CAR-T cell therapy. Although some preclinical studies have proven the efficacy of these strategies, such as combining IDO inhibitors with CAR-T cells, CAR-RIAD T-cells, or CAT-modified CAR-T cells, they have not been tested in any clinical trial. In general, a better understanding of tumor metabolism in the future may provide new strategies to improve the efficacy of CAR-T cells.

TARGETING IMMUNE SUPPRESSIVE CELLS AND CYTOKINES

Although the immune system has complicated regulatory mechanisms to balance the activation and suppression of immune cells, tumor cells can utilize negative regulatory mechanisms to inhibit the antitumor activity of the immune system, and there are many immunosuppressive cells and cytokines within the tumor microenvironment. Myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) are two major types of immunosuppressive cells that accumulate in many cancers.[26,152,153] These cells exert their inhibitory functions by secreting negative immune-regulatory cytokines, such as IL-10 and TGF-β.

Several approaches have been applied in preclinical and clinical studies to target these immunosuppressive cells and cytokines. Studies found that all-trans retinoic acid (ATRA) is able to reduce MDSCs in cancer patients, as it induces immature myeloid cell differentiation into mature dendritic cells and is able to stimulate T cells.[154,155] Kusmartsev and colleagues reported that ATRA administration induced a reduction in MDSCs and improved CD4-

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and CD8-mediated tumor-specific immune responses and the effect of vaccination in tumor-bearing mice.[156] Mirza and colleagues also reported that ATRA treatment resulted in significant MDSC reduction and DC expansion in patients with renal cell carcinoma.[157] Since then, many other studies have explored the therapeutic efficacy of ATRA in treating various solid tumors, including lung, breast, cervical cancer, and nonsmall-cell lung cancer.[158] Phosphodiesterase-5 (PDE5) inhibitors can revert MDSC-mediated immunosuppression, increase tumor-infiltrating T cells, and induce an increased immune response in patients with myeloma and head and neck squamous cell carcinoma (HNSCC).[159,160] In addition, vitamin D3 derivatives have been shown to reduce MDSCs in the tumor microenvironment and enhance the antitumor activity of T cells.[161–163] MDSCs have been proven to impair T cell trafficking through downregulation of CD62 L on CD4 and CD8 T cells and chemokine nitration;[164,165] thus, MDSC-targeting therapeutic strategies are expected to significantly improve T cell trafficking and the efficacy of adoptive immunotherapies.

Depletion of Tregs is another effective approach to enhance antitumor immunity that has been achieved in different ways. Shimizu and colleagues showed that depleting CD25$^+$ Treg cells in tumor-bearing mice by using anti-CD25 monoclonal antibody administration increased tumor-infiltrating CD8$^+$ T cells and enhanced tumor eradication.[166] Other molecules expressed on Tregs, such as CTLA-4, OX40, GITR, and CCR4, have also been developed as targets for Treg depletion. Several groups demonstrated that an anti-CTLA-4 antibody was able to induce antibody-dependent cell-mediated cytotoxicity (ADCC) and deplete Treg cells in tumor tissues, thereby enhancing tumor-control activity in mice.[167–169] A clinical study showed that ipilimumab treatment significantly reduced FOXP3$^+$ Treg cells in tumor tissues, particularly in clinical responders among melanoma patients.[170] In addition to CTLA-4, antibodies against CD25, OX40, GITR, and CCR4-depleted Treg cells specifically in an ADCC-dependent manner and to enhanced antitumor immune responses.[167,171–173] In addition, Litzinger and colleagues showed that a fusion protein of interleukin 2 (IL-2) and diphtheria toxin could be used to reduce Treg cells and enhance vaccine-mediated T-cell immunity in mice.[174]

Other strategies use immunosuppressive cytokines as targets, and some approaches have been utilized to modify CAR-T cells, either by limiting suppressive signaling induced by cytokines or converting suppressive signals into activating signals. Foster and colleagues engineered Epstein–Barr virus-specific T cells via a dominant-negative mutation of the TGF-β type II receptor (DNRII), and the DNRII-expressing CTLs showed resistance to the inhibitory effects of tumor-derived TGF-β both in vitro and in vivo as well as enhanced antitumor activity.[175] By using DNR-transduced papillomavirus-E7-specific T cells, the same group also demonstrated that genetic modification with DNR did not lead to a spontaneous proliferation of CTLs in vivo.[176] Zhang and colleagues reported that murine T cells that were engineered to express TGF-β-DNRII or soluble TGF-β receptors resisted TGF-β-induced inhibitory signaling both in vitro and in vivo and had markedly improved killing efficacy against B16 tumors.[177] By employing the same strategy, Kloss and colleagues generated dominant-negative TGF-βRII-modified CAR-T cells specific for prostate-specific membrane antigen (PSMA), and these CAR-T cells exhibited increased proliferation, enhanced cytokine secretion, resistance to exhaustion, long-term in vivo persistence, and the induction of tumor eradication in aggressive human prostate cancer mouse models; this approach is now under investigation in phase I clinical trial (NCT03089203).[178] Other studies have reported that TGF-β-insensitive CD8$^+$ T cells induce robust tumor-specific T cell responses in vitro and cause superior tumor regression and prolong mouse survival in vivo.[179,180]

The inhibitory Th2 cytokine IL-4 is elevated levels in many tumors, including Hodgkin's lymphoma and breast, prostate, and pancreatic cancer. IL-4 has been reported to favor tumor growth by inhibiting tumor-directed Th1-polarized effector T-cell responses.[181–185] To reverse the inhibitory effects induced by IL-4, Vera, Leen et al. fused the IL-4 receptor exodomain to the signaling endodomain of the IL-7 receptor; thus, binding of the IL-4 receptor to IL-4, which is enriched in the tumor microenvironment, transmitted therapeutic signals through an IL-7-induced downstream pathway. The researchers expressed the chimeric receptor in EBV-directed T cells and found enhanced proliferation and superior antitumor activity of the T cells in mouse models.[186] They also applied this strategy for prostate stem cell antigen (PSCA)-directed CAR-T cells and found similar results in a xenograft pancreatic tumor model.[187]

In brief, inhibitory immune cells or cytokines that are enriched in the tumor microenvironment may serve as additional and promising targets for improving the efficacy of CAR-T cells. Among all the strategies mentioned above, CAR-T cells targeting TGF-β are considered to be the most promising. The efficacy of TGF-β-dominant-negative receptor-modified CAR-T cells or combining antibodies targeting soluble TGF-β with CAR-T cells has been tested in several clinical trials (NCT00889954, NCT04227275, NCT03089203, NCT03198546), but the efficacy has not yet been reported.

CONCLUSION

Improving the efficacy of CAR-T cells in treating solid tumors is a matter of great urgency. In contrast to hematologic malignancies, solid tumors exist in an intricate microenvironment in which a variety of factors, including inhibitory receptors/cells/cytokines, physical barriers, hypoxia, and tumor metabolism, suppress immune responses and promote tumor growth. Innovative strategies have been developed to overcome these barriers. Coadministration of CAR-T cells with checkpoint blockades targeting PD-1, PD-L1, and CTLA-4, the most widely studied strategy, has shown superior tumor-control efficacy in some cancer patients. Nevertheless, the overall response rates are limited due to variations in the expression levels of these inhibitory receptors/ligands. Predictive biomarkers such as the tumor mutation load should be of great help in selecting patients who might respond to treatment. Moreover, due to the discovery of other inhibitory receptors, such as TIM-3, LAG-3, and B7-H3,[55] different combinations of immune checkpoint blockades with CAR-T cells may achieve better efficacy. In addition to inhibitory receptors, suppressive cells such as MDSCs and Tregs or inhibitory cytokines (TGF-β/IL-10) need to be considered. Given that anti-TGF-β antibodies show promising antitumor efficacy in clinical settings, the combination of TGF-β-targeting strategies with CAR-T cells has great prospects, and such a combination has been tested in clinical trials. Chemokine-receptor networks and other physical barriers also have great potential as targets to enhance the trafficking and tumor infiltration of CAR-T cells, but such combination therapies still need to be assessed in clinical studies. Intratumoral administration offers an additional option to overcome the trafficking barriers of CAR-T cells in solid tumors. Targeting hypoxia and metabolism is another potential strategy to address the hostile tumor microenvironment and enhance the therapeutic efficacy of CAR-T cell therapy.

Taken together, the solid tumor microenvironment is highly complicated and heterogeneous, and a single therapy may have limited clinical benefit. CAR-T cells possess the capacity to directly eliminate tumor cells. To overcome various obstacles in the tumor microenvironment, combination with other strategies has great potential to produce excellent tumor-killing results.

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AUTHOR CONTRIBUTIONS

G.L. and X.L. conceptualized this review, G.L. wrote the manuscript, and W.R., X.Z., and X.L. edited and revised the manuscript. All authors approved the manuscript to be published.

ADDITIONAL INFORMATION

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