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CAR T Cell Therapy: Challenges to Bench-to-Bedside Efficacy

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Abstract

Immunotherapy with T cells genetically modified to express chimeric antigen receptors (CARs) that target tumor-associated molecules have impressive efficacy in hematological malignancies. The field has now embraced the challenge of applying this approach to treat common epithelial malignancies, which make up the majority of cancer cases but evade immunologic attack by a variety of subversive mechanisms. Here, we review the principles that have guided CAR T cell design and the extraordinary clinical results being achieved in B cell malignancies targeting CD19 with a single infusion of engineered T cells. This success has raised expectations that CAR T cells can be applied to solid tumors, but numerous obstacles must be overcome to achieve the success observed in hematologic cancers. Potential solutions driven by advances in genetic engineering, synthetic biology, T cell biology, and improved tumor models that recapitulate the obstacles in human tumors are discussed.

Introduction

Innovations in gene transfer and adoptive T cell transfer (ACT) have converged in a novel approach to cancer therapy in which a patient's T cells are genetically modified to express synthetic chimeric antigen receptors (CARs) that redirect T cell specificity toward tumor-associated antigens. CAR T cells have shown remarkable success in some hematologic malignancies and serve as an example of how advances in immunology can inform a new class of cancer therapeutics (1). Here, we review the principles underlying CAR T cell therapy and discuss obstacles to further improve results in hematologic cancers and extend this approach to common cancers that are the major cause of cancer mortality.

Principles of CAR Design and T Cell Engineering

A CAR is a synthetic construct that, when expressed in T cells, mimics T cell receptor activation and redirects specificity and effector function toward a specified antigen. For cancer therapy, this is accomplished by linking an extracellular ligand-binding domain specific for a tumor cell surface antigen to an intracellular signaling module that activates T cells upon antigen binding. The earliest “first-generation” CARs contained only a CD3ζ or Fc receptor gamma signaling domain (2), and the addition of one (second generation) or more (third generation) costimulatory domains such as CD28, 4-1BB, or OX40 induced more cytokine production and T cell proliferation (3-5). The constellation of signaling modules in a CAR is usually selected based on analysis of tumor recognition *in vitro* and in preclinical *in vivo* models(6-8), and advances in synthetic biology are likely to improve

upon constructs currently in clinical trials. For example, strategies for small molecule-mediated regulatory control of CAR expression (9), combinatorial antigen sensing (10), targeted integration of the CAR transgene into defined loci (11), logic gating of CAR recognition to improve tumor selectivity (12, 13), and suicide mechanisms for targeted elimination of transferred T cells (14, 15) have been described and could provide more potent and safe CARs.

The immune cell chassis used to express a CAR is most commonly a T cell derived from the peripheral blood. Peripheral T cells can be broadly divided by surface phenotype into naïve (T_N), memory (T_M), and effector (T_E) subsets. T_M are further subdivided into memory stem (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and tissue resident memory (T_{RM}) cells, each of which has a distinct role in protective immunity (16-18). Current data supports a progressive differentiation model such that activation of T_N by antigen gives rise to long-lived T_{SCM} and T_{CM} that can self-renew and provide proliferating populations of shorter-lived T_{EM} and T_E cells (19-21). This understanding has led several groups to focus on defining the starting population of T cells that are genetically modified with CARs and used for ACT, initially in preclinical models and subsequently in clinical trials (22-27). Accumulating data suggest that engineering less differentiated T_N and/or T_{CM} cells, or culturing T cells in conditions that preserve these phenotypes, provides CAR T cell products with superior persistence *in vivo* (22-28). Thus, as with CAR design, cell product composition can be manipulated to improve potency and potentially reduce toxicity by providing consistent proliferation and persistence after ACT.

Clinical Efficacy: B Cell Malignancies and Beyond

Clinical trials of CAR T cells have proceeded rapidly in B cell malignancies. B cell malignancies are an attractive target for CAR T cells because they express B cell lineage-specific molecules such as CD19, CD20, and CD22 that are not expressed on other tissues, and preclinical data demonstrated that human B cell tumors could be eradicated in immune-compromised mice treated with CAR T cells (29-32). To prepare CAR T cell products for treatment of patients, T cells are obtained from the blood, activated *in vitro* to facilitate gene insertion, and modified to express the CAR by viral or non-viral gene delivery. CAR T cells are then re-infused into the patient, often after the administration of lymphodepleting chemotherapy to promote engraftment and proliferation of transferred cells (Figure 1). Initial reports in patients with relapsed and/or refractory chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL) and non-Hodgkin lymphoma (NHL) showed remarkable antitumor effects of CD19-specific CAR T cells (33-36). Subsequent larger phase 1/2 trials at a number of centers confirmed the high level of efficacy of CAR T cells, particularly in ALL where complete remission (CR) rates of 70 to 93% are achieved (26-28, 37-40). CAR T cells administered in these studies varied in T cell subset composition, method of gene delivery, cell manufacturing platform, and used either CD28/CD3ζ or 4-1BB/CD3ζ costimulatory domains. Further studies are necessary to determine optimal product characteristics and CAR design.

A majority of patients with CLL and NHL demonstrate tumor regression after treatment with CD19 CAR T cells; however, the CR rates for these lymph node based malignancies

are lower than for ALL (27, 28, 34, 41-43). Defining the reasons for incomplete response in CLL and NHL is important to improve outcomes and is the subject of ongoing research. Combination therapy with checkpoint inhibitors, cytokines, modulators of the tumor microenvironment, improved CAR design, and/or further genetic modifications of the T cells are being studied to improve efficacy. The initial response rates in patients with refractory leukemia and lymphoma are impressive, but the durability of responses will only be established with longer follow-up. CRs have been reported for up to 56 months after CD19 CAR T cell therapy; and CR continues even after the disappearance of functional CD19 CAR T cells and recovery of normal B cells(44). Understanding the factors that correlate with long-term CR or with relapse will be critical to enhancing the efficacy of CAR T cell therapy.

The eradication of large tumor burdens by CAR T cells is not accomplished without toxicity. Cytokine release syndrome (CRS) is a common complication initiated by release of IFN-γ, TNF-α, and IL-2 by activated CD19 CAR T cells is associated with fever, hemodynamic compromise, and macrophage activation with production of IL-6 and additional cytokines (45). The severity of CRS correlates with tumor burden, and interventions to block IL-6 signaling, or to suppress cytokine production by immune cells with dexamethasone, are the mainstays of therapy. Algorithms for timing interventions based on clinical and laboratory parameters are rapidly evolving. Neurologic adverse events are observed concurrent with or following CRS in a subset of patients treated with CD19 CAR T cells, and rare fatal cases have occurred. The pathogenesis of neurotoxicity remains to be elucidated: current data suggest that cytokines released by activated T cells play a role by affecting endothelial integrity(46). Finally, an anticipated side effect of targeting CD19 is that normal $CD19^+$ B cells are eliminated. Transient and even prolonged loss of normal B cells can be managed clinically. However, vector designs that permit elimination of CAR T cells and restoration of B cell numbers are effective in animal models and could be applied in patients that achieve durable remission of their malignancy and have persisting CAR T cells (14).

The success of CAR T cells in ALL, CLL, and NHL has encouraged translation of this approach to other malignancies. CARs have been designed to target molecules such as CD123 and Lewis Y on acute myeloid leukemia (AML) (47, 48). However, none of the targets are as attractive as CD19 due to their expression on other critical hematopoietic cells and/or lack of uniform expression on the tumor. Multiple myeloma expresses several candidate molecules to target with CAR T cells including BCMA and CS1(49, 50), and early clinical data targeting BCMA is promising (51). The application of CAR T cells to common solid tumors has proceeded cautiously following a fatal toxicity in a patient treated with a high dose of ERBB2-specific CAR T cells due to recognition of normal epithelial cells (52). Subsequent studies in patients with glioblastoma and sarcoma suggested it may be possible to target ERBB2 safely, although antitumor efficacy was limited in these studies (53, 54). In glioblastoma, a dramatic response was observed after local intracranial administration of CAR T cells specific for IL13Ra2, and activity of systemically administered CAR T cells specific for EGFRvIII has been reported (55, 56). CAR T cells targeting gD2 have shown activity in patients with Ewing's sarcoma and neuroblastoma (57, 58). Trials using CAR T cells to target mesothelin, Muc16, Muc1, and ROR1 are in progress

(59-63) but, as discussed below, may need to overcome unique obstacles compared to hematologic malignancies.

Barriers to CAR-T Cell Efficacy and Potential Solutions

Tumor Antigen Loss

A challenge for CAR T cell therapy in solid tumors is identifying target antigens expressed homogeneously throughout the tumor and not on normal vital tissues. The success of CAR-T cells in B cell malignancies targeting CD19 is tempered by outgrowth of $CD19^-$ tumor cells in some ALL patients (26, 64, 65). Few targets with homogeneous expression on epithelial cancers have been identified, and outgrowth of antigen-null tumor cells after CAR T cell therapy is an anticipated resistance mechanism. A strategy to circumvent tumor escape is to target multiple antigens simultaneously, such that only tumor cells that lack expression of all target molecules would escape an anti-tumor immune response (66). One way to target multiple antigens is to use promiscuous receptors as the antigen-binding portion of the CAR. NKG2D CARs, for example, target multiple ligands expressed on both tumor cells and immunosuppressive cells, while CARs using the promiscuous ErbB ligand T1E as the extracellular domain can bind multiple ErbB1-based homo- and hetero-dimers that are often overexpressed in tumors(67, 68). Another strategy is to link multiple scFvs in tandem. Several groups have demonstrated that co-targeting CD20 or CD123 in addition to CD19 with bi-specific CAR T cells eliminates CD19 loss variants and is superior to targeting CD19 alone in xenograft models (69, 70). Bi-specific CAR T cells were also superior to mono-specific CAR T cells when targeting antigens with non-uniform expression on solid tumors, such as Muc1 and PSCA for pancreatic tumors, or Her2 and IL-13Ra2 for glioblastoma (71, 72). Of interest, bi-specific T cells showed superior activity *in vivo* compared to 1:1 mixtures of mono-specific CAR T cells targeting the same antigens, although the mechanism behind functional superiority remains unclear. Bi-specific CARs showed enhanced ZAP70 phosphorylation and downstream signaling when both target antigens were engaged, suggesting that dual-positive tumor cells activate bi-specific CAR T cells more efficiently (72). CD19, CD20 and CD22 are attractive for multivalent targeting because they are often co-expressed on B cell malignancies, but identifying other pairs of tumor-associated antigens that are co-expressed on common epithelial tumors but not normal tissues remains a challenge.

Minimizing the escape of antigen-null tumors may also depend on the ability of CAR T cells to induce epitope spreading and engage an endogenous immune response against other tumor-associated antigens. It is possible that CAR T cell-mediated lysis of tumor cells will result in release and cross-presentation of other tumor antigens to endogenous T cells, resulting in a more effective polyclonal anti-tumor response. Some preclinical studies using CAR T cells have demonstrated epitope spreading and even resistance to rechallenge with antigen-null tumors, suggesting the development of immunological memory to other tumor-associated antigens, although this has yet to be demonstrated in patients(73, 74). Mesothelin-targeting CAR T cells were reported to induce humoral epitope spreading in some patients, though not to antigens overexpressed by the tumor or involved in tumorigenesis(60). Co-treatment of CAR T cells with modulators that enhance cross-presentation or activation of

the endogenous immune system may enhance the probability of epitope spreading. For example, CAR T cells secreting IL-12 were able to activate macrophages that mediated elimination of antigen-negative tumor cells in preclinical models(75). Likewise, T cells engineered to express CD40L may better activate cross-presenting $CD8a^+$ dendritic cells, while those expressing 4-1BBL can provide direct co-stimulation to bystander tumor-specific T cells(76, 77). Future studies will be needed to determine whether CAR T cells can be engineered to better engage an endogenous anti-tumor response and whether this can help combat tumor heterogeneity more effectively.

Toxicity to Normal Tissues

Because few truly tumor-specific targets have been identified, applying principles in synthetic biology that might enable CAR T cells to discriminate between tumor and normal cells expressing the same antigen could improve both the efficacy and safety of therapy. Tuning the affinity of the CAR scFv can allow T cells to distinguish between antigens that are overexpressed on tumor cells but expressed at lower levels on normal cells (78). Tumor antigens thought to be unsafe to target due to wide normal tissue expression, thus, may be targetable if expression levels are sufficiently higher on tumor versus normal cells. CARs designed from scFvs targeting either CD38 or EGFR with ~1000-fold reduced affinity conferred effective lysis of tumor cells but spared antigen-positive normal cells (79, 80). Whether this could truly achieve discrimination of tumor and normal cells based on level of antigen expression in clinical settings without the outgrowth of antigen low tumor cells remains to be determined.

Another strategy to increase tumor-specificity is to use “AND” logic gates that require recognition of two different antigens on the same target cell to elicit full CAR T cell activation (13). The success of this strategy requires identifying antigen pairs that are selectively co-expressed on tumor cells but not normal tissues. One implementation of this strategy is to split the CD3ζ signaling and CD28 co-stimulatory domains across separate receptors, with each signaling domain linked to an scFv specific for a different antigen (81). However, several studies employing such split-receptor systems have found that CD3ζ signaling alone is sufficient to induce some T cell effector functions, including lysis of single-positive cells(66, 81), suggesting toxicity to single-positive normal tissues may not be avoided. This problem could be solved by using a low-affinity scFv that is incapable of inducing T cell activation when linked only to the CD3ζ signaling domain (13). Development of such dual-signaling CAR T cells is likely to require further optimization of each individual scFv. Other aspects of antigen pairs, such as size and ability to co-localize in the synapse, might also affect their ability to properly activate T cells.

Several groups have built constructs in which CAR expression is regulated by a drug-inducible promoter or in which the recognition domain and signaling domain are only associated together in the presence of a small molecule dimerizer (9, 82, 83). CAR T cells can be transiently activated *in vivo* by drug administration, and their activity can theoretically be halted if toxicity occurs by withdrawal of the drug. Alternately, timing and location of drug delivery can be adjusted to minimize toxicity. For example, Her2-specific CAR T cells induced rapid pulmonary toxicity as a consequence of recognition of $Her2^+$

cells in the lung (52). If a CAR-inducing drug was delivered several days after infusion, when the majority of intravenously infused T cells have migrated out of the lung, toxicity to normal cells in the lung might be diminished or averted. Likewise, by delivering the drug locally rather than systemically, CAR T cell activity could be restricted to particular tissue compartments.

Engineering T cells in which CAR expression is regulated by input signals found primarily in the tumor microenvironment (TME) is another potential strategy. Tumors are often hypoxic, and oxygen-sensitive CAR T cells have been designed by fusing the CAR to a subdomain of the hypoxia transcription factor HIF-1α that is sensitive to protein degradation under normoxic conditions (84). Likewise, Roybal et al. engineered a synthetic Notch ("synNotch") receptor that upon recognition of one tumor-associated antigen releases a transcription factor that induces expression of a CAR specific for a second tumor-associated antigen (10). The advantage of these approaches is that CAR expression is restricted to the local tumor environment, minimizing the potential for off-tumor toxicity. However, as with "AND" gate CAR T cells, clinical application of the synNotch strategy requires the identification of two tumor-associated antigens that are co-expressed in the tumor but do not overlap in their normal tissue expression. It is also uncertain whether the kinetics of CAR degradation after signal 1 is disengaged will be sufficiently rapid to minimize off-tumor toxicity. Despite these limitations, both approaches can theoretically improve tumor-selectivity, and may prove advantageous in settings where antigen-positive tumor cells and normal tissues are spatially segregated, such that CAR expression is fully degraded before T cells that leave the tumor site encounter antigen-positive normal cells.

Trafficking to Solid Tumors

Analysis of the TME has identified a variety of obstacles such as trafficking, immunosuppressive molecules and cells, and immune checkpoints that CAR T cells will need to overcome to be effective in solid tumors (Figure 2). The efficacy of CAR T cells in hematological malignancies in part may reflect efficient access to tumor cells in the bone marrow and lymph nodes where T cells normally traffic. Recognition of solid tumors requires egress from the blood into the tumor site, and many malignancies evolve such that T cell infiltration is actively impeded (85-87). In situations where the tumor is localized, regional rather than systemic administration of CAR T cells might be effective. Intracranial delivery has been shown to be safe and to have antitumor activity in glioblastoma (56), and intra-pleural delivery of CAR T cells was superior to systemic administration in preclinical studies of human pleural malignancy (88).

Improved understanding of mechanisms that promote or exclude T cell infiltration into tumors are likely to create opportunities to improve CAR T cell trafficking, either by additional genetic modification of T cells (89), or by combining CAR T cells with oncolytic viruses or other strategies that promote inflammation at the tumor site (90),(91). CAR T cells can be engineered to express receptors like CCR2 and CCR4 that are specific for chemokines naturally overexpressed by tumors, enabling them to traffic more efficiently to tumors (92, 93) (94). Rather than custom engineering T cells to the chemokine profile of individual tumors, a more generalizable strategy is to induce tumors to secrete chemokines

that CAR T cells are already responsive to. An oncolytic virus has been used to deliver the chemokine CCL5 (RANTES) to the tumor. CAR-T cells already express receptors (CCR1, CCR3, and CCR5) for CCL5, and combination therapy with CCL5-expressing oncolytic virus and CAR T cells synergistically improved survival and tumor clearance in preclinical models (90).

CAR T cell access to tumors might also be improved by combining adoptive therapy with drugs that induce immunogenic cell death (ICD) of tumor cells. Unlike physiological cell death, in which dying cells are cleared without an inflammatory response, ICD induces release of damage-associated molecular patterns (DAMPs), which directly activate dendritic cells to secrete T cell-attracting chemokines and cross-present tumor antigens (95). Local radiotherapy and certain chemotherapeutic agents induce ICD and activate endogenous T cell responses to tumor antigens (96, 97). These modalities also inhibit or eliminate immunosuppressive cell subsets in the TME, resulting in an overall shift to a pro-inflammatory state and improved immune responses (96-98) (99). A similar regimen may improve CAR T cell infiltration by inducing production of chemokines and creating a favorable environment for CAR T cell function. Unlike genetic engineering-based approaches, such combination therapy has the advantage of modulating multiple immune pathways at once.

The differentiation state of the T cells selected for CAR modification can also influence CAR T cell function and migratory properties *in vivo*. Central memory T cells (T_{CM}) have superior anti-tumor function relative to effector memory T cells (T_{EM}) in xenograft models of hematological malignancies due to superior persistence and proliferation (22, 24, 25). However, T_{EM} express higher levels of chemokine receptors and adhesion molecules required for homing to inflamed peripheral tissues and may be better poised to enter solid tumor sites. Despite these attributes, a recent study demonstrated that *in vitro*-generated T_{EM} expressing a gp100-specific TCR were less effective on a per-cell basis than T_{CM} of the same antigen-specificity against B16 tumors (23). *S*uperior activity was dependent on the ability of T_{CM} to traffic first to secondary lymphoid organs rather than peripheral tissues, which may be necessary to engage antigen-presenting cells (APCs) in tumor-draining lymph nodes. CAR T cells, however, do not depend on interactions with APCs for activation, and one study demonstrated that CAR T cells engineered from $CCR7^-$ T cells accumulated better within solid tumors than those derived from $CCR7^+$ T cells (100). These CAR T cells were more prone to activation-induced cell death (AICD), but when CD28 and OX40 co-stimulation were incorporated into the CAR construct, AICD was reduced such that $CCR7^-$ CAR T cells were more effective at clearing tumors than $CCR7^+$ CAR T cells. Thus, the best T cell subset for CAR T cell therapy for solid tumors may differ from the subset suited for hematological malignancies, or from the subset used for TCR-based T cell therapy. Further research is likely to define genetic manipulations of specific T cell subsets that endow the cells with homing and functional properties needed to infiltrate and effectively target solid tumors.

Overcoming the Immunosuppressive Tumor Microenvironment

Migration of CAR T cells into tumor sites is not sufficient to ensure tumor destruction because of the immunosuppressive TME (Figure 2). Low pH, hypoxia, an absence of vital nutrients, and stromal and immune cells that release suppressive factors are characteristic of the TME and inhibit T cells. Additionally, tumor and infiltrating cells may express inhibitory receptor ligands like PD-L1 that can directly suppress tumor-specific T cells.

Several groups have attempted to enhance CAR T cell activity by combining ACT with modulators of the TME. A promising avenue is the use of checkpoint inhibitors that target the PD-1/PD-L1 or CTLA-4 pathways, which alone have shown efficacy in some cancers (101). Responsiveness to checkpoint blockade was improved by enhancing priming of tumor-specific T cells and might logically be combined with adoptive transfer of CAR T cells, although the risk of toxicity to normal tissues may be increased (99) (102, 103). Other groups have engineered CAR T cells to secrete anti-PD-L1 antibodies (104), knocked out PD-1 and Lag3 using CRISPR (105-107), or co-expressed “switch receptors” linking the PD-1 ectodomain to the CD28 endodomain such that engagement of PD-L1 delivers an activating rather than inhibitory signal to the T cell (108, 109). Anti-CTLA-4 antibodies can also boost endogenous T cell responses to tumors, but the context in which they might improve CAR T cell responses is unclear. CTLA-4 inhibits T cell responses in part by competing with CD28 for binding to CD80/CD86 on dendritic cells and by physically excluding CD28 from the synapse (110). Thus, CAR T cells with a CD28 signaling endodomain may not be intrinsically affected by CTLA-4 regulation. This is supported by a study demonstrating that shRNA-mediated knockdown of CTLA-4 improved the function of first generation CAR T cells *in vivo* but not second generation CAR T cells with CD28 signaling domains (111). However, anti-CTLA-4 antibodies also promote immune responses in a cell extrinsic fashion by depleting CTLA-4^+ Treg cells(112, 113), which may benefit CAR T cells. In addition to inhibitory receptor expression, T cell dysfunction may be acquired in the TME by dysregulation of signaling pathways through upregulation of SHP-1 or diacylglycerol kinase (DGK), and pharmacologic inhibition of these enzymes can improve the anti-tumor function of CAR T cells(114, 115).

Overcoming immunosuppressive cells in the TME is likely to be necessary for CAR T cell efficacy. Depletion of regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) with blocking antibodies or genetic manipulation has improved the efficacy of T cell therapy in animal models(116-118). Cancer-associated fibroblasts (CAFs), which comprise a majority of tumor stromal cells and express high levels of fibroblast activation protein (FAP), play a central role in establishing the immunosuppressive microenvironment and depositing extracellular matrix (ECM) proteins to limit T cell penetration. Targeting CAFs with FAP-specific CAR T cells or engineering CAR T cells to secrete ECM-degrading enzymes improves their ability to infiltrate and lyse tumors (73, 119). Alternatively, engineering CAR T cells to express the pro-inflammatory cytokine IL-12 can modulate the TME and promote recruitment and activation of macrophages (73, 75, 120, 121).

A number of studies have focused on improving CAR T cell activity by altering their metabolic profiles to enhance cell function in hostile environments. Tumors are often characterized by high levels of adenosine and reactive oxygen species (ROS), both of which

directly impair T cell responses (122, 123). Knocking down the adenosine 2A receptor with shRNA or co-transducing T cells with catalase to enable breakdown of ROS significantly improved CAR T cell persistence and function *in vivo* (124, 125). Likewise, tumors display elevated levels of extracellular potassium that directly impair TCR-driven Akt-mTOR phosphorylation and effector function. Engineering T cells to overexpress a potassium channel to enable greater potassium efflux effectively undoes this mode of suppression and improves T cell function within the tumor (126).

Overall, CAR T cells face a number of hurdles in combating solid tumors, but some obstacles may be easier to overcome than others. Advances in genetic engineering are proceeding rapidly, and our ability to engineer CARs that, for example, target multiple antigens to overcome tumor heterogeneity and antigen-loss or that co-express modulators of the TME are now undergoing clinical evaluation. On the other hand, one of the highest barriers to success may be the ability of CAR T cells to infiltrate solid tumors. A number of studies have shown that the success of checkpoint inhibitors depends on the presence of tumor-infiltrating T cells, and strategies aimed at enhancing T cell infiltration can overcome tumor resistance to checkpoint inhibition(99, 127). Increasing CAR T cell migration to tumors could potentially increase the response of tumors to other combination therapies as well, such as those aimed at targeting immunosuppressive cells, enhancing CAR survival, and activating the endogenous immune response. Thus, efficient infiltration of tumors is likely to be a rate-limiting step for CAR T cell therapy.

Moving Beyond Empirical Testing

Given the myriad ways in which tumors can suppress T cells, the number of genetic manipulations and combination therapies that could be tested in the clinic are seemingly limitless. A challenge for the field is the need for faithful preclinical models to screen therapeutic combinations before clinical translation. Better tools to analyze post-treatment biopsies will also help maximize our understanding of what resistance mechanisms may evolve and inform the design of future combination therapies.

Models for CAR T Cell Therapy

A challenge for preclinical studies evaluating the efficacy of CAR T cells is having clinically relevant models that recapitulate the obstacles in human solid tumors (Figure 3). Most studies have relied on transplanted human tumor xenografts in immune-compromised NOD/SCID/$\gamma c^{-/-}$ (NSG) mice that lack T cells, NK cells and B cells. The NSG model allows rapid analysis of human T cell recognition of tumor cells *in vivo* and is useful to evaluate T cell persistence and effector function. However, NSG models fail to develop a clinically relevant TME and do not inform the safety of targets that lack epitope homology and/or normal tissue expression. ACT is also studied in immune-competent syngeneic mouse models but a majority of these models implant tumor cells into foreign anatomical sites where the tumors grow rapidly and do not co-evolve with the host immune system in the same way as human tumors (128). Moreover, endogenous anti-tumor immune responses are higher in transplanted than autochthonous tumors for the same disease, suggesting the implantation process artificially increases immunogenicity (129, 130).

An alternative to transplantable models is to induce malignant transformation in normal cells *in situ* with defined oncogenic events. Such genetically-engineered mouse (GEM) models recapitulate tumor initiation, progression, and the genetic and histopathological characteristics of human cancers (128). Oncogenic mutations in Kras and p53 can be introduced at birth through a Cre/lox system, such as in the KPC ($Kras^{LSL\text{-}G12D/+}$; $p53^{f/f}$; Pdx1-Cre) model of pancreatic adenocarcinoma, where a Pdx1-driven Cre restricts the mutations to the pancreas (131). A disadvantage of tissue-restricted Cre expression is that cancer is induced throughout the entire tissue, and the presence of mutations from birth may affect central tolerance and influence the evolution of immune responses differently than if mutations were acquired postnatally. To address this, Tyler Jacks' group developed an inducible “KP” GEM model of lung adenocarcinoma in which intratracheal infection with Cre-expressing lentivirus initiates p53 deletion and $Kras^{G12D}$ activation in individual lung epithelial cells (132). Importantly, this model mimics both the development and therapeutic response of human lung adenocarcinomas (99). A drawback of GEM models, however, is their relative lack of CAR targets and neoantigens relative to carcinogen-induced models and human cancer(133). However, model antigens can easily be introduced in the KP model by engineering the lentivirus, and exposing Kras-mutant GEM mice to tobacco smoke can induce a more realistic mutational landscape.

GEM models that reflect the TME of human tumors may give more accurate estimates of treatment efficacy and offer insight into resistance mechanisms that evolve and pathways to target with combination therapy (134). For example, recent studies have used GEM models to study Tregs in tumor development and test strategies for Treg inhibition. One approach to Treg inhibition may be to block IL-35, an immunosuppressive cytokine secreted by tumor-resident Tregs that promotes effector T cell exhaustion in part by promoting expression of inhibitory receptors like PD-1, Tim3, and Lag3 (135). Interestingly, this model accurately predicted that IL-35 and PD-1 blockade would not synergize since IL-35 overexpression and PD-1 upregulation are part of the same suppressive pathway. Thus, it is anticipated that GEM models will be useful for studying impediments to CAR T cell therapy of solid tumors and for identifying rational combination therapies for clinical translation.

Advances in Immune Monitoring of Clinical Trials

Identifying methods to enhance CAR T cell therapy will be assisted by discovery-driven approaches to clinical trials. Collecting tumor biopsies and blood pre- and post-treatment, enables thorough analysis of tumors by flow cytometry, immunohistochemistry, and unbiased genome-wide RNA sequencing and can identify correlates of clinical success or failure. Response to anti-CTLA-4 therapy of localized bladder cancer, for example, was associated with up-regulation of ICOS on T cells(136, 137); subsequent studies in mice demonstrated that ICOS expression was required for the efficacy of anti-CTLA-4 *in vivo* and that activation of the ICOS/ICOSL pathway synergistically enhanced response to anti-CTLA-4 (138, 139). Similar analysis of pre- and post-treatment biopsies and transcriptomic and epigenetic analysis of CD19 CAR T cells are being performed to identify mechanisms of resistance in the approximately 50% of NHL patients that do not achieve a complete remission, and where CD19 loss is not the mechanism of escape (27, 28, 42, 140).

New technologies with improved sensitivity and systems-analysis will facilitate the identification of pathways associated with therapy response or resistance. Single-cell RNA sequencing can provide unbiased insight into tumor responses, revealing differences in gene transcription that may be obscured by heterogeneity at the cell population level. At the protein level, methods such as CyTOF allow analysis of 40 (and up to 100 theoretically) proteins simultaneously, providing high resolution of cell phenotype, and can be coupled to immunohistochemical methods to obtain spatial information of proteins and protein modifications at subcellular resolution (141). Additionally, the development of multiplex immunohistochemistry allows detection of multiple biomarkers simultaneously on tumor biopsies and visualization of cell subsets with tissue architecture preserved. Integrating longitudinal data from gene expression, epigenetics, flow and mass spectrometry, and IHC will provide a comprehensive understanding of patient responses to therapy and should guide the development of rational, rather than empirical, combinations.

Conclusions

Progress in immune-based therapies is improving outcomes for many patients with advanced malignancies. The development of CAR T cells represents a convergence of insights from multiple scientific fields, but success has thus far been limited to B cell malignancies. Extending this approach to other cancers will require the development of strategies based on understanding the obstacles posed by tumor heterogeneity and the tumor microenvironment that is emerging from sophisticated analytic tools and superior models. These strategies will take advantage of our unprecedented ability to genetically manipulate T cells to confer novel functions, enabling them to target tumor cells and persist and function in hostile circumstances.

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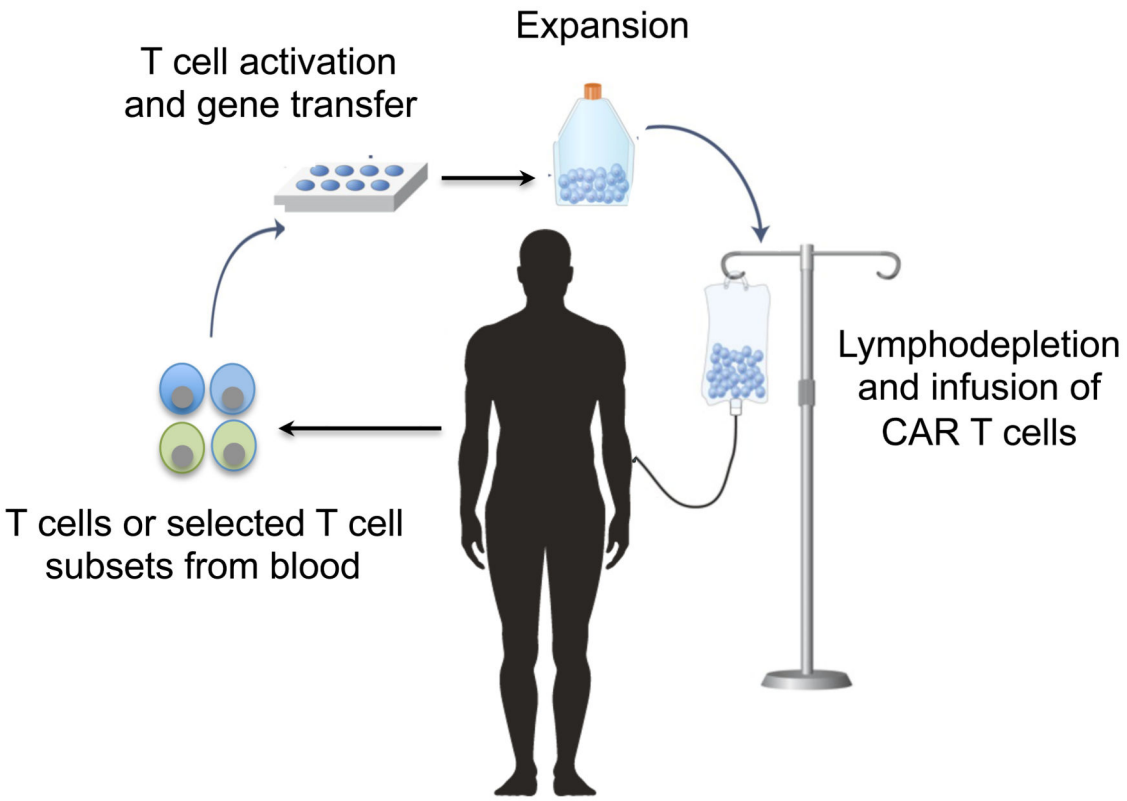


Figure 1. Adoptive cell therapy with chimeric antigen receptor (CAR)-modified T cells

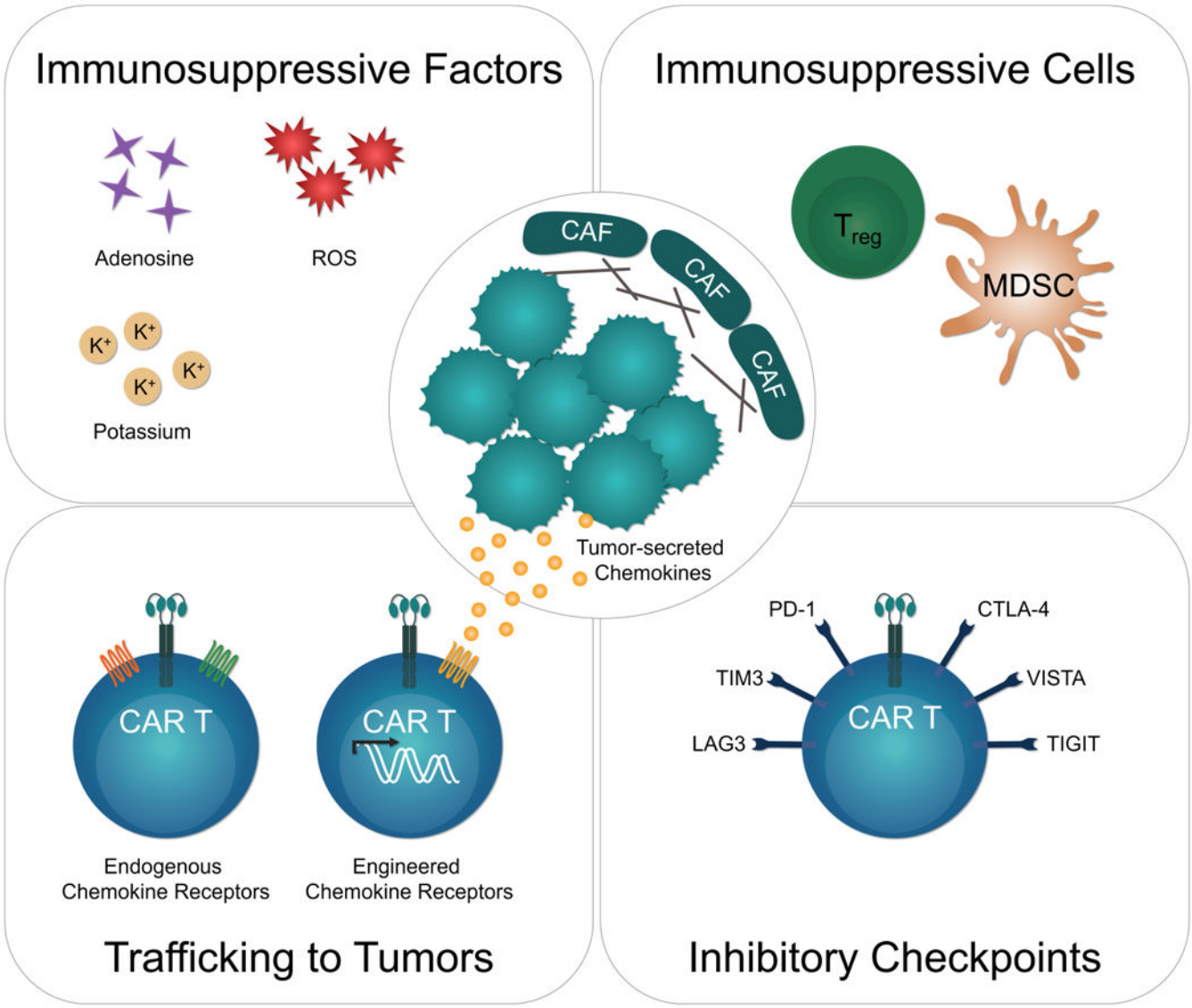


Figure 2. Barriers to CAR T cell therapy for solid tumors

Bottom left: CAR T cell trafficking depends on expression of receptors for chemokines secreted by the tumor. CAR T cells endogenously express chemokine receptors like CXCR3 and CCR5, but their cognate ligands are often not highly expressed by solid tumors. CAR T cells can be engineered to express receptors (e.g. CCR2, CCR4) for chemokines naturally secreted by the tumor to improve trafficking to tumors. **Bottom right:** Antigen-activated CAR T cells in the tumor microenvironment up-regulate expression of inhibitory receptors which can lead to T cell dysfunction. **Upper left:** Tumor microenvironments are rich in factors like adenosine, extracellular potassium, and reactive oxygen species (ROS), which can inhibit T cells directly or indirectly. **Upper right**: Immunosuppressive cells like regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) can promote tumor growth and inhibit T cell activity both directly and indirectly. Cancer-associated fibroblasts (CAF) deposit extracellular matrix to limit T cell penetration and can recruit other immunosuppressive cells.

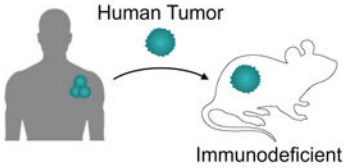
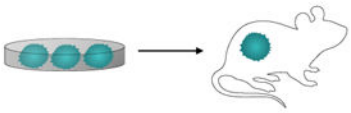
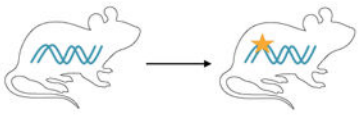
	Pros	Cons
Xenogeneic Transplantable 	• Predicts persistence and lytic ability of human CAR T cells • Predicts drug response of human tumors • Technically easy to use	• No host immune system • No tumor microenvironment • Cannot answer questions about T cell trafficking, function within the tumor microenvironment, or interaction with host immune cells
Syngeneic Transplantable 	• Intact host immune system • Some TME develops • Cell lines can be engineered to express neoantigens or tumor targets • Technically easy to use	• Tumors are fully mature at implantation and do not co-evolve with host immune system • Transplantation process can be artificially immunogenic • TME is present but artificial
GEM Spontaneous 	• Tumors develop from clinically relevant mutations and evolve naturally with host immune system • Realistic TME develops	• Oncogenic mutations are present from birth; central tolerance may develop to mutations or neoantigens • Model is slow and variable • Time to tumor development is variable • Introducing neoantigens or tumor targets requires complex breeding
GEM Inducible 	• Tumors develop from clinically relevant mutations and evolve naturally with host immune system • Realistic TME develops • Tumor initiation can be synchronized by inducible event • Neoantigens/tumor targets can be introduced easily	• Model is slow and variable • Technically complex

Figure 3. Comparison of mouse models for human solid tumors