CAR T Cell Therapy for Solid Tumors

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Abstract

The field of cancer immunotherapy has been re-energized by the application of chimeric antigen receptor (CAR) T cell therapy in cancers. These CAR T cells are engineered to express synthetic receptors that redirect polyclonal T cells to surface antigens for subsequent tumor elimination. Many CARs are designed with elements that augment T cell persistence and activity. To date, CAR T cells have demonstrated tremendous success in eradicating hematologic malignancies (e.g., CD19 CARs in leukemias). However, this success has yet to be extrapolated to solid tumors, and the reasons for this are being actively investigated. We characterize some of the challenges that CAR T cells have to surmount in the solid tumor microenvironment and new approaches that are being considered to overcome these hurdles.

INTRODUCTION

CEA: carcinoembryonic antigen

FAP: fibroblast activation protein

TME: tumor microenvironment

Treg: regulatory T cell

MDSC: myeloid-derived suppressor cell

TAM: tumor-associated macrophage

TAN: tumor-associated neutrophil

Infusion of T cells directed against specific antigens has demonstrated promise in HIV and cancer therapy. Along with immune checkpoint blockade (1), this approach is triggering a paradigm shift in cancer immunotherapy. Perhaps the most exciting of these approaches has been the use of peripheral blood T cells that have been genetically modified to express chimeric antigen receptor (CAR) genes. CARs are composed of an extracellular single-chain variable fragment (scFv), which serves as the targeting moiety; a transmembrane spacer; and intracellular signaling/activation domain(s). The CAR constructs are transfected into T cells using plasmid transfection, mRNA, or viral vector transduction to direct them toward surface-exposed tumor-associated antigens (TAAs). CAR structure has evolved from an initial composition involving only the CD3ζ signaling domain (dubbed a "first-generation CAR") to more complex forms in which costimulatory endodomains have been added, giving rise to second-generation (e.g., CD3ζ plus 41BB or CD28 signaling domains) and third-generation (e.g., CD3ζ plus 41BB and CD28 signaling domains) CARs that have augmented T cell persistence and proliferation. CARs have also been constructed to target specific peptides within the context of human leukocyte antigen molecules, potentially allowing the targeting of intracellular molecules (2).

The adoptive transfer of CAR T cells has demonstrated remarkable success in treating hematologic cancers; prominently, the use of CD19 CARs in acute and chronic B cell leukemias (3) and indications in patients with lymphoma and myeloma are being explored. Given this "proof of principle," a growing number of clinical trials have now focused on solid tumors, targeting surface proteins including carcinoembryonic antigen (CEA), the diganglioside GD2, mesothelin, interleukin 13 receptor α (IL-13Rα), human epidermal growth factor receptor 2 (HER2), fibroblast activation protein (FAP), and L1 cell adhesion molecule (L1CAM) (reviewed in References 3 and 4). Unfortunately, the clinical results in solid tumors have been much less encouraging. To date, the most positive trials reported have used GD2 CARs to target neuroblastoma (3 of 11 patients with complete remissions) (5), HER2 CARs for sarcoma (4 of 17 patients showing stable disease) (6), and HER1 CARs for lung cancer (2 of 11 patients with partial responses) (7).

The reason for this differential is unknown but is likely multifactorial. Solid tumors present barriers that are absent in hematologic malignancies. Finding specific tumor antigens that are highly and uniformly expressed has been difficult. Unlike the situation in hematologic malignancies, CAR T cells must successfully traffic from the blood into solid tumor sites in spite of potential T cell chemokine receptor-/tumor-derived chemokine mismatches. They must then successfully infiltrate the stromal elements of solid tumors in order to elicit TAA-specific cytotoxicity, regardless of antigen loss or heterogeneity. Even after successful trafficking and infiltration, T cells become rapidly dysfunctional owing to (*a*) a hostile tumor microenvironment (TME) characterized by oxidative stress, nutritional depletion, acidic pH, and hypoxia; (*b*) the presence of inhibitory soluble factors and cytokines; (*c*) suppressive immune cells, namely regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs) or neutrophils (TANs); and (*d*) T cell–intrinsic negative regulatory mechanisms, such as upregulation of cytoplasmic and surface inhibitory receptors. Lastly, the CAR T cells themselves may be problematic given their potential immunogenicity and toxicity.

In this review, we discuss some of the key immunosuppressive barriers and other factors within solid tumors that ultimately neutralize the function of antitumor T cells, and CAR T cells in particular (**Figure 1**), and we discuss possible solutions (**Table 1**).

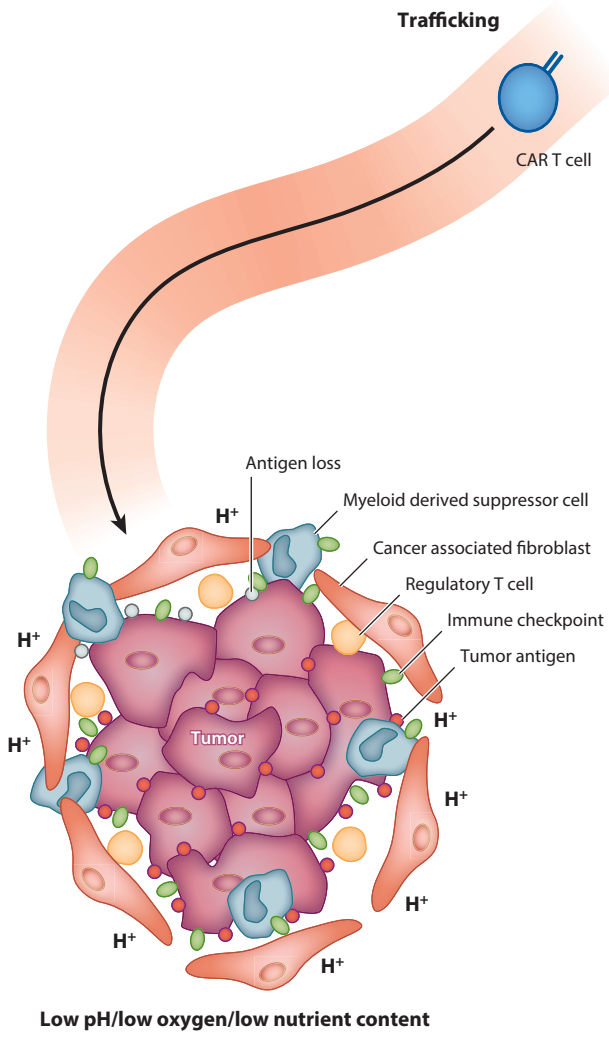

Figure 1

Immunosuppressive tumor microenvironment. This diagram depicts the multiple challenges for chimeric antigen receptor (CAR) T cells. In addition to proper trafficking and successful infiltration, there are additional hurdles presented upon their arrival in a solid tumor microenvironment. Failure to overcome the presence of these negative elements results in inhibition of the CAR T cell activity and unabated tumor growth.

TARGET ANTIGEN SPECIFICITY

The first step in successful adoptive T cell therapy is selecting an optimal TAA for CAR T cell targeting. The ideal target should meet at least two criteria. First, the TAA needs to be selectively expressed on tumor cells at high levels but not be expressed on the surface of important normal tissues (or, if expressed, it should be at a very low level). Second, the ideal TAA would be expressed on 100% of the tumor cells. Because the CAR can only attack cells having the targeted antigen, success would be unlikely unless almost all the tumor cells expressed the TAA.

Table 1 Summary of challenges that ultimately neutralize the efficacy and function of CAR T cells, and strategies that have been designed to surmount these challenges

Obstacle	Strategies to overcome obstacle (references)
Target antigen heterogeneity	Screen patients for expression of tumor antigen, and only enroll them if the proportion of TAA-expressing tumor cells exceeds a certain threshold of expression Target multiple antigens at once (e.g., CAR targeting both CD19 and CD20 in B cell leukemias) (24) Use multifunctional CARs—in addition to specific recognition of target, these CARs also encode byproducts (such as cytokines) to augment killing (28–33)
Trafficking	Use CARs that coexpress chemokine receptors (35–37) Use oncolytic viruses encoding tumor target and chemokines (38, 39) Local instillation of CARs is being explored in clinical trials (NCT02498912, NCT02414269, NCT01818323)
Hostile tumor microenvironment: physical and metabolic barriers	Use CARs that deplete fibroblast cells (26) or CARs that degrade the extracellular matrix (40)
Hostile tumor microenvironment: tumor-derived soluble factors and cytokines	Antigen-specific CARs that simultaneously interrupt inhibitory adenosine and PGE2 signaling (37) and CARs expressing dominant negative TGFβ have shown promise in animal models (37, 51–54)
Hostile tumor microenvironment: immunosuppressive immune cells	Simultaneous depletion of $GR1^+$ cells augmented the efficacy of CEA CAR T cells (57) In ovarian cancer, CARs induced the production of nitric oxide by TAMs, which augmented tumor lysis (58) Simultaneous PDL1 blockade and depletion of Tregs augmented T cell adoptive transfer (60) Inhibiting Tregs augmented mesothelin CAR activity (L. Wang, et al., submitted) IL-7 and IL-21 administration boosted CAR efficacy without stimulating Tregs (65)
Intrinsic regulatory mechanisms of T cells	Combining CAR therapy and PD1 blockade was efficacious in breast cancer and mesothelioma models (67, 68) Use of PD1 switch receptors was shown to neutralize inhibitory PD1 signaling (69, 70) Blocking CTLA4 enhanced adoptive transfer (71) Engineering CAR T cells lacking inhibitory molecules (e.g., diacylglycerol kinase) led to enhanced function (73)

Abbreviations: CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; TAM, tumor-associated macrophage.

CD19 fits both of these criteria as a target antigen for B cell malignancies. Virtually all acute lymphoblastic leukemia cells express CD19 at high levels, and the other targets of CD19—that is, normal B cells—are relatively dispensable, with intravenous immunoglobulin support. These characteristics are a large reason for the tremendous success of CD19 CARs in leukemias (although it should be noted that immune editing with loss of CD19 has been implicated in relapses). To date, roughly 30 solid tumor antigens are being evaluated for CAR T cell therapy (see above); these include neoantigens (e.g., mutated sequences), oncofetal or developmental antigens, and tumor-selective antigens (i.e., those with enriched expression on neoplastic cells but low basal expression on normal cells). A recent list of CAR targets that are currently being evaluated in clinical trials is available (3). It should be noted that the scFv avidity to TAA may also be important, and immunoediting and subsequent removal of the most immunogenic epitopes may lead to tumor escape (8).

So far, none of these antigens has been ideal. Cancers arising from virus transformation that express viral products are attractive targets for therapy because these products are not displayed

on normal tissues; human papillomavirus (HPV)-transformed ovarian cancers are an example of this type (9). Unfortunately, most of the antigens are intracellular and not accessible to CARs. Oncofetal antigens on the surface of solid tumors represent an especially good target for CAR therapy as their expression is largely restricted to tumor cells, making them highly specific. CAR T cells targeting the mutated epidermal growth factor receptor (EGFR) illustrate this approach; EGFR variant 3 (EGFRvIII) is only expressed on malignant tumor cells (mostly glioblastomas). EGFRvIII CARs have shown promise in treating animal models of glioblastomas (10, 11), and clinical trials testing the efficacy of EGFRvIII CAR in patients with glioblastomas are under way (NCT02209376, NCT01454596). Abnormal glycosylation of the extracellular glycoprotein MUC1 is also seen in a large variety of cancers; MUC1-targeted CAR T cells against MUC1-overexpressing breast cancer xenografts were shown to significantly delay tumor progression (12). A similar success was reported for CAR T cells targeting MUC16, which is overexpressed in many ovarian carcinomas (13).

Tumor-selective (versus tumor-specific) antigens include targets that are overexpressed on transformed cells but expressed at low levels on normal tissues. One example is mesothelin, a glycoprotein whose overexpression in mesothelioma and in ovarian and pancreatic carcinomas, combined with low expression on peritoneal, pleural, and pericardial surfaces, has made it an attractive target for CAR therapy (14, 15). Two mesothelin-specific CARs have been reported. One, based on the SS1 antibody (16), is a mouse antihuman scFv, which is currently being evaluated in a clinical trial at the University of Pennsylvania (NCT02159716); the other, designated P4, is a fully human scFv (17). A fully human scFv targeting mesothelin was recently described by another group (18) and is currently being tested in a clinical trial (NCT02414269). Treatment using T cells electroporated with the mRNA encoding SS1 CAR, though promising, raised concerns about potential immunogenicity-related toxicity (see below). The search for optimal CAR targets is an area of active investigation.

Ultimately, the degree of specificity will be critical for safety. The most feared complication of CAR therapy, a catastrophic and rapid "on target–off tumor" event, has been documented. A fatal event occurred shortly after infusion with a high-affinity HER2 CAR and was attributed to low-level expression of the antigen on normal endothelium and epithelium (19). Despite preclinical animal studies, safety can only really be established in careful clinical trials. Approaches to avoid this type of event include the use of "self-limited CARs," which employ mRNA rather than lentivirus to transiently express the CAR receptor (16), and dose-escalation trial designs. Some groups are also advocating the insertion of suicide genes that can be activated in case of adverse events. Success in preclinical models has been shown with use of the herpes simplex virus thymidine kinase (HSV-TK) gene or an inducible caspase 9 (iCasp9) gene. Another approach could be to increase the specificity of CARs by requiring the CAR to recognize two antigens to promote activity (20, 21).

TARGET ANTIGEN HETEROGENEITY

A major limitation to all of the proposed TAAs for solid tumors is antigen heterogeneity, that is, variability in the expression of the antigen on the cells within a given tumor. For example, although mesothelin is expressed on >90% of epithelial malignant mesothelioma tumor cells, it is expressed on lower percentages of tumor cells in ovarian, breast, and lung cancer tumors. In addition, it is likely that immunoediting with subsequent removal of the most immunogenic epitopes may lead to tumor escape, as has been shown with CD19-negative escape variants in leukemia (8).

A critical (but unanswered and underexplored) question with high relevance to solving the heterogeneity problem is the extent to which CAR therapy can induce indirect tumor killing

and/or can trigger "antigen spreading." Indirect killing could result from activation of tumoricidal neutrophils, macrophages, or natural killer (NK) cells by cytokines released after CAR engagement. Antigen or epitope spreading is a process in which CAR T cells induce the generation or activation of other endogenous antitumor CD8 T cells. This is postulated to occur when CAR T cells engage tumor cells, secreting stimulatory cytokines (e.g., tumor necrosis factor and interferon-γ) and killing the tumor cells, resulting in the release of tumor antigens in an "immunostimulatory microenvironment" that then allows cross presentation by dendritic cells and generation of endogenous CD8 responses against tumor antigens that were not originally targeted by the CAR. These endogenous T cells could then eliminate the remaining tumor cells.

The extent to which these processes occur will likely be critical in setting the thresholds of antigen expression that will be used to define eligible candidates. Unfortunately, it is not really known what percentage of a tumor needs to express the target antigen for efficacy. Despite the importance of this question, it has been studied relatively little. Answering it will require experiments in mice with intact immune systems so that antigen-presenting cells (i.e., dendritic cells and other normal myeloid cells) are present or careful studies from clinical trials where known tumor antigens can be assessed after CAR therapy. One encouraging study that indirectly supports the possibility of antigen spreading was reported by Sampson et al. (22). They showed that after treatment of brain tumors by CAR therapy directed at EGFRvIII in syngeneic mice, the cured mice were resistant to rechallenge with EGFRvIII-negative tumors, suggesting generation of host immunity against additional tumor antigens. Data in humans are very scarce. A study of mesothelin-directed CARs transduced with mRNA and injected into mesothelioma patients showed induction of new antibody responses after treatment using a high-throughput serologic analysis of a protein array and using tumor cell lysates (23). These data show that antitumor humoral immune responses were induced.

What can be done to address the problem of tumor antigen heterogeneity? Three approaches are currently under investigation. First, under the assumption that more target is better, patient tumors can be screened with immunohistochemistry for expression of tumor antigen, with subjects being enrolled only if the percentage of TAA-expressing tumor cells exceeds a certain threshold in the intensity and number of cells that stain.

A second approach being studied is to target multiple tumor antigens at once (a well-established concept in infectious diseases), to provide better "killing coverage" and possibly prevent resistance from developing. For example, in B cell leukemias, CARs that target both CD19 and CD20 are being administered. It is unclear if it will be more advantageous to use T cells that express two CARs on their surface (24), two different CAR T cell lines (each expressing a different antigen), or more complex designs, such as CARs where two scFvs targeting different antigens are engineered into the same CAR—TanCARs (25). A similar two-pronged approach is to combine a CAR that targets a tumor antigen with a CAR that eliminates tumor stroma. CARs targeting the stromally expressed FAP (26) or the vascular endothelial growth factor (VEGF) receptor on tumor endothelium have been explored (27).

A third approach is to extend the CAR T cell's ability to kill tumor cells beyond the traditional antigen-activated T cell killing pathways (i.e., perforin/granzyme or Fas). CARs could thus be used to secrete or to carry additional molecules that could either directly or indirectly lead to tumor cell death. One strategy has been to introduce activating cytokines to augment CAR function (and perhaps also enhance antigen spreading). This approach has been successfully tested with CARs or T cells that release the stimulatory cytokine IL-12 upon T cell receptor engagement (28). Although the approach worked extremely well in animal models, a recent clinical trial in which the IL-12 gene [driven by a nuclear factor of activated T cell (NFAT) promoter] in adoptively transferred tumor-infiltrating lymphocytes (TILs) resulted in unacceptable toxicity (29). Tighter

control of IL-12 release or the use of less toxic cytokines (i.e., type 1 interferons) might allow this strategy to proceed in the clinic. Despite the issues in the TIL–IL-12 trial, a clinical trial using IL-12 expressed in CD19 CAR T cells (30) is under way. Another possibility to augment efficacy has been to engineer the T cells to secrete "T cell engager molecules" such as bispecific antibodies (BiTEs). These could activate endogenous T cells that do not express the CAR. Proof of principle using T cells that secrete a BiTE targeting both CD3 and the tumor antigens Eph2A or CEA has been provided (31, 32). The CD40 ligand (CD40L) has been expressed on the CAR T cell surface (33). CD40L was found to enhance activation of T cells and also to stimulate and activate other TME cells, such as dendritic cells. Other potential ways to arm CAR T cells could include expression of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) and perhaps even prodrugs that could activate chemotherapeutic drugs within the TME.

TRAFFICKING

Once a CAR targeting an appropriate tumor antigen is generated and infused into a patient, it must successfully target and infiltrate the solid tumor. Successful trafficking depends on the appropriate expression and pairing of adhesion receptors on both T cells and the tumor endothelium, as well as a match between the chemokine receptors on the CAR (primarily CXCR3 and CCR5) and the chemokines secreted by the tumors. Unfortunately, there is often a chemokine/receptor mismatch, with tumors producing very small amounts of CXCR3 and CCR5 ligands, leading to inefficient targeting of the $CXCR3^{high}$ $CD8^{+}$ CAR T cells to tumor sites (34). One approach to overcome this problem is to design CAR T cells that coexpress better-matched chemokine receptors. For example, two groups have shown that introducing CCR2b into CARs and injecting these CARs into tumors that made large amounts of CCL2 lead to enhanced intratumoral migration of CAR T cells and better tumor eradication (35, 36). Our group has also discovered that the genetic inhibition of protein kinase A (PKA) activation in CAR T cells increases their ability to infiltrate tumors in vivo owing to higher baseline expression of CXCR3 (37).

Several groups have also demonstrated the successful use of oncolytic viruses armed with chemotactic chemokines in attempts to attract CAR T cells to tumor sites. Oncolytic viruses have been shown to successfully and specifically infect tumor cells and lyse them. The use of an oncolytic adenoviral vector expressing CCL5 and GD2 CAR T cells robustly controlled neuroblastoma progression in mice and improved CAR T cell influx (38), and similar observations were attained with the use of HER2 CAR T cells loaded with modified oncolytic viruses (39).

Because of poor trafficking after intravenous injection, local instillation of CARs is also being explored; clinical trials (NCT02498912, NCT02414269, NCT01818323) are evaluating the merits of site-specific (i.e., systemic versus regional versus intratumoral) administration of CAR T cells in solid tumors. One potential limitation is that local instillation is often more technically challenging than simple intravenous administration. Another potential issue is that although site-specific injection of CAR T cells will likely result in higher T cell levels locally, the ability of these CARs to exit the tumor, enter the blood, and then traffic to other tumor sites (which presumably exist in advanced cancer patients) is uncertain, although at least one study supports systemic trafficking (18). The ongoing studies will help to address these issues.

THE HOSTILE TUMOR MICROENVIRONMENT

Physical and Metabolic Barriers

Once the T cells arrive in the solid tumor, the microenvironment presents many problems for CAR T cells. Purely physical/anatomical barriers generated by stroma characterize many types of

cancers, and the associated high tissue pressure prevents extravasation. Countering these barriers by reducing tumor fibroblast numbers through the use of FAP CAR T cells (26) or by having the CARs secrete an enzyme that degrades matrix (40) have both shown some success in augmenting CAR T cell function in animal models.

The metabolic landscape within the TME is markedly stressful and inhospitable toward T cells (41). One prominent feature of the TME is hypoxia. Although the literature is a bit contradictory (41), most investigators believe that hypoxia dampens lymphocytes' activation, diminishes their proliferation, and reduces their effector activity. A recent supportive study showed that exposing tumor-bearing mice to hyperoxia augmented antitumor immunity and reduced tumor growth (42).

A second key factor is likely nutrient starvation. In the TME, elevated lactate generation (leading to acidosis) and the lack of glucose and other metabolites inhibit T cell proliferation and cytokine production (43, 44). Nutrient depletion can trigger intracellular cell sensors that include 5′ adenosine monophosphate-activated protein kinase (AMPK), the mammalian target of rapamycin (mTOR), and activators of the integrated stress response (such as the amino acid–sensing kinase GCN2). Low levels of glucose or amino acids such as tryptophan, arginine, and lysine can thus cause protein translation shutdown or autophagy responses in effector T cells as a means of survival in order to generate an intracellular source of nutrients (45). As an example, the amino acid tryptophan is essential for many biological functions, but it cannot be synthesized and hence must be obtained via dietary means. Tryptophan metabolism as catalyzed by tumor- and MDSC-expressed indolamine-2,3-dioxygenase (IDO) leads to T cell anergy and death, as well as Treg accumulation. In a solid tumor xenograft model of CD19-expressing tumor cells transduced with IDO, Ninomiya et al. showed the failure of adoptively transferred CD19 CAR T cells to control progression of IDO-expressing tumors (46). MDSCs may also reduce the bioavailability of the key amino acid arginine (see below). Preliminary work has suggested that manipulation of key cellular regulators of protein synthesis (i.e., mTOR) might augment the efficacy of adoptively transferred cells (47). Sukumar et al. also showed that inhibiting glycolysis promoted the formation of memory cells, and enhanced antitumor activity (48).

Tumor-Derived Soluble Factors and Cytokines

Many studies have found immunosuppressive soluble factors in sera, ascites fluid, and tissue extracts from cancer patients. Prostaglandin E2 (PGE2), a small-molecule derivative of arachidonic acid produced by the inducible cyclooxygenase 2 (COX2) enzyme, is generated by both tumor cells and macrophages. Many studies have reported PGE2-mediated inhibition of T cell proliferation, suppression of CD4 help, and subversion of CD8 differentiation (49). Adenosine, a purine nucleoside seen at high levels during hypoxia, is another potent inhibitor of T cell proliferation and activity. Both PGE2 and adenosine elicit immunosuppressive effects via signaling through their own G-coupled receptors, which activate PKA in a cyclic AMP (cAMP)-dependent manner (50). A recent study demonstrated that genetic inhibition of PKA activation in CAR T cells can enhance their antitumor efficacy (37).

Cytokines, implicated in inflammatory responses at tumor sites, may bolster or inhibit the antitumor response. One of the most important inhibitory tumor cytokines is transforming growth factor β (TGFβ). In addition to its ability to promote epithelial-to-mesenchymal transition, enhance matrix production, promote metastasis, and skew the immune response toward a Th2 phenotype (51), TGFβ has direct negative effects on T cell effector functions (52). Several approaches have been used to counteract this effect. Systemic blockade of TGFβ using soluble receptors to antibodies was efficacious in augmenting adoptive T cell therapy (53). To specifically

counteract TGFβ effects in T cells, CAR T cells expressing a dominant negative TGFβ receptor have been created. These CAR T cells were resistant to TGFβ suppression and demonstrated augmented efficacy in animal models (54).

Immunosuppressive Immune Cells

Within the TME, various suppressive surveilling immune cells, Tregs, MDSCs, and TAMs/TANs with the so-called M2 and N2 phenotypes are known to present a barrier against antitumor immunity. Although there is extensive literature describing the immunosuppressive nature of these cells, to date, their effects on CAR T cell therapy have not been extensively examined. One technical factor to consider is that in order to study these cell–cell interactions, mouse CAR T cells must be injected into immunocompetent mice. Given the major differences between the behaviors of mouse and human CAR T cells (e.g., mouse CAR T cells are much more sensitive to activation-induced cell death and have much shorter persistence), the relevance of these studies to human CAR T cells is not certain.

MDSCs, M2 TAMs, and N2 TANs are well-known producers of TGFβ, PGE2, reactive oxygen/nitrogen species, and arginase (55, 56). As discussed above, all these factors probably blunt the efficacy of CAR T cells. In addition, TAMs can express high levels of programmed death ligand 1 (PDL1), which could potentially interact with PD1 on CAR T cells and inhibit them (see below). MDSCs may also recruit Tregs. On the other hand, TAMs and TANs activated in the proper fashion (the M1 and N1 phenotypes) can work to eliminate tumor cells.

The role of myeloid cells in CAR therapy is not yet clear. Burga et al. found that depletion of $GR1^+$ cells (targeting TANs and MDSCs) augmented the ability of CEA CAR T cells to control colorectal cancer liver metastases (57). In contrast, Spear et al. found in an ovarian cancer model that CARs activated $F4/80^{high}$ TAMs and enhanced production of nitric oxide by TAMs, leading to tumor lysis (58). Further studies are needed to more precisely define the role of myeloid cells in CAR efficacy.

$CD4^+/FOXP3^+$ Tregs are well-documented suppressors of T cell activity that act through multiple mechanisms including cell–cell contact inhibition and via soluble factors such as TGFβ and IL-10 (59). It has been difficult to study the effects of Tregs on CAR therapy because it is difficult to selectively deplete Tregs. For example, depletion using anti-CD25 antibody will also deplete activated CAR T cells. Nonetheless, some studies have been performed using genetic depletion approaches or adoptive transfer of Tregs along with CAR T cells. Zhou et al. (60) studied adoptively transferred cytotoxic T lymphocytes in a mouse leukemia model and found that antibody blockade of PDL1, combined with genetic depletion (using a diphtheria toxin model) of Tregs, markedly increased efficacy of T cell adoptive transfer, although depletion of Tregs alone had relatively minor effects. Our lab has recently conducted studies using a selective inhibitor of Tregs (61) and shown augmentation of mouse CAR T cells targeted to mesothelin (L. Wang, S. Kumar, S. Dahiya, F. Wang, K. Newick, et al., submitted).

Some data in humans suggest an inhibitory effect of Tregs on adoptive T cell transfer. Perna et al. (62) described a model in which the efficacy of human GD2 CARs was inhibited by coinjection of human Tregs with IL-2. An analysis of four T cell adoptive therapy clinical trials employing nonmyeloablative chemotherapy with or without total body irradiation before adoptive T cell transfer revealed that the percentage and number of reconstituting $CD4^+/FOXP3^+$ Tregs observed in the peripheral blood were higher in nonresponders than in responders (63). In addition, the number of administered doses of IL-2 was found to be positively associated with peripheral Treg reconstitution. These latter data highlight the complex role of IL-2 in CAR therapy. Although IL-2 can support CAR T cells in vivo and has been used preclinically and in many

clinical trials (63), it also, and perhaps preferentially, activates and induces proliferation of Tregs (64). Thus, the use of alternative homeostatic cytokines, such as IL-7 and IL-21, was explored and was shown to enhance CAR efficacy (65). The effect of IL-2 in selectively stimulating Tregs may also be an issue in CAR constructs containing the CD28 cytoplasmic domain, which produces much higher levels of IL-2 than do CARs with the 41BB cytoplasmic domain (66).

INTRINSIC REGULATORY MECHANISMS OF T CELLS

In order to maintain tolerance, T cells express activation-induced surface molecules, such as CTLA4 and PD1, that can have antagonistic effects on the overall antitumor immune response, generally restricting the extent and strength of the immune response upon receptor ligation. The importance of these inhibitory receptors has now been established in multiple clinical trials (1). Because these receptors are upregulated on infused CAR T cells and are even further increased on CAR tumor-infiltrating lymphocytes (67), a number of groups have shown that blockade of these receptors can augment therapy. For example, using mouse T cells, a combinatorial strategy of HER2 CAR T cell adoptive transfer and PD1 blockade led to significant tumor regression (68). In experiments studying human CAR T cells in an immunodeficient animal tumor model, our group showed that PD1 blockade using antihuman antibodies enhanced antitumor effects of human mesothelin-directed CARs (67). It is also possible to reverse the inhibitory effects of PD1 by transducing T cells with a PD1 "switch receptor"—that is, the extracellular domain of PD1 fused to the cytoplasmic domain of an activating receptor like CD28 (69, 70). Antibodies against CTLA4 have also been shown to augment adoptive T cell transfer (71).

In addition to surface inhibitory receptors, T cells activate a range of intracellular negative feedback loops after T cell receptor stimulation that work to shut down T cell activity (72). Examples include enzymes, such as diacylglycerol kinase; phosphatases, such as SHP1; ubiquitin ligases, such as Cbl-B; and transcription factors, such as Ikaros. Augmenting CAR T cells' function by reducing the expression or function of these inhibitors is an active area of investigation; for example, CAR T cells lacking expression of diacylglycerol kinase showed markedly increased efficacy (73).

Another process that can limit CAR function is activation-induced cell death. In many cases, this is affected by activation of Fas (CD95) on the T cells through the engagement by Fas ligand that is upregulated in most tumor cells, in tumor vasculature, and on activated T cells. Engagement of Fas induces T cell apoptosis, thereby dampening T cell–mediated immunity. Along these lines, engineering T cells to express higher levels of antiapoptotic proteins has been undertaken (74).

FUTURE PERSPECTIVES AND CONCLUSIONS

Although early trials of CAR T cells for solid tumors have not shown the same success as seen in the leukemia trials, a better understanding of the multiple barriers seen in solid tumors will drive advances in CAR engineering and in clinical trial design. For example, it is currently unclear if the aggressive lymphodepletion suggested for tumor-infiltrating lymphocyte therapy (8) will also be needed for CAR T cell infusion. In preliminary studies from our institution, the use of cyclophosphamide appears to increase blood levels of CARs after infusion, suggesting that some sort of lymphodepletion may be needed in solid tumor therapy.

A number of approaches to overcome solid tumor barriers are discussed in this review, and many other strategies are being tested. To mention just a few, the use of alternative cytoplasmic activation domains, such as ICOS, 41BB, OX40, and CD27, are being explored (75, 76). Wang et al. (77) have fused a scFv for antigen recognition to the transmembrane and cytoplasmic domains of

KIR2DS2, a stimulatory killer immunoglobulin-like receptor (KIR). This KIR CAR, when linked to the adaptor DAP12, proliferated in an antigen-specific manner and demonstrated enhanced effector function.

The compelling success of CAR therapy in hematologic malignancies and the success of tumor-infiltrating lymphocyte infusions in melanoma are propelling the development of CARs that can show similar efficacy in solid tumors. The ability to genetically manipulate infused CAR T cells provides almost limitless opportunities for additional changes and improvements, and thus provides strong hope for future success.

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