Using adoptive cellular therapy for localized protein secretion

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Abstract

Redirection of T cell cytotoxicity by the CAR structure may not be sufficient for optimal anti-tumor function in the patient tumor microenvironment. Co-modifying CAR T cells to secrete different classes of proteins can be used to optimize CAR T cell function, overcome suppressive signals and/or alter the tumor microenvironment milieu. These modifications aim to improve initial responses to therapy and enhance the durability of response. Furthermore, CAR T cells can deliver these molecules locally to the tumor microenvironment, avoiding systemic distribution. This approach has been tested in preclinical models using a variety of different classes of agonistic and antagonistic proteins and clinical trials are currently underway to assess efficacy in patients.

Keywords

Adoptive cell therapy; CAR T cells; immunotherapy; cellular immunotherapy; tumor microenvironment

Introduction

CAR T cells have demonstrated impressive clinical response against certain types of leukemia and lymphoma[12]. However, despite the high rates of complete response induced by CAR T cells in these malignancies, obstacles remain to improve and expand CAR T cell efficacy to other cancer types, overcome toxicities related to therapy, and increase the durability response[13]. Many of these challenges to effective therapy can be addressed by co-modifying CAR T cells to secrete different classes of proteins. These modifications allow CAR T cells to be morphed into living "micropharmacies" capable of producing proteins

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that, when secreted, can work in an autocrine or paracrine manner to improve CAR T cell phenotypes and/or function in the tumor microenvironment (TME)[4]. Expression of genes alongside the CAR construct allows for the expression of proteins, even those not naturally made by T cells. Also referred to as "armored" CAR T cells, these cells can secrete proteins that antagonize suppressive signals in the TME. Alternatively, armored CAR T cells can secrete agonists that can activate and improve of the function of CAR T cells or other proinflammatory cells in the TME.

Localized secretion of proteins by CAR T cells at the tumor site is a major advantage associated with this approach. Systemic infusion of proinflammatory molecules can lead to significant toxicities. CAR T cells are engineered to traffic to and proliferate at site of tumor and result in localized delivery of these immunomodulatory agents to the TME.

As a mode of drug delivery, CAR T cells have the ideal physiological functions and pharmacokinetics. These cells precisely localize to the tumor area, activate, and then expand and contract with tumor burden. Proteins engineered to be secreted by CAR T cells are delivered to the tumor area in concentrations correlated with tumor size. These cells also have structurally large volume/surface area, allowing for the expression of more therapeutic cargo compared to exosomes or nanoparticles[4]. Therefore, CAR T cell-mediated drug delivery of therapeutic agents allows for potentially cheaper and more efficacious combination immunotherapies.

Diverse classes of proteins can be secreted by armored CAR T cells for a variety of functions. This review discusses the different classes and their intended mechanism to modulate the TME.

Cytokines

Inhibitory signals in the TME create immunosuppressive conditions which arrest endogenous immune functions. Armored CAR T cells co-modified to secrete cytokines have demonstrated promise in preclinical studies in hematological and solid tumors. CAR T cells expressing cytokines are also referred to as T cells redirected for universal cytokine killing (TRUCKS)[5]. The cytokines secreted by these cells are usually those not endogenously expressed by T cells but are required by T cells for optimal function. These cytokines can stimulate the CAR T cells that secrete them in an autocrine fashion, leading to enhanced T cell proliferation, survival, and anti-tumor responses. The localized delivery of the immunostimulatory cytokines by CAR T cells can also promote the recruitment of proinflammatory endogenous immune cells that can aid in combating antigen escape to CAR T cell therapy (Figure 1).

IL-12

Interleukin (IL)-12 is a pleiotropic, pro-inflammatory cytokine produced by dendritic cell, macrophages, neutrophils and B cells and plays a vital role in the immune response to microbial pathogens through T and NK cell-mediated immunity[6]. In addition to increasing the cytotoxic and cytokine producing capacity of T and NK cells, IL-12 can inhibit or reprogram suppressive cells in the TME[6]. While IL-12 has demonstrated potent anti-tumor

effects in preclinical models of cancer, systemic infusion of recombinant IL-12 in patients has lethal dose-limiting toxicities and minimal efficacy at tolerated doses[789]. Localized delivery of the IL-12 to the TME by CAR T cells is a promising approach as it may allow for the effective anti-tumor effects of IL-12 therapy but with a reduction in systemic exposure and toxicities. Furthermore, IL-12 expression by armored CAR T cells can be engineered to secrete inducible or lower levels of IL-12, further decreasing the risk of systemic toxicities[1011]. Localized IL-12 secretion by CAR T cells has been demonstrated to work in an autocrine manner to enhance CAR T cell function and in a paracrine manner to enhance activation of local immune cells while reducing immunosuppression in the TME.

CAR T cells that secrete IL-12 benefit from this modification in a number of ways. In a "conditioning-free" syngeneic model of CAR T cell therapy, armored anti-CD19 CAR T cells that constitutively express IL-12 demonstrated increased cytotoxicity against tumor cells compared to parental CAR T cells through increased expression of interferon gamma (IFN-γ, granzyme B, and perforin[12]. These CAR T cells also exhibited enhanced expansion and resistance to regulatory T cell (Treg)-mediated suppression[12]. *In vivo*, IL-12-secreting armored CAR T cells had superior engraftment and eradicated established systemic hCD19+ tumor cells[12]. In this model, autocrine IL-12 stimulation of both CD4+ and CD8+ CAR T cells was needed for the elimination of tumor. Subsequent studies of IL-12-sereting CAR T cells in a solid tumor model further demonstrated enhanced CAR T cell proliferation and persistence, as well as higher systemic IFN-γ levels and increased anti-tumor efficacy in tumor-bearing mice as compared to parental CAR T cells[1314]. Furthermore, the IL-12 armored CAR T cell were more resistant to programmed death receptor ligand 1 (PD-L1) induced dysfunction[14] and exhibited an enhanced pro-inflammatory gene expression profile[15]. Dramatic expansion of CAR T cells after IL-12 expression led to the ability to generate CAR T cells from small numbers of cells isolated from umbilical cord blood samples[16]. These armored CAR T cells retained a unique central-memory effector phenotype, exhibited increased anti-tumor efficacy *in vitro*, and significantly enhanced the survival of CD19+ tumor-bearing mice[16].

IL-12 secreted by CAR T cells can also enhance the proliferation of endogenous T cells in the TME. In a xenograft mouse model of gastric cancer, CAR T cells directed against ICAM-1 were co-modified to express IL-12 downstream of the NF-κB-NFAT promoter[17]. Inducible IL-12 expression by CAR T cells resulted in higher levels of IFN-γ and TNFα and enhanced reduction of tumor burden as compared to parental CAR T cells. Analysis of blood from these mice revealed significant expansion of CD3+ T cells in sample groups treated with IL-12-secreting CAR T cells as compared to parental CAR T cells[17]. Interestingly, this expansion was not limited to transduced CAR T cells, as the total percentage of T cells was increased in these mice, suggesting paracrine effects of IL-12 on all T cells[17].

IL-12 locally secreted by CAR T cells can have effects on the cells of the TME. In syngeneic mouse models of ovarian carcinoma, IL-12 secreting armored CAR T cells mediated the depletion of tumor associated macrophages[14]. Carcinoembryonic antigen [18]-targeting CAR T cells modified to secrete IL-12 demonstrated increased penetration of metastatic tumors such as brain and inner organ metastases. These cells recruited and

activated innate immune cells such as macrophages in mice xenografted with CEA-positive colon cancer cell lines[19]. In this model, macrophage numbers increased after treatment and were shown to be critical in the anti-tumor response against antigen positive and negative tumors[19]. IL-12 can also decrease or reprogram suppressive cells in the TME. VEGFR-2-targeted CAR T cells secreting IL-12 reduced immunosuppression in the TME by decreasing myeloid suppressor cell subsets expressing VEGFR-2[20]. These armored CAR T cells penetrated solid tumors, proliferated, and persisted for extended periods in multiple mouse models of well-vascularized solid cancers and increased the anti-tumor effects of endogenous immune cells expressing IL-12 receptors[20]. Finally, Glypcian-3-specific T cells modified to express IL-12 in an inducible manner had superior anti-tumor efficacy in immunocompetent hosts without the need for preconditioning and decreased infiltration of Treg in established tumors[21].

The success of these preclinical studies has led to the clinical translation of IL-12 secreting CAR T cells (Table 1) and supported the commencement of clinical trials testing the efficacy of MUC16ecto-targeting, IL-12 armored CAR T cells in patients with ovarian cancer[18] (NCT02498912). Another study is testing Nectin/FAP-targeted CAR T cells expressing IL-7, CCL19, or IL-12 in Nectin4-positive advanced malignant solid tumors (NCT03932565). It is important to note that given the toxicities associated with prior IL-12 therapies, the clinical translation of these CAR T cells is not without danger. For example, a clinical trial testing localized delivery of IL-12 by tumor-infiltrating lymphocytes in patients with metastatic melanoma demonstrated liver dysfunction, high fevers, and life-threatening hemodynamic instability with increasing cell doses[11]. While this trial did not specifically use CAR T cells, it suggests that researchers still need to proceed with caution when delivering IL-12 locally to the TME.

IL-15

IL-15 is a pleiotropic cytokine produced mainly by monocytes, macrophages and dendritic cells that plays a role in innate and adaptive immunity[22]. IL-15 signaling in T cells and NK cells results in differentiation, lymphocyte homeostasis, and survival[22]. Preclinical research in murine and rhesus macaque models suggested an anti-tumor role for IL-15. In clinical trials, recombinant IL-15 therapy in patients with hematologic and solid tumors led to significant increases in circulating T cells and NK cells but did not translate to clinical efficacy[23]. Furthermore, high doses of IL-15 are associated with toxicities such as thrombocytopenia and hypotension[22]. Similar to the rational for armored CAR T cells secreting IL-12, CAR T cells secreting IL-15 are intended to deliver potent signaling locally to the TME.

IL-15 leads to robust expansion of T cells. This phenotype has been observed in CAR T cells that have been modified to secrete IL-15. CD19-targeting CAR T cells modified to secrete IL-15 demonstrated a 10-fold expansion post antigen stimulation *in vitro* and up to a 15-fold increase in *in vivo* expansion in SCID-lymphoma xenograft mouse models when compared to parental CD19-targeting CAR T cells[24]. These CAR T cells exhibited reduced cell death, lower PD-1 receptor expression, and increased anti-tumor efficacy[24,25]. In orthotopic glioma and metastatic neuroblastoma xenografted mouse models, IL-13Ra2-targeting or GD2-

targeting CAR T cells modified to secrete IL-15 demonstrated enhanced survival and anti-tumor activity[2627]. A CLL-1-targeting, IL-15 armored CAR T cell was less terminally differentiated and had increased expansion of AML cell lines *in vitro*[28].

IL-15 expression by CAR T cells has led to severe acute toxicity in preclinical models. For example, treatment with CAR T cells modified to secrete IL-15 led to acute toxicities related to high levels of TNFα, IL-15, and IL-2 in xenograft mouse models of aggressive AML[28]. These toxicities were managed successfully with anti-TNFα antibody treatment and an inducible caspase-9 safety switch to arrest T cell expansion. These strategies led to an increase in tumor-free survival of mice treated with IL-15-secreting CAR T cells[28].

In addition to toxicities associated with treatment, uncontrolled proliferation is a major concern with armored CAR T cells constitutively expressing IL-15. Logarithmic growth and persistence in the absence of exogenous cytokine support have been observed in mature human T lymphocytes following transduction with a retroviral vector to promote IL-15 expression[29]. Additionally, transgenic mouse models engineered to over-express IL-15 exhibited proliferation of NK and CD8+ T lymphocytes that led to the development of leukemia[30].

Given the association of constitutive IL-15 expression with uncontrolled proliferation and toxicity, researchers have developed methods to provide IL-15 signaling to CAR T cells without effecting other cell types. T cells engineered to co-express a CD19-targeting CAR and a membrane-bound chimeric IL-15 exhibited improved persistence and anti-tumor activity in xenograft leukemia mouse models and retained a phenotype with similarities to T memory stem cells[31]. Furthermore, engineering a CD19-targeting CAR T cell with an IL-15 receptor signaling domain fused with the CD28 co-stimulatory domain resulted in increased rates of proliferation cytotoxic activity and effector cytokine secretion as well as causing complete remission of one ALL patient after treatment with a CAR T cell infusion[32]. Finally, preclinical studies with nanogel engineering strategies have successfully loaded large dosages of IL-15 onto CAR T cells for tumor site delivery without systemic toxicities[33].

Clinical use of IL-15 secreting CAR cells has been limited to CAR-expressing NK cell or NKT cells. A clinical trial using cord blood-derived NK cells modified to express CD19-targeted CAR and secrete IL-15 in patients with CD19+ lymphoid tumors demonstrated a 64% complete response rate without major toxic effects[34]. Another trial tested the safety and anti-tumor response of GD2-specific NKT cells modified to secrete IL-15 in children with relapsed or resistant neuroblastoma[35]. No dose-limiting toxicities occurred in this trial and the armored CAR NKT cells induced an objective response in 1 out of the 3 patients treated. Current ongoing clinical trials are testing the efficacy of IL-15 expressing conventional CAR T cells (Table 1).

IL-18

IL-18 is a proinflammatory cytokines released from epithelial cells or macrophages that facilitates IFN-γ production, contributes to multiple immune regulatory effects on T cells, and can alter the phenotypes of T cells and NK cells[36]. IL-18 has been shown to stimulate

both innate and adaptive immunity alone or in conjunction with other cytokines such as IL-12 and IL-13[36]. IL-18 has demonstrated antitumor activity in preclinical models of cancer[36]. However, pro-IL-18 is required to be cleaved by proteases in order to converted into the active form of cytokine[36]. IL-18 binding protein (IL-18 BP) is a soluble factor found in the TME that can inhibit the function of IL-18[37]. These factors may play a role in why systemic infusion of recombinant IL-18 led to no major toxicities or efficacy[38,39].

CAR T cells modified to secrete IL-18 exhibit increased expansion, persistence, and survival in mouse models of hematological and solid malignancies[40,41,42]. These armored CAR T cells can also alter the inhibitory conditions of the TME and recruit endogenous anti-tumor immune cells. This includes recruitment of endogenous immune cells to the TME and suppression of Treg cells and anti-inflammatory M2-polarized macrophages[40,41,42]. Further engineering of IL-18 has resulted in a form of IL-18 resistant to IL-18BP binding[37], and if secreted by CAR T cells may be more effective in the TME.

However, as IL-18 may play a pathogenic role in autoimmune diseases[43,44], caution is warranted with clinical translation of IL-18-secreting CAR T cells.

Other cytokines

Armored CAR T cells that secrete IL-21, IL-7, IL-33, or IL-36γ have demonstrated preclinical efficacy. IL-21 is mainly secreted by activated CD4+ T cells and NK T cells and has broad proinflammatory effects on the immune cells that express IL-21 Receptor[45]. These include B, T, and NK cells, dendritic cells, and macrophages. IL-21-secreting CAR T cells were superior to CAR T cells modified to express IL-2 or IL-15 in clearing systemic in a xenograft model of lymphoma[46]. These armored CAR T cells had effector memory phenotypes and long term persistence in mice. In a separate model of solid and hematologic malignancies, IL-21 armored CAR T cells exhibited increased CAR T cell expansion and reduced apoptosis *in vitro* as well as increased tumor infiltration and tumor growth reduction *in vivo*[46].

IL-7 is mainly produced by epithelial and endothelial cells and is critical for every stage of T cell development[47]. Similar to IL-21-secreting CAR T cells, CAR T cells modified to constitutively secrete IL-7 were superior than IL-2 or IL-15 secreting CAR T cells in eradicating lymphoma in xenograft mice[45]. These armored CAR T cells demonstrated long term persistence and central memory phenotype. A separate study engineered CAR T cells to express both IL-7 and the chemokine CCL19 to mimic the function of T-zone reticular cells and recruit T cells and dendritic cells to the TME. These armored CAR T cells increased the numbers of endogenous T cells and dendritic cells in the TME and lead to a regression of pre-established solid tumors in mice[48]. This was due to a collaborative anti-tumor effort by these cells that resulted in memory responses.

Similar to IL-18, IL-33 and IL-36 are members of the IL-1 superfamily of cytokines that pay a role in immune regulation. Damaged tissue cells and activated immune cells release IL-33 to trigger inflammation and cell-mediated responses in damaged tissues[49]. Tumor cells down-regulate IL-33, suggesting a role for IL-33 in the inhibition of tumor progression or immune avoidance[50]. In preclinical models, IL-33-secreting armored CAR T cell have been

used to deliver this cytokine to the TME[50]. Similar to IL-33, IL-36 is produced by epithelial cells in response to tissue damage. It binds to epithelial cells as well as dendritic cells, monocytes, neutrophils and T cells to initiate and amplify an immune response[51]. Armored CAR T cells modified to secrete IL-36γ exhibit significantly enhanced CAR T cell expansion, persistence, and anti-tumor function *in vivo*, as compared to second generation CAR T cells[51]. IL-36γ delivered to the TME also activated endogenous antigen presenting cells and T cells to mediate a secondary anti-tumor response.

Chemokines

Chemokine proteins are the largest subfamily of cytokines and are divided into four highly conserved groups: C, CC, CXC, and CX3C. Produced by stromal, tumor or immune cells, chemokines function as signals for immune cell trafficking[52]. Chemokines play an important role in the TME by regulating tumor cancer progression and metastasis[53]. Strategies to equip CAR T cells to express chemokines in the TME aim to enhance immune cell homing and anti-tumor function (Figure 1).

As mentioned in the previous section, Adachi et al. modified CAR T cells to secrete IL-7 and CCL19 in order to mimic the function of T-zone reticular cells[48]. CCL19 is highly expressed in the thymus and lymph nodes and functions to attract dendritic cells. In this preclinical model, the armored CAR T cells indeed recruited endogenous dendritic cells and T cells to the TME, which resulted in superior anti-tumor efficacy[48].

CXCL11 expression in leukocytes, endothelial cells, and other cell types is induced by IFNγ[54]. CXCL11 is chemotactic for activated T cells. Intratumoral delivery of CXCL11 with oncolytic vaccinia virus along with CAR T cell therapy demonstrated potent anti-tumor efficacy and enhanced T cell infiltration in a mouse xenograft model[55]. However, CAR T cells modified to express CXCL11 in the TME did not demonstrate improved T cell trafficking to the tumor[55]. While both approaches resulted in increased levels of CXCL11 at the tumor site, the oncolytic virus strategy may prime the TME to be a more favorable environment for CAR T cells and enhance antitumor potential[55,56,57]. Furthermore, endogenous chemokine receptors on CAR T cells may act as a sink for the expressed chemokine.

A complementary strategy to chemokine expression by CAR T cells is the surface expression of chemokine receptors to enhance T cell migration to the tumor site. While this approach does not result in a secreted protein, it has demonstrated enhanced CAR T cell migration to the TME in preclinical models[58]. Clinically, armored CAR T cells that express combinations of chemokines and cytokines are currently being tested for safety in phase I trials (Table 1).

Antibodies

While antibodies are naturally produced by B cells, recent advances in technology have led to the engineering and characterization of smaller antibody-like molecules that retain similar functions as antibodies but can be produced by different cell types. These molecules include single chain variable fragments (scFv), nanobodies, and BiSpecific T cell Engagers (BiTE).

When expressed by armored CAR T cells these molecules can function as agonists or antagonist in the TME and can also broaden target recognition (Figure 1).

Secretion of Antibody-based molecules to overcome suppressive TME

Antibodies that block the interaction of suppressive ligands on tumors, such as PD-L1, and inhibitory receptors on T cells, such as programmed death receptor (PD-1), are broadly referred to as immune checkpoint inhibitors. Engineering CAR T cells to secrete these inhibiting antibodies locally in the TME is an attractive approach as it overcomes the hurdle of tumor site localization and may decrease the toxicities associated with systemic administration. Furthermore, these agents can enhance the efficacy of CAR T cells against immunosuppressive tumors[59]. One example of this platform is an armored CAR T cell modified to secrete PD-1-blocking scFv that demonstrated efficacy against PD-L1+ tumors in syngeneic and xenogeneic mouse models of leukemia and ovarian cancer[60]. This approach demonstrated that checkpoint inhibiting scFv secreted by CAR T cells function in an autocrine manner to prevent suppression of CAR T cells, as well as a paracrine manner to block suppression of endogenous bystander T cells. Finally, localized delivery of checkpoint blockade to the TME by CAR T cells was demonstrated, as no PD-1-blocking scFv was detected systemically in tumor-bearing mice treated with scFv-secreting CAR T cells[60].

Additional studies utilizing armored CAR T cells secreting checkpoint inhibitors to PD-1 mainly focused on testing this approach in xenograft models and the autocrine function of this strategy. Anti-CD19 CAR T cells modified to secrete anti-PD-1 nanobodies produced more inflammatory cytokines after antigen-dependent stimulation in artificial CD19-expressing solid tumor cells[61]. These armored CAR T cells exhibited enhanced antitumor activity in a xenograft mouse model. Recently, Zhou et al. showed that the PD-1 blocking enhanced the long-term cytotoxic capacity of EGFR-targeting CAR T cells *in vitro* against gastric cancer cells and significantly improved anti-tumor efficacy in a xenograft mouse model.[62]. Similar results of enhanced antitumor efficacy were also demonstrated with anti-mesothelin CAR T cells modified to secrete anti-PD-1 scFv[63].

Suarez et al. adopted a slightly different strategy to develop CAR T cells expressing checkpoint inhibitors by targeting PD-L1 on tumor cells. This strategy has the advantage of blocking inhibition of any effector cells in the TME. Anti-carbonic anhydrase IX (CAIX)-directed CAR T cells modified to secrete anti-PD-L1 antibodies demonstrated reduced tumor growth and volume in xenograft models of clear cell renal cell carcinoma[64]. Armored CAR T cell secretion of anti-PD-L1 antibodies with IgG1 or IgG4 Fc domains were additionally tested and the anti-PD-L-1 IgG1 isotype recruited NK cells to the tumor site.

CTLA-4 is a checkpoint molecule expressed by activated T cells. When bound to CD80/CD86 on antigen presenting cells or tumor cells, CTLA-4 suppresses T cell function. CAR T cells modified to secrete an anti-CTLA-4 minibody demonstrated superior efficacy to second generation CAR T cells in syngeneic models of mouse and canine glioma[65]. These data were used to design a pre-clinical trial to evaluate the safety of anti-IL-13Ra2 CAR T cells that secrete anti-CTLA-4 minibody in dogs with spontaneous glioma.

CD47 is another checkpoint molecule that has been targeted by armored CAR T cells that secrete antibody-based molecules. CD47 is ubiquitously expressed on many cell types and overexpressed in certain cancers. Expression of CD47 on cells delivers a signal to macrophages to prevent phagocytosis. Blockade of this signal can result in clearance of tumor cells. Anti-PD-L1 CAR T cells modified to secrete anti-CD47 single-domain antibody fragment nanobodies in the TME demonstrated blockade of CD47 signaling and improved engagement of the innate immune system[66].

Clinical applications of antibody-based molecules secreted from CAR T cells with checkpoint blockade are currently being tested against solid tumors and leukemia (Table 1). These are all testing CAR T cells at secrete antibody-based proteins against the checkpoint molecules PD-1 or PD-L1.

Secretion Antibody-based molecules to prevent antigen escape

Antigen escape is a currently a major hurdle to the durability of response to current CAR T cell therapy. One way to overcome this is to increase the number of antigens recognized by CAR T cells.

Bispecific T cell engagers (BiTE) are one way to direct T cells to cancer cells in the TME. Composed of two scFv-based binding domains, BiTE bind to CD3 on T cells and a tumor-associated antigen on cancer cells. In 2017, the FDA approved blinatumomab, an anti-CD3/CD19 BiTE for patients with relapsed or refractory B-cell acute lymphoblastic leukemia. Designing CAR T cells to secrete BiTE in the TME is a promising approach to increase the number of antigens being targeted on tumor cells and prevent antigen escape. For example, although EFGRvIII is a gliobastoma-specific tumor antigen, heterogenous expression of this antigen on glioblastoma results in antigen-negative relapse of disease in mice treated with anti-EGFRcIII targeting CAR T cells[67]. However, when these CAR T cells were modified to a secret BiTE specific for wild-type EGFR, antigen escape was prevented in mouse models of glioblastoma. BiTE secreted by CAR T cells were capable of redirecting CAR T cells as well as bystander T cells to eliminate heterogenous tumors. This dual-targeted platform demonstrated the engagement of bystander T cells *in vitro* and a complete and durable response *in vivo* glioma mouse model[67]. Localized delivery of the BiTE is a key aspect to this therapy, as systemic delivery of a BiTE specific for wildtype EGFR would result in on-target, off-tumor toxicity of normal tissues.

Enzymes

In addition to immunosuppressive ligands and receptors, the structure and oxidative stress environments of some solid tumors can prevent optimal T cell function. CAR T cells can be modified to secrete particular enzymes in the TME to locally alter the structure or stress (Figure 1).

For example, a stroma-rich extracellular matrix (ECM) surrounds many solid tumors and prevents immune cell penetration, which decreases anti-tumor efficacy during immunotherapy. Armored CAR T cells have been engineered to locally deliver ECM modifying enzymes directly to the solid tumor in attempts to increase penetration. Anti-GD2

CAR T cells cultured *in vivo* were compared to T lymphocytes isolated from patients, and researchers found downregulation of heparinase, an enzyme capable of degrading heparan sulfate proteoglycans in the ECM, in the CAR T cells[68]. Anti-GD2 CAR T cells were engineered to express heparinase and resulted in degradation of the ECM and increased tumor infiltration and antitumor activity in xenografted mouse models compared to anti-GD2 CAR T cells without heparinase expression[68].

Furthermore, tumor cells have high levels of oxidative stress and reactive oxygen species (ROS). ROS in the TME can impair antitumor activity by inducing oxidative stress-mediated repression of immune cells. Anti-CEA or anti-Her2 CAR T cells were engineered to coexpress a catalase enzyme to increase their antioxidative capacity[69]. Known as CAR-CAT T cells, these T cells demonstrated increased efficacy compared second-generation CAR T cells due to increased levels of intracellular catalase and reduced oxidative stress[69]. The CAR-CAT T cells also exhibited substantial bystander protection to other immune effector cells even at high H_2O_2 concentrations[69].

Lastly, a novel application of enzyme secreting CAR T cells converts these cells to synthetic enzyme armed killer (SEAKER) cells. This approach delivers pro-drug activating enzymes to the TME with CAR T cells[470]. Systemic administration of a nontoxic pro-drug converts to potent chemotherapeutic drug only in the TME where the SEAKER cells are located, allowing for localized delivery of toxic agents.

Peptides

Peptides secreted by armored CAR T cells have widespread functions and potential applications. These applications range from enhancing CAR T cell function to combating CAR T cell therapy-associated toxicities (Figure 1).

CAR T cell activation and proliferation after infusion can lead to systemic inflammatory toxicities referred to as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS). Interactions between tumor cells and CAR T cells activate myeloid cells to produce IL-1 and IL-6, levels of which determine the severity of CRS[70,71]. Systemic administration of an IL-1 receptor antagonist (IL-1Ra) protected against CRS and ICANS-related mortality in leukemia/lymphoma mouse models, without effecting CAR T cell efficacy[71,72]. Anti-CD19 CAR T cells modified to express IL-1Ra were engineered and administered to NSG mice with CRS[71]. Results indicated the IL-1Ra armored CAR prevented CRS in the mice without compromising the CAR T cell's anti-tumor efficacy[71].

Armored CAR T cells modified to secrete peptides have also been used to overcome immunosuppressive mediators in the TME surrounding solid tumors. Prostaglandin E2 (PGE2) and adenosine in the TME activate protein kinase A (PKA), which inhibits T-cell receptor activation by binding to the membrane protein ezrin in immune synapses. Armored CAR T cell engineered to secrete a regulatory subunit 1 anchoring disruptor (RAID) peptide competitively inhibits binding of PKA to ezrin[73]. This disruption increases T cell receptor

signaling, cytokine release, and the anti-tumor efficacy of armored CAR T cells in synergistic mouse models[73].

Peptides secreted from CAR T cells can also bind to tumor cells and restore inhibitory signals mutated on cancer cells. In germinal center lymphomas, the HVEM receptor gene is the most frequently mutated genes. This leads to the disruption of the inhibitory cell to cell interaction between HVEM and BTLA (B and T lymphocyte attenuator), resulting in B cell proliferation and the development of lymphoma. Soluble HVEM delivered by CAR T cells binds to BTLA and restores the inhibitory signal and leads to lymphoma cell death[74].

Finally, peptides secreted from CAR T cells can engage the endogenous immune cells to combat cancer. Recently, Lai et al. developed CAR T cells to secrete dendritic cell[32] growth factor Fms-like tyrosine kinase 3 ligand (Flt3L), which substantially promoted expansion of intertumoral conventional type 1 DC cells[75]. When combined with immune agonists, these armored CAR T cells inhibited tumor growth and induced epitope spreading to combat antigen-negative tumor escape[75].

Discussion

CAR T cells modified to secrete proteins have great potential to enhance the anti-cancer efficacy of cellular therapies. These cells allow for local protein delivery in a controlled manner by immune effector cells. Future innovations to this platform include inducible gene expression to control protein secretion only when the CAR is activated in the TME, logic gating to determine which activation signals in the TME control protein expression, and synthetic biology circuits that can elegantly modulate protein expression by CAR T cells[34]. However, as these cells have increasing genetic modifications, suicide switches or controlled expression of the CAR construct may be needed for safe translation of these engineering strategies into patients.

There are further considerations and applications for the use of armored CAR T cells for cancer therapy. Current limitations to this technology include gene size and number of proteins that can be expressed in viral constructs. Future application with CRISPR may allow multiple proteins to be expressed and, with directed insertion, in a predictable manner. Non-genetic engineering methods such as liposome fusion or phagocytosis in addition to CAR transduction can also be employed to engineer these micropharmacies[4].

CAR T cells modified to secrete proteins locally in the TME can deliver a myriad of different protein to improve the elimination of cancer cells. While clinical translations of CAR T cells modified to secrete proteins is still in its infancy (Table 1), this natural next step for CAR T cell therapy has great potential to positively impact patient care. Furthermore, with the capability of overcoming the suppressive TME, improving CAR T cell function, and eliciting an endogenous immune response, armored CAR T cells may be the key to making cellular immunotherapy effective against solid tumors.

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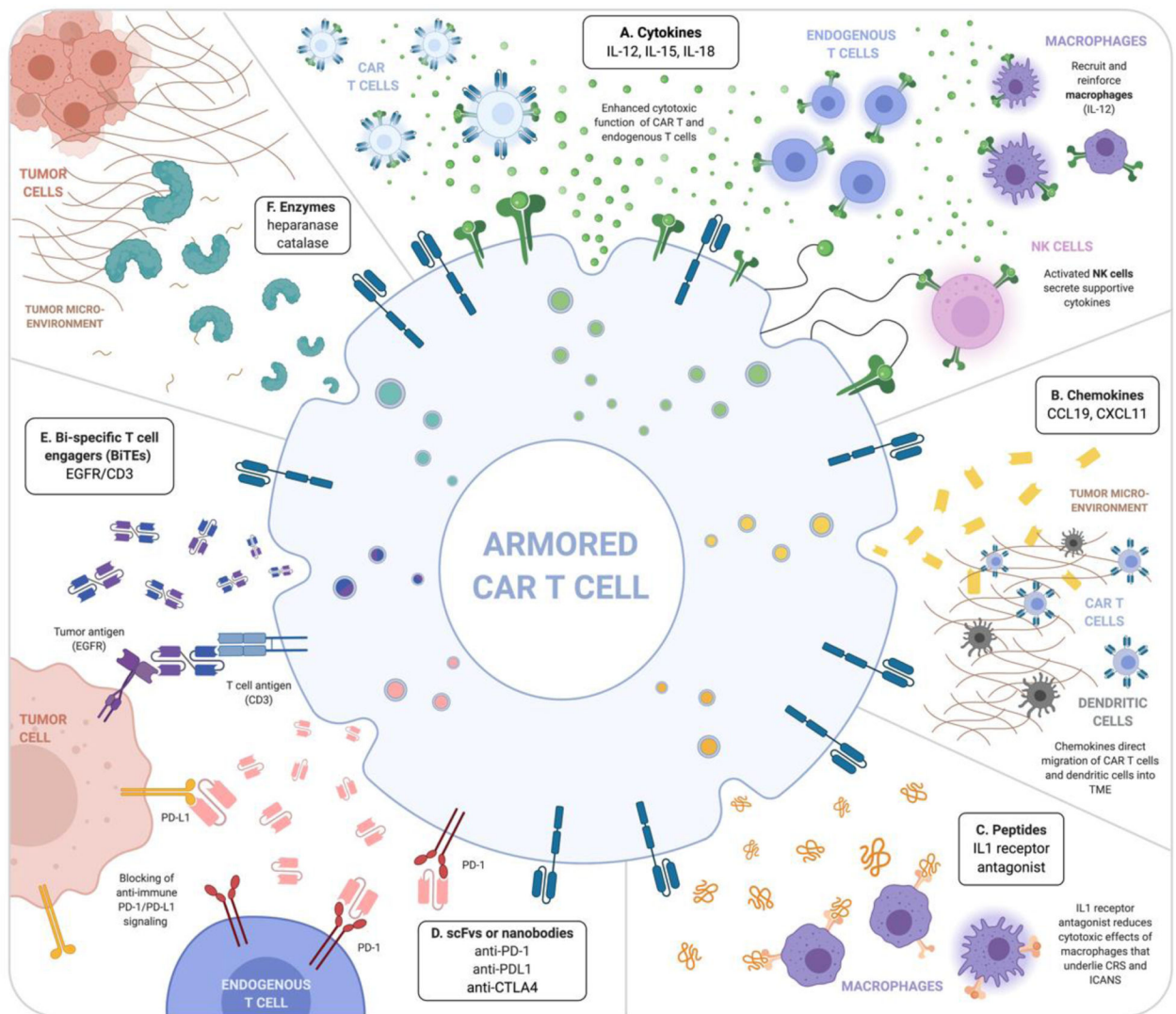
Figure 1. Localized delivery of secreted proteins by CAR T cells can alter the tumor microenvironment.

A) Cytokines (i.e. IL-12, IL-15, IL-18) enhance self and other CAR T cell function as well as endogenous T cells, NK cells, and macrophages. B) Chemokines alone or in conjunction with cytokines can direct migration of CAR T cells and dendritic cells into the tumor microenvironment (TME). C) Peptides, such as IL-1 receptor antagonist, can work to inhibit undesirable cytotoxic effects from macrophages that mediate life-threatening complications such as CRS and ICANS. Antibody-based molecules such as D) scFvs and nanobodies can target and inhibit anti-immune surface signaling molecules, such as PD-1or PD-L1. E) Bi-specific T cell engagers (BiTEs) bind a T cell antigen, often CD3, and a tumor antigen (i.e. EGFR) to bring the cells into close proximity to enhance effector function. F) Enzymes, such as heparanase and catalase, digest extracellular matrix present in the TME to allow accessibility to the tumor cells. Created with BioRender.com.

Table 1.

Clinical trial currently open with CAR T cells modified to locally secrete proteins.

	Armor	Target antigen	Disease	Clinical Trial ID, Phase	Sponsor
	IL-12	MUC16ecto	Solid Tumors	NCT02498912, Phase 1	Memorial Sloan Kettering Cancer Center
		EGFR	Metastatic Colorectal Cancer	NCT03542799, Phase 1	Shenzhen Second People's Hospital
	IL-15	GD2	Neuroblastoma	NCT03721068, Phase 1	UNC Lineberger Comprehensive Cancer Center
		GD2	Neuroblastoma	NCT03294954, Phase 1	Baylor College of Medicine
		GPC3	Liver Cancer, Rhabdomyosarcoma, Malignant Rhabdoid Tumor, Liposarcoma, Wilms Tumor, Yolk Sac Tumor	NCT04377932, Phase 1	Baylor College of Medicine
CYTOKINES	mbIL-15	CD19	Lymphomas and Leukemias	NCT03579888, Phase 1	M.D. Anderson Cancer Center
	PD-1	Mesothelin	Advanced Solid Tumor	NCT03615313, Phase 1/2	Shanghai Cell Therapy Research Institute
		CD19	Lymphoma, B-Cell	NCT04163302, Phase 2	Chinese PLA General Hospital
		BCMA	Multiple Myeloma	NCT04162119, Phase 2	Chinese PLA General Hospital
		Mesothelin	Colorectal, Ovarian cancer	NCT04503980, Phase 1	Shanghai Cell Therapy Group Co., Ltd
		Mesothelin	Non-small-cell Lung Cancer, Mesothelioma	NCT04489862, Phase 1	Wuhan Union Hospital, China
	PD-L1	CD22	Solid Tumor, Cervical Cancer, Sarcoma, NSCLC	NCT04556669, Phase 1	Hebei Senlang Biotechnology Inc., Ltd.
	PD-1 or PD-L1	EGFRviii	Glioblastoma Multiforme of Brain	NCT03170141, Phase 1	Shenzhen Geno-Immune Medical Institute
ANTIBODY-BASED MOLECULES			Sarcoma, Osteoid Sarcoma, Ewing Sarcoma	NCT03356782, Phase 1/2	Shenzhen Geno-Immune Medical Institute
	IL-7/CCL19	CD138, Integrin B7, CS1, CD38, BCMA	Refractory/recurrent multiple myeloma	NCT03778346, Phase 1	The Sixth Affiliated Hospital of Wenzhou Medical University
		CD19	B Cell lymphoma	NCT03929107, Phase 2	Wenbin Qian
		CD19	Diffuse Large B-cell Lymphoma	NCT04381741, Phase 1	Second Affiliated Hospital, School of Medicine, Zhejiang University
	IL-7/CCL19 or IL-12	Nectin4	Nectin4+ Advanced Malignant Solid Tumor	NCT03932565, Phase 1	The Sixth Affiliated Hospital of Wenzhou Medical University
COMBINATIONS	IL-7/CCL19 +/− PD-1, CTLA4, Tigit	GPC3, TGFβ	Hepatocellular Carcinoma, Squamous Cell Lung Cancer	NCT03198546, Phase 1	Second Affiliated Hospital of Guangzhou Medical University

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