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Strategies for enhancing adoptive T-cell immunotherapy against solid tumors using engineered cytokine signaling and other modalities

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Abstract

Introduction: Cancer therapy has been transformed by the demonstration that tumor-specific T-cells can eliminate tumor cells in a clinical setting with minimal long-term toxicity. However significant success in the treatment of leukemia and lymphoma with T-cells using native receptors or redirected with chimeric antigen receptors (CARs) has not been recapitulated in the treatment of solid tumors. This lack of success is likely related to the paucity of costimulatory and cytokine signaling available in solid tumors, in addition to a range of inhibitory mechanisms.

Areas covered: We summarize the latest developments in engineered T-cell immunotherapy, describe the limitations of these approaches in treating solid tumors, and finally highlight several strategies that may be useful in mediating solid tumor responses in the future, while also ensuring safety of engineered cells.

Expert opinion: CAR-T therapies require further engineering to achieve their potential against solid tumors. Facilitating cytokine signaling in CAR T-cells appears to be essential in achieving

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Declaration of Interest

T Shum has filed patents related to adoptive T-cell therapy from Celgene Corporation. RL Kruse has filed a patent on CAR T-cell technology from Celgene Corporation. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed. Peer reviewers on this manuscript have no relevant financial or other relationships to disclose

better responses. However, the engineering of T-cells with potentially unchecked proliferation and potency raises the question of whether the simultaneous combination of enhancements will prove safe, necessitating continued advancements in regulating CAR-T activity at the tumor site and methods to safely switch off these engineered cells.

Keywords

Cancer; chimeric antigen receptor (CAR); cytokine; immunotherapy; synthetic biology; T cell

1. Introduction

Steady preclinical and clinical progress has moved adoptive T-cell immunotherapy into the spotlight of cutting edge medicine. The first evidence of efficacy with adoptive immunotherapy was achieved with tumor-infiltrating lymphocytes (TILs) [1]. Supporting the principle that native T-cell receptors (TCRs) can recognize tumor antigen fragments presented on major histocompatibility class (MHC) molecules, TILs extracted from patient tumors, expanded in *ex vivo* culture conditions, and re-infused into patients could achieve objective tumor responses. Recently, T-cells expressing chimeric antigen receptors (CARs) have become a popular technology platform to attack leukemia and lymphoma. With the FDA approval of Novartis's tisagenlecleucel, and Kite's axicabtagene ciloleucel, CD19 CAR T-cell therapy for pediatric B-cell precursor acute lymphoblastic leukemia (ALL) and adult diffuse large B-cell lymphoma, respectively, interest has grown around the possibility of achieving similar success against solid tumors. Relapsed and metastatic solid tumors continue to resist treatment with current medical practices, but breakthrough results with CAR T-cell therapies against solid tumors have not been achieved. We have focused our review on the successes of adoptive immunotherapy, its shortcomings when applied to solid tumors, and the combinatorial solutions that are likely necessary to increase clinical efficacy in treating cancer.

2. Overview of Chimeric Antigen Receptors

CARs were first described as a fusion of an extracellular single chain fragment variable chain (scFv) with the ζ intracellular signaling domain from the T-cell receptor [2]. This invention, when introduced into T-cells through retroviral vector transduction, permitted the facile manufacture of large quantities of T-cells that recognize tumor-associated antigens (**Figure 1**). It was soon recognized, however, that these engineered T-cells required additional signals to proliferate, release inflammatory cytokines and orchestrate an effective immune response [3,4], since clinical evaluation of first generation CARs revealed limited efficacy [5–7]. This led to the incorporation of costimulatory endodomains into CARs, beginning with CD28 and subsequently extending to molecules such as OX40 and 41BB from the tumor necrosis factor (TNF) receptor family. These new CARs were dubbed “second generation” if they included a single costimulatory endodomain addition (such as CD28. ζ or 41BB. ζ), or “third generation” if they included two costimulatory endodomains (such as CD28.41BB. ζ or CD28.OX40. ζ) [8–11]. With these improved functional CAR backbones, the immunotherapy community could interrogate different cell surface target

antigens for CAR T-cell recognition of human tumors. These targets are extensively reviewed elsewhere [11].

2.1 Efficacy of tumor-redirection CAR T-cells against leukemia

Targeting CD19, a B-cell antigen, with CAR T-cells has produced highly effective responses in patients with treatment refractory leukemia. In a pioneering case report, a single infusion of 10 million CD19-specific CAR T-cells (modified with a lentiviral vector and including a 41BB endodomain) expanded 1000-fold after infusion and eradicated chronic lymphocytic leukemia (CLL) in a patient who had already failed multiple drug regimens [12]. Additional CLL patients treated with this therapy experienced similarly dramatic and long-lasting remissions [13]. CD19-CAR T-cells using an identical design were then shown to produce complete remissions in 90% of cases of drug-refractory pediatric acute lymphoblastic leukemia [14]. Similarly, CD19-CAR T-cells generated with a retroviral vector and utilizing a CD28 endodomain produced robust results in relapsed adult ALL patients [15]. However, complete responses were not always long-lasting, as antigen-loss escape was seen due to the emergence of leukemia cells expressing CD19 molecules that had *de novo* frameshift or missense mutations, as well as alternatively spliced CD19 variants, allowing the target cells to escape recognition by the CD19-CAR scFv [16]. These breakthrough results serve as a powerful validation for the therapeutic potential of immunotherapy and would suggest that similar breakthroughs may happen for CAR T-cell therapies against solid tumors.

2.2 Efficacy of tumor-redirection CAR T-cells against solid tumors

In contrast to the success seen in liquid tumors so far, aggressive adoptive cell therapy regimens have been needed to achieve even modest results in solid tumors. The first of these studies utilized TIL technology because CAR molecules had not been developed at the time. High doses of TILs with concurrent infusions of high-dose IL-2, in conjunction with extensive preconditioning of patients with cytotoxic chemotherapy and even radiation therapy, have generally been necessary for adoptive cell therapy to succeed in solid tumors [17]. Ultimately these early studies concluded that in order to achieve objective immune reduction of tumor burden in melanoma, colorectal, renal, and lung malignancies [1], expanded TILs must be administered with high-dose cytokine support and preceded by aggressive lymphodepletion regimens. Furthermore, while long lasting complete remissions could be achieved in patients with local disease, metastasized lesions were more difficult to treat and only partial remissions could be obtained. Since then, other adoptive cell therapies for solid tumors have not provided an exception to this rule.

Consistent with this trend, our clinical studies found that Epstein-Barr virus (EBV) specific T-cells could generate long-lasting complete remissions in patients with locoregional nasopharyngeal carcinoma without using lymphodepletion [18]. However, long-term follow-up of patients in subsequent studies revealed that clinical remissions were eventually followed by disease relapse in the majority of cases [19,20]. Some improvement was observed in a subsequent study using lymphodepletion prior to adoptive cell transfer in Singapore, achieving a 40% patient survival at 48 months (compared to the 11–22 months of overall survival in patient receiving conventional treatment) [20].

T-cells expressing a first generation CAR have shown some promise against solid tumors [6], but were in general limited in efficacy, as reviewed elsewhere [21]. To determine if second generation CAR modified T-cells have improved efficacy due to *in situ* provision of costimulation, our group used HER2/neu-redirected CAR T-cells, which delayed sarcoma progression in several patients, allowing them to receive surgical resections of residual tumor burden and consequently enter remission [22]. A modification of this method was later made for clinical evaluation in glioblastoma patients by expressing HER2-CAR in T-cells specific for CMV, adenovirus and EBV with the hope that TCR stimulation by endogenous viruses would increase T-cell persistence *in vivo*. This resulted in disease stabilization in three patients who were alive with stable disease 24, 28, and 29 months post infusion at the time of reporting [23].

A common finding from these studies was that HER2 CAR T-cells did not expand *in vivo*, at least as measured in peripheral blood, although they persisted at low levels for up to 42 days. However these studies did not use cytotoxic lymphodepletion to enhance the expansion of the infused CAR T-cells. Similar results were seen in targeting glioblastoma with EGFRVIII CAR T-cells[24]. Interestingly, analysis of glioblastoma tissue retrieved from patients after treatment with EGFRVIII CAR T-cells revealed that the majority of tumors had downregulated EGFRVIII antigen density, which again highlights antigen-escape as a mechanism for CAR T-cell therapy failure. One strategy to compensate for poor *in vivo* expansion and persistence is local delivery of CAR T-cells, as opposed to peripheral infusion of CAR T-cells. Intraventricular delivery of multiple doses of IL13R α 2 CAR T-cell in patients with relapsed glioblastoma produced a 7 month complete remission in one patient, although relapse was eventually observed [25]. Taken together, these clinical trials have made promising inroads, but there is still ample room for improvement in the efficacy of T-cells against solid tumors.

In addition to tumor antigen escape, tumor antigen heterogeneity is also a challenge for CAR T-cell therapy. Unlike CD19, many tumor-associated antigens have varying levels of expression within the bulk tumors found in a clinical population. Since CAR T-cells cannot form effective immunological synapses with tumor targets if the target antigen is expressed at insufficient levels, this provides a mechanism of tumor escape. Bivalent or trivalent CAR T-cell strategies have been studied as a promising mechanism to optimize CAR T-cell activation even against tumors expressing heterogeneous or limited levels of antigen [26], but this remains to be evaluated in a clinical trial. While it has been shown that the inflammatory response caused by tumor-specific T-cell therapy can enable endogenous T-cells to eliminate antigen-loss tumor variants, the results from several CAR T-cell clinical studies do not suggest that this effect is sufficiently induced, if at all[16,24,25]. Indeed, cytotoxic lymphodepletion may eliminate TILs and hence prevent epitope spreading. Therefore, to counter tumor antigen escape as well as tumor antigen heterogeneity, effective methods to drive tumor neoantigen recognition during and after CAR T-cell administration must be developed.

2.3 Costimulatory molecule and cytokine differences between leukemias and solid tumors

Clinical success using adoptive cell therapies against solid tumors will likely require large numbers of tumor-specific lymphocytes to persist within a therapeutic window. This may be achieved by increasing the numbers of infused T-cells and the frequency of dosing, or by enhancing their capacity for expansion and persistence *in vivo*. The latter may be achieved by providing immunostimulatory cytokines and by enabling T-cells to resist the physical and soluble components of the immunosuppressive tumor stroma.

To understand how to increase the persistence of T-cells in solid tumors, a comparison of the cytokines and costimulatory molecules available in solid tumors and in the B-cell malignancies that have been treated successfully by CD19-CAR T-cells may be helpful. The importance of immunostimulatory cytokines is evident if one revisits the three signals required for optimal T-cell activity: Signal 1, activation through the T-cell receptor (TCR); signal 2, activation through costimulatory receptors; and signal 3, activation through immunostimulatory cytokine receptors [27]. The immune system uses regulation of these three signals to modulate the strength and potency of immune responses. Current CAR T-cell designs provide only signals 1 and 2, and while they can transiently produce IL-2 after the initial antigen stimulation, CAR T-cells generally lack the long-term ability to activate sufficient signal 3. A reasonable question to ask, then, is how the CD19-CAR T-cell therapies have gained so much success in clinical trials without external cytokine support?

The first crucial source of cytokines is provided by lymphodepletion, which has proved essential for the initial burst of CAR T-cell expansion by liberating homeostatic cytokines that would otherwise maintain homeostatic levels of naïve and memory T-cells. Second, lymphoid organs, including the bone marrow, which is the physiologic location of B-ALL, are costimulation and cytokine-rich. Normal and malignant B cells can furnish signals 1 and 2 in tandem through their expression of costimulatory molecules, and thus supplement CAR-T signaling, which is non-physiological and may be suboptimal. CD19-CAR T-cell engagement of B-ALL blasts and normally developing B-cells in the bone marrow puts them in the proximity of homeostatic cytokines, IL-7 and IL-15, produced by the fibroreticular cells within the bone marrow stroma [28–33]. Altogether, this theater of combat may reproduce the triumvirate of T-cell activating signals [27], and likely provides an ideal environment to incubate CD19-CAR T-cell growth. Accordingly, approximately 1,000–100,000 fold expansion of CD19-CAR T-cells *in vivo* after adoptive transfer has been observed in patients [12,34,35].

Such favorable conditions are not present within solid tumors. Immunostimulatory cytokines are likely absent or present at insufficient quantities, a conclusion inferred by the fact that immune-mediated tumor shrinkage or eradication is facilitated when immunostimulatory cytokines are expressed as transgenes by tumor cells at the tumor site [36]. Without the presence of immunostimulatory cytokines, T-cells that have trafficked to the tumor microenvironment lack the cytokine support to expand and persist in their fight against the tumor. Furthermore, most solid tumors do not present activating costimulatory ligands. The tumor microenvironment is instead filled with immunosuppressive cytokines and ligands. Tumor cells actively drive the formation of an immunosuppressive milieu that fosters the

growth and expansion of cell types such as macrophages, neutrophils, myeloid derived suppressor cells, and endothelial cells, which can inhibit adoptively transferred T-cells [37].

While lymphodepletion can provide an initial immunostimulatory boost, the resultant CAR T-cell expansion is transient as endogenous T-cell reconstitute [38]. For adoptively transferred T-cells to persist and be effective in the solid tumor microenvironment, additional immunostimulatory approaches may be necessary. The maintenance of high numbers of TILs, for example, has required the maximum tolerated doses of IL-2. This supports an aggressive treatment approach in the case of solid tumors, and progress in this area will probably be seen only if cytokine support is sufficient to produce prolonged expansion of functional tumor-specific T-cells at the tumor site.

3. Restoration of Signal 3 to improve the persistence of T-cells

The ability of cytokines to sustain the expansion of adoptively transferred T-cells has been demonstrated using a number of different strategies. Tumors expressing a membrane-bound IL-15 stimulated the proliferation of T-cells specific for a low affinity tumor epitope, suggesting that cytokines lower the activation threshold in T-cells [36]. In a study of injected cytokines to enhance T-cell activation by a dendritic cell vaccine, IL-7 or IL-15 (but not IL-2) enhanced the expansion of memory T-cells specific for subdominant tumor antigens [39]. The cytokines, IL-7, IL-12, IL-15, and IL-21, have all been studied in the context of adoptive immunotherapy [40–42]. In combination, IL-15 and IL-21[43] enhanced the formation of memory T-cell populations that were associated with control of the B16 melanoma in mice.

Unfortunately, clinical studies lag behind preclinical studies, in part because of the difficulty of balancing systemic side effects versus local antitumor efficacy. The complexity of cytokine administration has been illustrated with a number of “cytokine only” clinical trials where patients received cytokines as a solo treatment. The therapeutic benefits of high-dose IL-2 for renal cell carcinoma and as a general adjunct in adoptive cell therapy has required careful management of its side effects, which include vascular leak syndrome, fever, and temporary organ failure [44]. This has often required interventional care of patients in intensive care units. Dose escalation trials of IL-7[45] and IL-15[46] were met with dose-limiting toxicity before any anti-tumor benefit was demonstrated, although IL-15 did increase the frequency of CD8 T-cell and NK cells in the circulation[46]. An IL-15 superagonist, comprising IL-15 bound to IL-15R α , demonstrated preclinical antitumor activity superior to that of IL15 alone, but remains to be clinically evaluated [47]. A Phase I clinical trial of IL-21 to treat renal cell carcinoma and metastatic melanoma showed promising anti-tumor efficacy, including one complete response; however, all patients that initially responded to therapy eventually relapsed [48]. Systemic injection of recombinant IL-12 induced profound lymphopenia [49]. Although this may have resulted from trafficking of T-cells out of the circulation into tissue, severe toxicities resulted in low patient tolerance for multiple doses of IL-12.

As an alternative to infused cytokines (**Figure 2A,B**), preclinical studies have shown that genetic modification of T-cells to secrete cytokines or express tethered cytokines can

enhance their antitumor activity and maintain high levels of cytokine preferentially at the tumor site [41,42,50] (**Figure 2C,E**). A recent clinical trial in patients with metastatic melanoma tested TILs expressing IL-12 from an NFAT promoter so that IL-12 would be produced only when the T-cells were activated, which would occur at the site of the tumor. The *in situ* production of cytokines at the tumor would in theory circumvent the toxicity of systemically distributed IL-12, while allowing tumor-concentrated IL-12 to provide signal 3 to the TILs and enhance the cytotoxic and inflammatory activity of endogenous tumor-specific CD8⁺ T-cells, CD4⁺ T-cells, NK cells present in the tumor microenvironment [42]. Preclinical testing showed promising antitumor efficacy and objective tumor reduction was indeed demonstrated in 33% of patients [51]. Interestingly, these objective responses were achieved at cellular doses 10–100 fold lower than the doses required by conventional TILs therapy (10–100 billion T-cells), and without the need high dose IL-2 infusion. However, despite the added potency of IL-12, no objective efficacy was observed if patients were treated with fewer than 1 billion TILs. Unfortunately, this trial was accompanied by serious adverse events due to the long half-life of IL-12 in the serum; IL-12 levels rose in parallel with IFN- γ levels and one patient developed massive macrophage infiltrates into the liver that in turn created multiple small foci of hepatic necrosis. While ICU intervention was ultimately able to stabilize and reverse the symptoms, it was concluded that the unpredictable nature of IL-12 spikes in the serum was too difficult to manage. The toxicity produced by IL-12, as a systemically administered product or as a cell-secreted agent, exemplifies the current challenges of confining cytokine activity to the tumor. It will be interesting to compare these results to a current ongoing clinical trial (NCT02498912) treating ovarian cancer patients with Muc-16-specific CAR T-cells that constitutively secrete IL-12.

To circumvent the bystander effects that likely contribute to the toxicities produced by systemic or locally provided cytokines, we recently explored the use of a constitutively active IL-7 cytokine receptor as an alternative to secreted cytokines (**Figure 2F**). Cysteine and proline insertions in the transmembrane domain of the IL-7 receptor, CD127, engenders the formation of functionally active IL-7 receptor homodimers, likely to due to helical twisting of the receptor chains that permit crosstalk of JAK1 kinases bound to the CD127 receptor endodomain [52]. Our studies have shown that these receptors sustain prolonged CAR T-cell activity in preclinical models and mimic the persistence advantage that IL-7 has demonstrated in the past when combined with adoptive immunotherapy [53].

Cytokine support may also be supplied through chimeric cytokine receptors that flip a negative signal produced by inhibitory cytokines within the tumor microenvironment (**Figure 2D**). For example, a chimeric receptor with the intracellular signaling domains of the IL-7 receptor or the IL-2 receptor and an extracellular IL-4 receptor not only circumvents the immunosuppressive effects of IL-4 on adoptively transferred T-cells, but actively produce stimulatory cytokine signals after engaging IL-4. Such receptors may also act as a cytokine sink, preventing the cytokine from promoting immunosuppressive components of the tumor microenvironment and from provide tumor support. These receptors have been shown to increase the anti-tumor activity of T-cells in both leukemia and solid tumor models [54–56]. A clinical study combining the IL-4/IL-2R β receptor with an

ErbB-specific CAR in T-cells to treat head and neck squamous cell carcinoma is currently ongoing (NCT01818323). A recent study also demonstrated that cytokine signaling may be provided within the CAR molecule itself, wherein the IL-2R β signaling domain was incorporated into a chimeric receptor with CD28 and ζ yielding a single molecule yielding Signal 1, 2, and 3 in response to antigen[57].

Beyond engineering T-cells themselves to produce cytokines, nanoparticle technology has been explored for providing short-term cytokine supplementation. Lipid-based nanoparticles containing IL-15 have been attached to T-cells, serving as “backpacks” that release their cytokine cargo over a period of 7 days [58]. Tumor-specific T-cells augmented with IL-15 backpacks have significantly enhanced anti-tumor activity in preclinical models. Another method is to incorporate cytokine-bearing nanoparticles into surgically implantable alginate-based tissue scaffolds [59], within which cultured T-cells can be loaded *ex vivo* and surgically implanted next to the tumor site. The alginate housing was embedded with nanoparticles coated with CD3-, CD28-, and 41BB-specific antibodies, as well as an IL-15 superagonist, to deliver signals 1, 2, and 3 to CAR T-cells; exodus of the expanded T-cells from the scaffold into the tumor was facilitated with collagen tracks. This strategy of using scaffold-launched T-cells avoids potentially ineffective trafficking by intravenously injected T-cells, and may be well suited for solid tumors that have been partially resected or are inoperable. Compared to intravenous or peri-tumoral T-cell injections, scaffold delivered T-cells demonstrated superior *in vivo* expansion and efficacy. These strategies highlight the use of temporary, site-specific cytokine bursts to improve T-cell persistence and retain immunostimulatory cytokines to a particular site of action. However, much longer-term cytokine support may be required to produce complete tumor responses and to prevent subsequent tumor relapse.

4. Engineering T-cells to combat the immunosuppressive tumor microenvironment

Reducing the impact of immunosuppressive cytokines and increasing immunostimulatory cytokines to help T-cells resist the tumor microenvironment may be crucial for successful adoptive tumor therapy against solid tumors. Even then, the tumor stroma, including cancer associated fibroblasts, myeloid derived suppressor cells, regulatory T-cells, macrophages, and neutrophils, creates a microenvironment that poses significant additional challenges to T-cells. The tumor microenvironment also alters T cell metabolism thereby promoting immunosuppression, representing another potential barrier [60]. Some or all of these issues may have to be addressed to enable the full potential of tumor-specific T-cells to be unleashed. Indeed, the clinical success of antibodies blocking PD1 and CTLA4, immune checkpoints that limit the anti-tumor activity of tumor-specific T-cells in melanoma [61], has validated the importance of suppressive pathways in pathogenesis of a range of tumor types [62]. This same principle likely applies to adoptively transferred T-cells.

One approach to combat the inhibitory tumor microenvironment is target it along with the tumor. This strategy was explored through simultaneous targeting of tumor parenchyma and tumor stroma with EphA2-CAR T-cells targeting tumor and FAP-CAR T-cells targeting

stroma, which together demonstrated improved tumor responses in murine xenograft models [63]. Blockade of T-cell responses to immunosuppressive cytokines may also improve immunotherapy potency. One such strategy provided resistance to TGF- β by overexpressing a dominant negative TGF- β receptor in EBV-specific T-cells [64,65]. This dominant negative receptor bound TGF- β and not only provided T-cell resistance to the cytokine, but also served as a physiologic sink that prevented TGF- β from interacting with functional TGF- β receptors on other cells [64].

The stroma of solid tumors is not limited to cells and soluble factors, but includes an extensive extracellular matrix (ECM) containing glycosaminoglycans, collagen, and laminin that may pose a physical barrier to T-cell invasion of the tumor. When human TILs were added to resected autologous lung tumors and analyzed for migratory behavior, they preferentially localized in the peritumoral stroma that contained fibrillar collagen, and showed minimal infiltration into areas with tumor parenchymal cells [66]. Moreover, high rigidity of the ECM could actively exclude T-cell infiltration, as T-cells would not traverse areas of collagen with increased fibrous density. These studies were expanded to include both lung and ovarian cancers in which the migration velocity of resident TILs was significantly slower in collagen-rich stroma relative to EpCAM-positive tumor parenchyma [67]. Thus, it appears that stromal ECM can trap tumor-specific T-cells and physically prevent their access to tumor cells. Poor infiltration may also be an artifact of cell manufacturing. T-cells cultured *ex vivo* for 10–12 days, which is a standard time for manufacturing time period, lose their ability to produce heparanase, an endoglycosidase necessary for the degradation of heparan sulfate in ECM [68].

Fortunately, strategies to enhance T-cell infiltration into solid tumors may already exist. Relevant strategies evaluated at our center have included genetic overexpression of chemokine receptors in T-cells to increase their migration to chemokine naturally produced by tumor cells [69,70], as well as forced production of chemokines in the tumor site using oncolytic viruses [71]. In addition, overexpression of heparanase in CAR T-cells was found to restore their invasive ability and significantly improved their efficacy in established solid tumors [68]. The inclusion of infiltration strategies may be necessary for adoptive cell therapy to overcome the physical barriers of some solid tumors.

Finally, the breakthrough efficacy produced by immune checkpoint inhibitors [61,72,73], whose function depends of the presence of endogenous tumor-specific T-cells, suggests great promise for synergistic combinations with adoptive T-cell therapies. Although generally infused systemically, PD1-blocking antibodies have also been supplied by oncolytic viruses, [74] likely providing greater concentrations of blocking antibodies at the tumor site and minimizing off target effects such as autoimmunity. An alternative strategy under exploration is to remove PD1 expression from the adoptively transferred T-cells, using gene-editing strategies such as CRISPR-Cas9 to delete essential domains of the PD1 gene. A recent report showed that the antitumor efficacy CAR T-cells was improved by CRISPR-Cas9 deletion of PD1 in a preclinical model [75]. This work is now being translated into clinical trials in China and the United States (NCT03399448, NCT03081715, NCT02863913). A potential concern with such strategies is that removing the break on T-cells with unknown native specificities may produce autoimmune toxicities.

5. Engineering safety and specificity into T-cell therapy

With the advent of genetically modified T-cells armed with cytokines and resisting the tumor microenvironment, safety will be an increasing concern. Furthermore, given the super-charging of CAR T-cells, off-tissue effects against antigens expressed at low-levels in non-tumor tissues may occur. Thus, new safety paradigms will be essential for CAR T-cell therapy.

Already, the field has witnessed significant toxicities from CD19-CAR-T therapies that efficiently clear B-cell malignancies, but trigger severe (though reversible) cytokine release syndrome [14] and neurotoxicities, along with deletion of normal B-cells [76]. The current generation of CD19-CAR-T therapies lack built-in safety mechanisms. Cytokine release syndrome can be managed with tocilizumab and B-cell deficiency with intravenous immunoglobulins, while neurotoxicities are so far managed with steroids and supportive care.

While significant side effects have remained limited in solid tumor studies likely due to the concurrent low anti-tumor efficacy, some toxicities have been observed. In a clinical trial using third generation HER2-CAR T-cells at high doses after non-myeloablative conditioning (NCT0092487), fatal on-target off-tissue reactions were observed against lung alveoli resulting in the death of a patient [77]. Similarly, pronounced bile duct toxicities were observed in a clinical trial using CAR T-cells targeting carbonic anhydrase [78]. In a future in which CAR T-cells may be super-charged against tumors, these toxicities threaten to become commonplace analogous to the surge in autoimmune disease secondary to checkpoint inhibitor therapy in patients today [79].

5.1 Safety Switches to Eliminate adoptively Transferred Cells

Several strategies to limit the toxicities of T-cells have been developed. Significant progress has been made with small molecule-inducible suicide systems. In one method, the herpes simplex virus thymidine kinase gene can be expressed in T-cells, inducing death after the administration of ganciclovir [80]. This strategy proved effective in human trials, but the immunogenicity of the transgene has ultimately limited its widespread use and precluded the development of other xenogeneic constructs. Toward creating a fully human safety switch, an inducible Caspase-9 (iCasp9) construct was created. In iCasp9, the endogenous apoptotic protease activating factor 1 (apaf-1)-inducible dimerization domain was replaced with a single human FK-binding domain, modified to bind a small molecule dimerizer with high affinity [81]. This system eliminated T-cells efficiently and rapidly. The caspase-9 domain proved superior to iterations with fas and caspase-8 domains since it resists upstream apoptosis inhibitors such as Bcl-2 and Bcl_{XL} [81,82]. In clinical trials, the iCasp9 system effectively eliminated GVHD-inducing T-cells in hematopoietic stem cell transplant recipients, eliminating up to 90% of T-cells within 30 minutes after infusion of the AP1903 dimerizer [83]. This system proved advantageous for haploidentical bone marrow transplants, allowing faster immune recovery, without clonal proliferation of gene-modified cells [84], and sparing virus and tumor-specific immunity after iCasp9 engagement [85]. When combined with a CD19 CAR preclinically, iCasp9 could reduce T-cell numbers in a dose-dependent manner, or completely eliminate them, potentially offering physician

flexibility [86]. Other safety switches relying on antibody-mediated T-cell elimination have been developed. The expression of a non-native human antigen such as CD20 or EGFR on T-cells, would allow elimination of gene-modified T-cells using a commercially available antibodies such as rituximab [87] or cetuximab [88], while sparing normal T-cells. The known safety profile of both antibodies facilitates their clinical use despite expression by normal tissues, but efficacy may be limited by reduced tissue penetrance compared to small molecules. A variant of this approach includes only the targeted CD20 epitope of rituximab in the CAR T-cell product [89]. Overall, given the possibilities of CAR T-cell toxicities in the future, these systems (**Figure 3**) and others need continued exploration for their efficacy in abrogating deleterious responses in patients.

5.2 Synthetic biology tools to control CAR function and recognition

Beyond safety switches, several strategies are under development for smart recognition of cell surface antigens to avoid on-target, off-tissue toxicity. The advantage of these strategies is the ability to target tumor antigens with some degree of expression in other tissues. Several strategies employ preclinical pattern recognition systems similar to Boolean gating of “AND” and “NOT” seen in computer science programming. Indeed, these Boolean gating systems hold promise for improving the selectivity of CAR T-cells against tumor cells while withholding cytotoxicity to normal tissues. An early example of the “AND” strategy was to separate the ζ chain (signal 1) and costimulatory (signal 2) into CAR molecules with different antigen specificities with the rationale that for maximal T-cell activation, both antigens must be present on the tumor cell (**Figure 4A**). Receiving only signal 1 would produce suboptimal activation and no expansion, while signal 2 alone would not induce T-cell activation. Initial exploration of the AND concept verified that Jurkat T-cells dually activated from a ζ -signaling CAR and a CD28-signaling CAR could produce IL-2 to a similar magnitude as antibody mediated CD3 and CD28 crosslinking [90]. A subsequent study employed the AND strategy to generate exquisite tumor specific killing by CAR T-cells, using a first generation prostate specific-membrane antigen (PSMA) CAR combined with chimeric costimulatory receptor (CCR) for prostate stem cell antigen (PSCA) that could mediate significant tumor destruction only when both antigens were present [91]. In a similar preclinical study, a combination of mesothelin and the α -folate receptor were targeted by CARs encoding CD3 ζ or CD28 endodomains, respectively. Again antitumor activity was observed only in the presence of both antigens [92]. In hindsight, the success of the CD19-CAR is due, in part, to the exquisitely restricted expression of CD19 in B cells (which can be expendably eliminated) and the uniformly high expression of CD19 antigen [93], properties not seen in the majority of tumor-associated antigens that have heterogeneous antigen density in tumors across the patient population [94], as well as basal expression in normal tissue [77]. Therefore “AND” Boolean gating strategies will be important to allow tumors to be destroyed selectively by adoptive cell therapies, despite targeting antigens expressed in normal tissues.

A similar strategy to the “AND” Boolean was creating a “NOT” Boolean (**Figure 4B**). In one example, a first generation tumor antigen-specific CAR was paired with a second CAR molecule connected to inhibitory domains (PD1 or CTLA4) [95]. The second CAR would be directed to a normal antigen in the off-target tissue of interest. If the T-cells migrated to

the off target tissue, then the inhibitor signal would prevent activation and proliferation of those T-cells, much in the same way that PD1 signaling naturally functions to inhibit T-cells and maintain tolerogenic signaling. This system was effective in murine models, but remains to be translated into clinical trials.

A different kind of Boolean operator was recently invented that does not require both CAR molecules to be expressed simultaneously, but rather coordinates the activation of one CAR molecule to induce the signaling of a second CAR molecule[96]. In this way, a two-antigen tumor recognition circuit is formed to selectively kill tumor cells at the tumor site. Briefly, the first CAR molecule was a derivative of the NOTCH receptor, which contains a self-cleaving endodomain that, upon receptor ligation, is delivered to the nucleus where it acts as a transcription factor. The native extracellular domain of Notch was replaced by a tumor antigen-specific scFv, so that notch function was dependent on tumor recognition. A second CAR specific for a different tumor antigen was expressed only as a result of signaling from the first tumor antigen, thereby creating a biphasic signaling response. Pre-clinical studies in xenograft murine models have shown that T-cells using the NOTCH-based Boolean operator are selectively primed at the tumor site and do not migrate elsewhere, creating another strategy to potentially bypass on-target off-tissue toxicity in patients. Beyond expressing a CAR as the second executioner molecule, the expression of bispecific antibodies and cytokines as the second molecule that could be delivered into the tumor has also been explored[97].

While Boolean-gated CAR schemes for more efficient tumor targeting are appealing, they ultimately present significant complexity that may limit their use. Every tumor must be validated for the expression of two different antigens, and consistent expression of both antigens on a specific tumor type may be difficult to identify. Because of this, simpler strategies that could regulate CAR-T function with exogenously supplied small molecules have been investigated. In these schemes, CAR T-cells that recognize a tumor become fully activated only when a small molecule is supplied. In one such scheme, the CAR molecule is split into two different component proteins that have the ζ and costimulatory domains, respectively. Both proteins include drug binding domains, such that the presence of the drug can co-localize the proteins together into a single functional complex, with both zeta and costimulatory domains for maximal signaling potency [98]. An alternative, but similar approach keeps the signaling molecules separate, with only costimulatory signaling regulated with a dimerizing small molecule drug [99]. In this strategy, T-cells express a CAR with a zeta endodomain engineered to recognize the PSCA receptor and co-express a separate molecule comprising MyD88 and CD40 endodomains linked to FKBP12 ligand-binding domains [100]. When the inducer drug AP1903 is added, the MyD88/CD40 domain oligomerizes and successfully signals to produce costimulation [101]. When this is combined with antigen recognition at the tumor site, maximal cytokine release and proliferation of the modified T-cells will occur.

6. Conclusion:

In conclusion, despite significant progress in the use of T-cells to treat solid tumors, much work remains if consistent clinical benefit in these malignancies is to be achieved. Closing

the gap in efficacy in adoptive cell therapy against liquid versus solid tumors will require safe solutions to counteract the physiological and physical barriers posed by the tumor stroma, excessive immunosuppressive cytokines, and lack of immunostimulatory costimulatory molecules and proinflammatory cytokines within solid tumors. Several novel preclinical cytokine delivery strategies await evaluation in the clinic. Simultaneously, to prevent the toxicities that may arise from more potent adoptive cell therapies, the selectivity of engineered immune cells can be refined using sophisticated antigen recognition systems, and T-cells can be outfitted with drug inducible safety switches. It will be interesting to see if integrating these technologies into an aggressive treatment strategy can produce meaningful and tolerable clinical improvements in solid tumor therapy.

7. Expert Opinion:

A pressing question in the field is whether CAR T-cell therapy can be made effective beyond B-cell malignancies. It is our opinion that these challenges are surmountable, and significant progress has already been made in preclinical and clinical studies. Effective T-cell therapy for solid tumors will require solving poor *in vivo* persistence of CAR T-cells, a common denominator in many solid tumor clinical trials, and overcoming multiple inhibitory pathways simultaneously. While a plethora of strategies to increase persistence in a tolerable manner are being evaluated by different investigators costimulatory signal integration, alleviation of inhibitory signaling, and selection of optimal T-cell subsets, it is our belief that engineered cytokine supplementation has shown the most promise in dramatically improving CAR T-cell responses against solid tumors and deserves priority in implementation.

As cytokine engineering strategies are translated into the clinic, safety will be a greater concern, as normal regulatory mechanisms are perturbed to enhance T-cell expansion, persistence and function. To fully assess and monitor these engineered T-cells at the solid tumor site, advanced radiographic imaging technology and biopsies will add an urgently needed dimension of understanding of safety and efficacy to clinical trial results.

Beyond monitoring, efficient schemes to regulate cytokine gene expression or function in engineered T-cells is likely required to orchestrate these responses and prevent uncontrolled cell proliferation. These would include safety mechanisms, beyond strategies that kill modified T-cells, or strategies that control signal 1 and 2. Given that many different cytokine pathways may be useful in stimulating engineered T-cells, new approaches will require significant preclinical testing and clinical trials in parallel, which maybe be undertaken in iterative fashion adding one extra element at a time to verify individual benefits. The most rapid and cost effective way to test these engineered cytokine strategies would be in small adaptive phase I trials performed in an academic setting, where vectors and constructs could rapidly be modified toward the most optimal designs.

While these challenges are many, we are optimistic for the field, as many more investigators, companies, and funding agencies join the battle to improve adoptive immunotherapies and help deliver them to patients. We hold firm belief that cellular immunotherapies can be engineered toward better responses in solid tumors, with optimized cytokine signaling playing a key role.

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* of interest

** of considerable interest

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Article Highlights:

- The initial success of CAR T-cells against B-cell malignancies was driven by unique contexts of antigen expression, costimulation, and microenvironment that are not operable in solid tumors.
- CAR T-cell efficacy against solid tumors has in particular been hampered by lack of signal 3 activation via cytokine stimulation, a deficiency not addressed in most CAR T-cell schemes today.
- Engineered cytokine signaling systems such as constitutively active cytokine receptors, chimeric cytokine receptors, and tethered cytokines have shown potential to endow engineered T-cells with potent persistence and antitumor properties.
- Genetic modifications to improve T-cell infiltration into solid tumors, circumvent extracellular matrix barriers, and provide resistance to immunosuppressive molecules will enhance the efficacy of adoptive immunotherapy strategies.
- Synthetic biology has led to the development of new methods to regulate apoptosis of modified T-cells as potential safety switches, while also providing methods of regulating CAR-T activation with small molecules.
- Split antigen recognition approaches are an exciting modality to bring specificity to CAR T-cell therapies and avoid responses in unwanted tissues.

Figure 1: Overview of Chimeric antigen receptors

The schematic shows successive iterations of chimeric antigen receptor design. First generation CAR molecules are composed of a single chain fragment variable (scFv) derived from a monoclonal antibody linked to an extracellular spacer and transmembrane domain (which can be derived from antibody components such as IgG1 and IgG4 or from other molecules such as CD8 and CD28), followed by the ζ chain signaling endodomain. Second generation CAR molecules and third generation CAR molecules incorporate one and two costimulatory molecules, respectively, to enhance T-cell expansion and cytokine release.

Figure 2: Genetic modification strategies of T-cell to provide cytokine signaling support

(A) A series of γ -chain cytokines and their native receptor systems that have been used in conjunction with adoptive cell therapies. IL-2 can bind a high-affinity heterotrimeric receptor (IL-2R α , IL-2R β , and γ c), or it can be trans-presented by IL-2R α on a T-cell to a recipient T-cell expressing IL-2R β and γ c. IL-15 binds to IL-15R α expressed by myeloid cells (such as dendritic cells) and is then trans-presented to IL-2R β and γ c on a T-cell. (B) Externally infused cytokines bind to cognate receptors of immune cells in the body to produce immunostimulatory signaling. (C) Cytokines, such as IL-12, secreted from genetically modified cells will accumulate in the vicinity of the cells and either bind to the cytokine receptors on the secreting cells in autocrine fashion as depicted or bind to other immune cells at the site of secretion or elsewhere in the body (not shown). (D)

Overexpression of chimeric cytokine “flip” receptors in T-cells results in their binding of an immunosuppressive molecule (such as IL-4) in the T-cell vicinity and then through the immunostimulatory endodomain (IL-7R α or IL-2R β) produce an immunostimulatory signal that dominates over immunosuppressive signaling from the native IL-4 receptor. (E) Tethered cytokines such as tethered IL-15 are positioned in an optimal conformation to be trans-presented to adjacent T-cells to provide support. (F) Constitutively active cytokine receptors operate without need for external ligand and thus are self-enclosed systems that can be further engineered with ectodomain modification to be completely unresponsive to external cytokines. This ensures that cytokine activity is restricted to the population of adoptively transferred cells.

Figure 3: Genetic modification of T-cells to include suicide systems

(A) Antigens that are not natively expressed on T-cells and which are recognized by commercially available antibodies can be employed as suicide systems. Antibody/antigen binding results in antibody dependent cell-mediated cytotoxicity. (B) The inducible Caspase-9 system is composed of inert molecules that only dimerize upon the addition of an external drug to initiate apoptosis.

Figure 4: Synthetic biological tools to enhance the specificity of T-cell activation

(A) Combinatorial antigen recognition systems are composed of co-expression of an attenuated first generation CAR with a costimulatory CAR to induce maximal T-cell activation only when the T-cells encounter a tumor cell expressing both tumor-associated antigens, while preventing activation at normal cells. (B) Inhibitory CARs can be coexpressed with CARs to prevent CAR activation at off-tissue locations while permitting CAR T-cell activation at the site of cancer cells. (C) Syn-Notch antigen recognition system selectively expresses a CAR molecule at the site of a cancer cell. (D) Inducible MyD88/CD40 molecules selectively provide costimulation only after the addition of external dimerizing drug.