Approaches for refining and furthering the development of CAR-based T cell therapies for solid malignancies

Caroline M Hull and John Maher

1. Current status of CAR T cells

Traditionally, cancer therapy has consisted of surgical removal of tumors, either alone or in conjunction with radiotherapy, chemotherapy or small-molecule pharmaceuticals. While great success has been achieved in improving survival, many patients respond unsatisfactorily to these approaches. Additionally, chemotherapy can lead to unwanted side effects due to a lack of specificity. The first targeted drugs for the treatment of cancer came with the approval of monoclonal antibody therapies that bind tumor associated antigens (TAA) and induce apoptosis or reduced proliferation of cancer cells. Employing the specificity of the immune system, the antigen recognition domains of antibodies have more recently been used to redirect T cell responses toward tumor cells. Physiologically, T cells rely on the T cell receptor (TCR) to recognize cancer cells and while genetic expression of tumor-antigen-specific TCRs in patient-derived T cells is feasible, a variety of issues concerning human leukocyte antigen (HLA) restriction hamper their application to large patient cohorts. Using a single chain variable fragment (scFv) comprised of a fusion of the light and heavy variable regions of antibodies and subsequent engraftment onto T cell signaling domains via a transmembrane or hinge region, T cell specificity can be redirected toward tumor antigens. 1st generation CARs typically included only the CD3ζ signaling domain; however, a key advance in the design of CARs was the inclusion of the co-stimulatory modules CD28 and 4–1BB within the cytoplasmic domain (summarized in Figure 1) [1]. In recent years the clinical potential of CAR T cells has been realized in striking fashion in selected hematological cancers. Response rates of up to 90% have been reported following the administration of CD19-specific CAR T cells to patients with B cell malignancies. This has resulted in the regulatory approval of five CAR T cell products that contain either a 4–1BB (KYMRIAH, Novartis; BREYANZI, Bristol Myers, Squibb; ABECMA, Bristol Myers, Squibb) or CD28 (YESCARTA and TECARTUS, Kite Pharmaceuticals) co-stimulatory domain [2–4].

1.1. Barriers to CAR T cell therapy in solid tumors

In contrast to the impressive response rates reported in hematological cancers, trials in solid tumors have produced disappointing results. Poor efficacy can be attributed to a number of factors summarized in Figure 2. Challenges that remain to be overcome fall into three main categories: antigen selection, CAR T cell homing to the tumor and maintenance of CAR T cell function within the TME. An ideal TAA target for CAR T cells would be highly specific to tumor cells and be expressed by numerous cancers. However, due to the spectrum of different solid tumors, numerous CARs are required with different specificities. Furthermore, TAAs are often expressed on healthy tissues thus increasing the risk of toxicity and their level of...
expression can vary between patients potentially impacting CAR T cell efficacy. An additional challenge to overcome in solid tumors is suboptimal CAR T cell trafficking to the TME. One advantage of treating hematological cancers by intravenous injection is that CAR T cells accumulate in lymphoid tissues and bone marrow while trafficking to solid tumors is limited. Even if T cells reach the TME, the stroma presents a barrier that they must navigate to reach their target cells. What’s more, once CAR T cells infiltrate the tumor, the surrounding TME is a hostile environment with many factors including: decreased nutrients, hypoxia and the presence of immunosuppressive cells that further impact CAR T cell survival and function [5].

2. Refining targeting of CAR T cells

Despite the success of CAR T cell therapy in hematological cancers, efficacy in solid tumors has been limited: truly tumor-specific antigens are rare, leading to risk of ‘on-target, off-tumor’ related toxicities and highlighting the requirement for careful CAR design.

2.1. Antigen selection

Members of the ErbB receptor family have been extensively tested as CAR T cell targets. The most studied of all is HER2, which is an attractive target for breast cancer and a number of other solid tumors. Clinical relevance of this target has been confirmed using the HER2-specific monoclonal antibody, Trastuzumab (Herceptin) [6], although not all HER2 amplified tumors respond. Logically therefore, the use of this antigen as a target for CAR T cells has been explored. T cells expressing a HER2-specific CAR specifically lyse a variety of different tumor cells in vitro, including breast and ovarian cancer cell lines [7,8]. Regional delivery of HER2-specific CAR T cells has also shown efficacy against metastatic breast cancer in the brain using mouse models [9]. Due to the safety profile of Herceptin, a high dose of 3rd generation HER2-specific CAR T cells were given in combination with low dose IL-2 to

![Diagram](image-url)
a patient with metastatic colon cancer. Following lymphodepletion, infusion of $10^{10}$ CAR T cells led to respiratory distress within minutes, multi-organ failure and death within 5 days despite administration of steroids [10]. Autopsy revealed infiltration of the lung by CAR T cells and it is thought toxicity may have occurred due to recognition of low level HER2 on the lung epithelium or microvasculature [11]. Although HER2 expression was not confirmed, the resulting toxicity emphasizes both the potency of CAR T cells and the potential risks when using a 3rd generation CAR. In hopes to avoid toxicity in a more recent phase 1 clinical trial testing 2nd generation HER2-specific CAR T cells in sarcoma, an ultra-low dose was administered in the absence of lymphodepletion or IL-2. Safety and CAR T cell persistence was demonstrated in this study, although efficacy was limited producing short term stable disease in a small patient cohort [12].

A more tumor selective ErbB receptor derivative, which arises through deletional mutation, is the epidermal growth factor receptor (EGFR) variant III. Johnson et al. generated a panel of humanized scFvs and compared their specificity for EGFRVIII with that of the wild-type EGFR protein, using a 2nd generation CAR backbone. Subsequent in vivo studies demonstrated that EGFRVIII targeted CARs were efficacious and safe in mouse models of glioblastoma [13]. However, neoantigen loss following EGFRVIII CAR T cell treatment has been reported in patients with glioblastoma [14] emphasizing the importance of broad-spectrum targeting of cancer cells.

Due to the selection pressures exerted by therapies that engage a single target, the ability to recognize two or more targets may avoid or lessen the risk of tumor escape. Successful broad-spectrum targeting of the ErbB receptor family has been achieved using a CD28-based 2nd generation CAR in which specificity was conferred by a chimeric polypeptide known as T1E. T1E consists of a chimera derived from transforming growth factor-α and epidermal growth factor and binds eight distinct ErbB dimer species [15]. Given the risk of toxicity with systemic administration, a phase 1 clinical trial has been initiated in head and neck cancer, employing intra-tumoral delivery to mitigate risk [16] and following treatment of 13 patients no dose limiting toxicity has been reported [17].

MUC1 is usually found in a highly glycosylated state and modification of this glycosylation along with increased expression of MUC1 by cancerous cells make it an attractive target. Alterations in MUC1 have been identified in a large number of cancers that have very limited treatment options, including triple negative breast cancer. Preclinical studies have demonstrated the capacity of MUC1-targeted CAR T cells to destroy tumors [18,19] leading to initiation of clinical trials. One such study utilized a 2nd generation CAR and achieved tumor
control in pancreatic cancer models, demonstrating the importance of neoantigens such as this when developing CAR T cell therapies [20].

T cells specific for mesothelin have been tested in the clinic using a CD28-based 2nd generation CAR in patients with malignant pleural disease. Although clinical results are preliminary, regional delivery of CAR T cells combined with anti-PD1 therapy was safe and led to complete responses in 2/19 patients [21]. Claudin 18.2, expressed highly in gastric and pancreatic adenocarcinoma, is another potential target. Treatment of 11 metastatic adenocarcinoma patients with 1–5 cycles of CAR T cells achieved a complete response in 1 patient, highlighting its potential as a target but also the limited efficacy achieved to date [22].

NKG2D binds an evolutionarily selected family of stress ligands, at least one of which is expressed by most cancers. The potential of NKG2D-targeted CAR T cells has been demonstrated in patients with acute myeloid leukemia (AML). Both safety and efficacy were reported following at least 3 infusions of CAR T cells, achieving an objective response in 4/11 patients [23]. In a second study NKG2D-based CAR T cells showed some efficacy in combination with oxaliplatin chemotherapy when administered to patients with metastatic colorectal cancer [24,25]. Of nine treated patients, stable disease was achieved in six and one patient achieved a partial remission. Due to the small study size the individual contribution of the chemotherapy or the NKG2D CAR T cells could not be determined.

2.2. Avoiding tumor escape and toxicity using dual targeting CARs

The potential for targeting two distinct antigens has been demonstrated in B-cell malignancies and multiple myeloma. CAR T cells targeting the B-cell maturation antigen (BCMA) produced rapid tumor responses; however, durable remissions required high doses and upon relapse loss of BCMA expression was reported [26]. To overcome this, a second antigen, transmembrane activator and calcium-modulator and cyclophillin ligand (TACI), was targeted alongside BCMA and prevented tumor escape when compared to targeting BCMA alone [27]. A similar story has been presented in glioblastoma using a single tandem (Tan)CAR to target both HER2 and IL13Rα2. To achieve dual specificity, the targeting domain of the CAR consisted of a high-affinity IL13 mitein linked to a HER2-specific scFV. In mouse models, tanCARs mitigated antigen escape and led to improved survival [28]. Dual targeting of CD19 and CD22 with two 2nd generation CARs has been trialed in patients with diffuse large B-cell lymphoma producing complete responses in 55% of patients dosed with at least 50 x 10^6 CAR T cells [29].

Dual specificity CAR T cells that utilize a chimeric coreceptor (CCR) to improve tumor selectivity (Figure 1) may also mitigate toxicities seen with single specificity CARs. Co-expression of a HER2-specific CD3ζ CAR and MUC1-specific CD28-containing CCR enabled the deployment of maximal anti-tumor activity against tumor cells that co-expressed both targets, refining tumor specificity [30]. Use of dissociated signaling domains has also been applied to co-target folate receptor α alongside mesothelin, producing comparable efficacy to 2nd generation CAR counterparts. In addition, the authors speculated that should the designer T cells receive signal 1 alone in healthy tissue, activation induced cell death or anergy would ensue [31,32], potentially reducing toxicity. In an attempt to further optimize the safety profile of dual specificity CAR T cells, the balance between the signal strength from the CAR and CCR has been interrogated. Using two prostate cancer derived TAAs, prostate-specific membrane antigen (PSMA) and prostate stem cell antigen (PSCA), the CAR activity was diminished to ensure its dependency on CCR engagement. By balancing signaling, recognition of single antigen positive cells was lost demonstrating improved selectivity and safety [33].

3. Co-stimulatory design in CAR T cells

3.1. Activating module selection in CAR T cells

CAR T cells are predominantly supplied with signal 1 using a portion of CD3ζ incorporating all three immunoreceptor tyrosine-based activation motifs (ITAMs) in combination with co-stimulation via CD28. However, it has been hypothesized that while the strength of signaling drives potent effector function it may also be detrimental to T cells due to increased exhaustion and differentiation. To alter T cell differentiation and exhaustion CD3ζ was mutated to include either functional ITAM 1, 2 or 3 alone, termed 1XX, 2XX and 3XX. The mutated CD3ζ produced a more balanced phenotype in terms of memory and effector T cells, maintaining a central memory population and decreasing effector T cells. A 2nd generation CAR incorporating 1XX also demonstrated superior efficacy in vivo when compared to standard CD3ζ [34].

An alternative approach to supply a single ITAM is to use DAP12 which associates with NKG2D. Fusing the NKG2D ectodomain to 4–1BB and the DAP12 cytoplasmic domain produced a CAR that, when compared to an NKG2D-CD3ζ CAR, induced lower levels of cytokine and proliferation and comparable in vivo efficacy. Additionally, toxicity was reported with NKG2D-CD3ζ and not NKG2D-DAP12 CAR T cells [35] highlighting the potential for DAP12-based CARs.

3.2. Commonly used co-stimulatory modules

Most 2nd generation CAR T cells utilize CD28 or 4–1BB as co-stimulatory modules (summarized in Figure 1) with the majority of studies focussing on CD28. However, each form of co-stimulation produces overlapping but, in some cases, distinct T cell phenotypes [36,37]. Addition of CD28 or 4–1BB domains leads to increased cytokine production [38,39]. CD28 co-stimulation may also prevent activation induced cell death [40] and while persistence is lacking, novel mutations to CD28 can be incorporated to promote CAR T cell survival in vivo. One study has demonstrated that an asparagine within the intracellular domain of CD28 drives T cell exhaustion and hinders persistence. Mutation of this residue to phenylalanine increased antitumour activity and reduced differentiation and exhaustion. Interestingly, the phenylalanine containing motif is found in inducible T cell co-stimulator (ICOS) where
mutation to asparagine abolished in vivo persistence and efficacy, highlighting the importance of this residue [41].

ICOS, a member of the B7 family to which CD28 belongs, can also deliver co-stimulation. Both ICOS and CD28 recruit PI3 kinase (PI3K) via a conserved cytoplasmic motif, with ICOS being the more effective of the two [42]. Unlike CD28, ICOS can recruit Lck to the immune synapse, vital for T cell activation [43] (e.g. delete the first two words “Unlike CD28.”). While ICOS co-stimulation induces lower levels of IL-2, alternative cytokines including IL-4 and IL-21 are produced, supporting development of Th2 and T follicular helper cells [44] and suggesting a tailoring toward use in CD4 T cells. The importance of different signaling requirements for particular cell types has also been proven by varying co-stimulatory combinations in mesothelin-specific CARs. Transducing CD4 and CD8 T cells separately demonstrated that using ICOS in CD4 T cells and a CD28, 4–1BB or ICOS co-stimulation domain in CD8 T cells improves efficacy. The optimal combination for persistence in vivo used ICOS in CD4 T cells and 4–1BB co-stimulation in CD8 T cells. Incorporation of either CD28 or 4–1BB co-stimulation in CD4 T cells led to minimal persistence of both CD4 and CD8 T cells, highlighting the intricacies of optimizing CAR design for different T cell populations and the interplay between T cell subtypes within the final cell product [45].

3.3. Members of the TNFR superfamily

While 4–1BB has been studied extensively, other members of the tumor necrosis factor receptor (TNFR) superfamily can supply co-stimulation. OX40 is a CD4 associated co-stimulatory molecule that induces signaling via NFκB pathways [46] with broadly similar co-stimulatory activity to 4–1BB [47]. CD27 which is constitutively expressed by CD4 and CD8 T cells and important for T cell memory formation [48] has also shown comparable efficacy to CD28 and 4–1BB in vivo. Similarly to 4–1BB, CD27 promotes increased CAR T cell persistence compared to CD28 [49]. Herpesvirus entry mediator (HVEM), which plays an important role in CD8 T cell memory formation [50] improved CAR T cell potency compared to CD28, reducing exhaustion and enhancing mitochondrial respiration [51].

3.4. Novel co-stimulatory modules

In hematological cancer models, CD28-based CARs tend to clear tumors faster while those incorporating 4–1BB achieve similar efficacies due to increased T-cell persistence. To combine speed with persistence, Zhao et al provided further co-stimulation to CD28-based 2nd generation CAR T-cells by co-expression of 4–1BBL (Figure 1) [52]. Addition of 4–1BBL reduced exhaustion marker expression when compared to 2nd generation CAR T cells. Gene expression analysis revealed that upregulated genes were enriched within the type-I interferon pathways which proved crucial for the improved anti-tumor response in vivo when compared to a 2nd generation CD28 CAR alone. It is yet to be seen whether a combined approach such as this would boost the function of 2nd generation CAR T cells in solid tumors.

Like 4–1BBL, CD40L also forms a trimer and its receptor CD40 is expressed by a number of cancers [53]. Constitutive expression of CD40L by CAR T cells resulted in increased proliferation and pro-inflammatory cytokine production (including IL-12) through engagement of monocye-derived dendritic cells (DC) [54]. Co-expression of CD40L alongside a CD19 targeting 2nd generation CAR increased expression of DC and macrophage activation or maturation markers including CD86 and MHC-II within the spleen and draining lymph nodes. In addition, increased infiltration of the lymphoid organs by CAR T cells was reported with a favorable CD8 + T cell to regulatory T cell (Treg) ratio in the spleen [55]. Together these results highlight how CD40L expression can help CAR T cells engage the endogenous immune response.

Another novel co-stimulatory module is based on TLR2 which signals via MyD88. Fusing the Toll/interleukin-1 receptor domain of TLR2 to the 3’ end of a CD19-targeted CD28 2nd generation CAR enhanced cytotoxicity and reduced T cell exhaustion. Furthermore, administration of a single dose of CAR T cells to a patient with relapsed B-cell acute lymphoblastic leukemia achieved complete remission suggesting both safety and potency, although this result has not been confirmed with a cohort of patients [56]. MyD88 signaling can also be delivered using a novel small molecule inducible MyD88:CD40 co-stimulatory receptor. Using tandem FKBP domains, dimerization and function of the co-receptor can be pharmacologically regulated [57], thereby improving safety. Co-stimulation via MyD88:CD40 improved CAR T cell function when compared to CD28 or 4–1BB co-stimulation, which may in part be due to maintenance of a less differentiated T cell phenotype [58]. Further development of this system has involved the construction of a constitutively active MyD88:CD40 chimera that can be used safely when co-expressed with an inducible Caspase 9 safety switch [59]. MyD88:CD40 signaling has also been coupled to ectopic IL-15 expression in CAR NK cells, enhancing expansion and cytotoxicity [60].

3.5. Switch receptors

Switch receptors can also provide co-stimulation by coupling the binding domains of inhibitory receptors such as PD-1 or TIGIT to co-stimulatory endodomains such as CD28 (Figure 1) [61–63]. Switch receptors convert an inhibitory signal into an activating one by competing with inhibitory ligands and providing a stimulatory signal. Introduction of a PD1:CD28 fusion protein into CD8 + T cells led to increased proliferation and enhanced production of effector molecules such as granzyme B [63]. A PD1-CD28 switch receptor also improved efficacy of a PSCA-targeted 2nd generation CAR T cell product in models of mesothelioma and prostate cancer [62]. Co-expression of a TIGIT:CD28 switch receptor led to improved function and cytokine secretion by T cells after multiple rounds of antigen challenge and improved efficacy in an in vivo melanoma model [64]. Expression of a CTLA-4:CD28 switch receptor has also similarly led to enhanced efficacy in tumor models. Notably, expression of this coreceptor in CD4 T cells significantly enhanced the antitumour effect of unmodified CD8 T cells [65].
4. Promoting T cell function within the tumor microenvironment

4.1. T cell homing

A crucial difference when compared to hematological cancers is the requirement for CAR T cells to successfully traffic to solid tumors. CAR T cells expressing receptors that do not match the chemokine profiles of the tumor lack the ability to home to the TME. Therefore, incorporation of chemokine receptors into CAR T cells may promote in vivo function. The proinflammatory chemokine CXCL8 (IL-8) is associated with disease burden and is elevated in the serum of patients with a variety of cancers including breast and ovarian cancer. Co-expression of the receptor for CXCL8, CXCR2 led to T cell migration toward tumor conditioned media and translated into superior efficacy in vivo [66]. T cell homing and activity is also suppressed in the TME by factors such as adenosine and prostaglandin E2 (PGE2) both of which activate protein kinase A (PKA). PKA localizes within the immune synapse, binding to ezrin, and dampening TCR signaling. Secretion of a peptide PKA inhibitor called regulatory subunit I anchoring disruptor by CAR T cells disrupted the interaction of PKA with ezrin. In vitro studies showed superior migration toward CXCL10 when compared to standard CAR T cells and increased efficacy and tumor infiltration in vivo.

4.2. Targeting tumor stroma

Once CAR T cells home to solid tumors an additional obstacle is the fibrous shield that develops to protect the tumor. Comprised of stroma and collagen, in addition to cancer associated stromal cells (CASCs) including cancer-associated fibroblasts (CAFs), this shield dampens the efficacy of CAR T cell therapies. To breakdown the stroma, CAR T cells have been designed that are specific for fibroblast activation protein (FAP) found on a major subset of CASCs. Destruction of CASCs by CAR T cells was shown to reduce extracellular matrix (ECM) proteins and tumor vascularization in addition to controlling tumor growth [67]. Disruption of the stroma can also be achieved by co-expressing enzymes such as heparanase [68]. Oncolytic viruses can also be engineered to deliver ECM degrading enzymes [69].

4.3. Counteracting the repressive immune response

In addition to the expression of checkpoint molecules such as PD-L1, cytokines, chemokines and small molecules help reinforce the immunosuppressive nature of the TME. Tumor associated macrophages (TAMs), involved in angiogenesis, cause Treg infiltration by secreting CCL-22 [70]. Tregs and TAMs then produce immunosuppressive cytokines such as IL-10 and TGF-β [71]. Myeloid derived suppressor cells (MDSCs) are also increased in frequency in tumors [72] and suppress CD8 T cells by producing arginine and nitric oxide synthase 2 (NOS2) while promoting Treg and TAM development [73,74]. Immunosuppressive mesenchymal cell types that are highly represented in the TME include CAFs and mesenchymal stromal cells [75]. Collectively, these elements render effective CAR T cell immunotherapy of solid tumors very challenging.

4.3.1. Secreted checkpoint inhibitors

Checkpoint blockade has been successful in treating numerous solid cancers using monoclonal antibodies targeting PDL-1, PD-1 and CTLA-4 [76–78], however, autoimmune toxicity has been problematic in some patients [79]. Together with the potential for toxicity, checkpoint blockade alone has only modest efficacy against some solid tumors such as non-small cell lung cancer potentially because systemic delivery does not produce the concentrations required within the TME [80]. Production of checkpoint inhibitors by CAR T cells could address this issue. Since the agent is produced in a more targeted manner this may permit higher concentrations of the checkpoint inhibitor to be reached within the TME, while mitigating against systemic toxicity. In vitro studies demonstrated that co-expression of a PD-1 blocking scFv preserved T cell cytolytic capacity and proliferation in the presence of PDL1+ tumor cells. Improved efficacy when compared to CAR T cells alone was demonstrated in a mouse model of ovarian cancer and importantly, the scFv could only be detected within the TME [81]. CAR T cells secreting an anti-PD-1 scFv also proliferate more and express less PD-1. Additionally, in mouse models of hematological cancer, CAR T cells secreting anti-PD-1 were more efficacious than CAR T cells alone or CAR T cells combined with systemic PD-1 blockade [82]. While CAR design mainly influences the response of the CAR T cell itself, targeted delivery of checkpoint inhibitors will also provide protection for endogenous T cells, enhancing their function.

4.3.2. Chimeric cytokine receptors

Converting suppressive cytokine signals into pro-inflammatory ones may boost function in the TME, building into the CAR T cells an innate resistance to inhibitory cytokines and acting as a ‘sink’ to help reduce cytokine binding to endogenous T cells. Anti-inflammatory IL-4 has been utilized in this way by fusing the extracellular domain of its receptor to the signaling domain of the IL2/15 [83] or IL7 receptor [84], leading to efficient T cell activation only within the TME. Illustrating this, pancreatic cancer forms a hostile TME in which immuno-suppressive cytokines including IL-4 are over-produced. Use of a chimeric IL4:IL7 receptor improved efficacy in preclinical models when compared to T cells expressing the CAR alone [85]. A chimeric IL4:IL21 receptor also demonstrated comparable efficacy to chimeric IL4:IL7 receptor CAR T cells in preclinical studies [86].

4.3.3. Armored CAR T cells

Armoring of CAR T cells with additional cytokines can promote their function and survival within the TME. So called TRUCK or 4th generation CAR T cells have been used to deliver various ‘payloads’ to the TME. IL-12 showed promise in preclinical studies [87] and the administration of tumor-infiltrating lymphocytes (TILs) with Nfat inducible expression of IL-12 achieved objective responses in 63% of patients with metastatic melanoma. However, responses were not maintained, and administered T cells showed limited persistence. In addition, when patients were treated with high doses of T cells,
toxicity was reported implying that a balance between toxicity and efficacy will be hard to find with IL-12 therapy [88]. IL-18 also promotes both CAR and endogenous T cell function by enhancing proliferation and inducing IFNγ production along with engaging the anti-tumor activity of monocytes [89,90]. In pancreatic and lung cancer models that did not respond to standard CAR T cell treatment, IL-18 producing CAR T cells demonstrated superior function. Within the TME, macrophages polarized toward an M1 phenotype along with increased NK cell frequency, while M2 macrophages and Tregs were reduced in number [90]. Secretion of both IL-15 and IL-21 has also been explored with combination secretion rather than secretion of either cytokine alone promoting expansion and persistence of CAR T cells in vivo and improving anti-tumor responses [91]. Another strategy involves tethering cytokines to the CAR T cell surface. Membrane bound IL-15 improved maintenance of a memory stem T cell phenotype and promoted survival in a leukemic mouse model [92].

4.4. Inclusion of decoy receptors

Immunosuppressive mechanisms within the TME can also be neutralized by expression of decoy receptors. Many tumors are known to secrete the immunosuppressive cytokine, TGF-β which can interfere with CAR T cell function. Blockade of TGF-β can be achieved by expression of a dominant negative TGF-β receptor-II (dnTGF-βRII) which acts as a ‘sink’ for TGF-β within the TME. Co-expression of dnTGF-βRII alongside a PSMA targeting CAR promoted T cell proliferation and enhanced anti-tumor responses in vivo [93]. The therapeutic potential of a dominant negative PD-1 decoy receptor has also been demonstrated. T cells that expressed dominant negative PD-1 and an anti-CD19 CAR showed superior cytotoxicity in vitro and induced remission in 3 diffuse large B cell lymphoma patients [94].

4.5. Targeting T cell metabolism

Tumors utilize large amounts of glucose, converting pyruvate to lactate via aerobic glycolysis [95,96], leading to an accumulation of lactate and reduced glucose availability. The importance of metabolism in oncogenesis is highlighted by the frequency of mutations found in cancer cells that target metabolic pathways including hypoxia inducible factor-1 (HIF-1), c-MYC and p53 [97,98]. Accumulation of lactate aids angiogenesis and in combination with hypoxia induces immunosuppression [99]. Another immunosuppressive mechanism is the production of enzymes including indoleamine-2,3-dioxygenase (IDO), tryptophan-2,3- dioxygenase (TDO) and arginase 1, which degrade amino acids required for T cell proliferation and survival and produce immunosuppressive by-products such as L-kynurenine [100,101].

4.5.1. Expansion procedures and effects on metabolism

Culture media often has a higher than physiological glucose content leading to dependency on glycolysis and even increasing T cell size, both of which may impact function within the low glucose TME. Expansion in low glucose conditions may therefore produce a T cell product with improved efficacy [102,103]. The metabolic requirements of T cells also depend on their memory phenotype with long-lived memory T cells having increased spare respiratory capacity and mitochondrial mass which aid survival [104]. More quiescent cells such as memory or naive T cells depend on fatty acid oxidation (FAO) and pyruvate oxidation and upon activation effector T cells reprogramme to use aerobic glycolysis. Reprogramming leads to an increased uptake of oxygen and glucose, which may be in limiting supply within the TME [105]. Alteration of expansion procedures to maintain a less differentiated T cell product may therefore be beneficial. A less differentiated T cell product could also be achieved using PI3K inhibitors. CAR tonic signaling leads to CD3ζ mediated activation of PI3K, promoting differentiation. Addition of PI3K inhibitors to expansion media suppressed effector T cell differentiation and increased frequency of central memory and naive T cells. PI3K inhibitors did not impact expansion and the resulting T cell population was more efficacious and persisted longer in vivo [106]. Another novel method for maintaining a less differentiated T cell phenotype involves expansion in the presence of human platelet lysate. Using a CD19 and PSCA-specific CAR, T cells exposed to human platelet lysate outperformed cells expanded in conventional media and displayed a less differentiated phenotype [107]. Recently, expansion of CAR T-cells in the presence of the S enantiomer of 2-hydroxyglutarate (S-2HG) favored generation of a memory enriched T cell product with superior therapeutic activity [108].

Addition of cytokines to expansion media can also improve CAR T cell function. Enrichment of memory stem T cells was achieved by addition of IL-7 and IL-15 to expansion media. Furthermore, IL-7 and IL-15 rescued the defective T cell expansion seen in some cancer patients [109]. Another study tested the effect of IL-15 alone on CAR T cell phenotype producing a less differentiated memory stem phenotype with reduced exhaustion and more proliferative potential. Interestingly, cells expanded with IL-15 were more metabolically fit than those expanded with IL-2. Analysis of enzymes involved in glycolysis and FAO revealed that IL-15 expansion promoted FAO, favoring CAR T cell function in the low oxygen TME [110].

IDO inhibitors may also overcome tumor mediated T cell suppression. In a lymphoma model, metabolites of tryptophan produced by IDO1 inhibited IL-2, IL-7 and IL-15 induced CAR T cell expansion while dampening their cytotoxicity. Using fludarabine and cyclophosphamide downregulated IDO1 expression by lymphoma cells and promoted CAR T cell function [111]. Using microRNAs to remove IDO1 expression in colon cancer cell lines also enhanced CAR T cell killing in vitro and suppressed growth of tumor xenografts in mice [112].

5. Effect of starting population on CAR T cell function and metabolism

Various studies have demonstrated the importance of the starting cell population and how it affects CAR T cell expansion and function. Differences in long-term efficacy of CAR T cells produced from CD4 versus CD8 T cells have been reported. One study demonstrated that CD8 but not CD4
CAR T cells when triggered via their TCRs exhibited loss of *in vivo* activity and were reduced in number likely due to increased apoptosis and exhaustion [113]. CD4- or CD8-targeted lentiviral *in vivo* generation of CAR T cells demonstrated that CD4 CAR T cells may be more efficacious at eliminating tumors when compared to their CD8 counterparts [114].

### 5.1. Naïve vs memory phenotype

It is becoming increasingly clear that the differentiation status of CAR T cells influences their potency. Comparing the transduction, expansion and cytotoxic potential of CD8 T cell populations derived from naïve, effector memory and central memory T cells showed that naïve T cells performed best with central memory superior to effector memory T cells. Naïve T cells transduced and expanded more efficiently than memory T cells and expressed less of the senescence markers KLRG1 and CD57, found on T cells during chronic infections [115]. An earlier study found a similar result using cytomegalovirus-specific T cell clones. T cell clones derived from a central memory rather than an effector memory T cell clone persisted longer when adoptively transferred into macaques [116]. Memory stem T cells which have stem cell like properties have also been tested for use in CAR T cell production. This T cell population expresses the stem cell marker c-KIT but discrepancies exist in terms of how they are identified. Using CD95 and IL2Rβ as markers of memory stem cells in human peripheral blood, both CD4+ and CD8+ memory stem T cells have been identified. Representing a less differentiated T cell population, they have an enhanced capability for self-renewal and are more multipotent than central memory or effector memory T cells. Corroborating previous findings, naïve and central memory T cell derived CAR T cells engrafted more efficiently than effector memory derived CAR T cells. However, memory stem T cells outperformed all other subtypes in terms of engraftment, anti-tumor response and survival [117]. Following discovery of memory stem T cells in human peripheral blood it has been reported that this cell population can be found up to 12 years after adoptive transfer of genetically modified peripheral blood lymphocytes or hematopoietic stem cells and that they retain their precursor potential [118].

### 5.2. CAR γδ T cells

Involved in the rapid stress response, γδ T cells have a natural tropism for the TME and not only can certain subsets engage the adaptive immune response by acting as professional antigen presenting cells [119], they also have an innate cytotoxicity toward cancerous tissue. Successful expansion of peripheral blood Vγ9Vδ2 T cells using the aminobisphosphonate Zoledronate is possible both *in vitro* and *in vivo* [120]. The Vδ1 subtype is also present, albeit at a lower frequency, in peripheral blood and may be more attractive for cell therapy due to a more naïve, tissue-resident phenotype and links to improved outcome in challenging solid tumors such as triple negative breast cancer [121]. However, some tumor infiltrating Vδ1 cells have demonstrated a regulatory phenotype, warranting caution with the clinical development of this approach [122]. From a genetic engineering perspective, both Vδ1 and Vδ2 T cells are amenable to transduction and expansion from healthy donor PBMC and Vδ1 T cells retained a more naïve less exhausted phenotype than their Vδ2 or αβ counterparts. Expansion in response to target cells was boosted when both the CAR and TCR were engaged and CAR γδ T cells retained their capacity for migration toward tumor cells [123]. CD19-specific CAR γδ T cells have also demonstrated the ability to recognize tumor cells after loss of the CAR target, an important characteristic when considering tumor immune escape [124].

### 6. Allogeneic CAR T cells

Despite approval of 5 CAR T cell therapies for clinical use, production costs remain high, leading to great financial burden when treating large cohorts of patient cohorts. Autologous CAR T cells present a number of restrictions in this regard owing to the need for prolonged and patient specific T cell expansion, leading to the generation of a batch of drug which generally consists of a single dosing unit. Not infrequently, CAR T cell production fails or does not yield the required target cell dose. Consequently, the generation of an allogeneic ‘off-the-shelf’ universal CAR T cell drug in which multiple doses are produced per batch is desirable but challenging.

The primary obstacle to the production of a safe allogeneic CAR T cell product is the potential for GVHD due to TCR-mediated recognition of peptides complexed to shared or foreign MHC molecules [125]. One approach that theoretically may address this entails the expression of the CAR in a T cell population of known specificity. Virus-specific T cells rarely cause GVHD in allogeneic recipients. Illustrating this, patients with B cell malignancies who received hematopoietic stem cell transplants were treated with donor derived virus-specific CD19 CAR T cells. Anti-tumor activity was reported and in 2/3 patients CAR T cells expanded without the induction of GVHD, supporting the safety of this approach albeit in small phase I clinical trial [126]. Further development of this approach has been undertaken to protect allogeneic CAR T cells from rejection by host-derived alloreactive T cells. β2 microglobulin is an obligate component of all HLA molecules and was fused to the endodomain of CD3ζ within virus-specific T cells. Since this complex is incorporated into cell surface HLA molecules, the β2 microglobulin-CD3ζ fusion triggered a cytolytic response if it were engaged by potentially alloreactive T cells. When co-expressed with a conventional CAR in virus-specific T cells, it was proposed that this would constitute an allogeneic CAR T cell product which would not cause GVHD and would resist rejection [127]. A similar veto type approach to protect against GVHD was described by Mamonkin et al using a CD3ζ fusion that had been engineered to recognize 4–1BB, a cell surface co-stimulatory receptor which is transiently expressed on activated T cells [128].

GVHD can alternatively be prevented by eliminating TCR expression in CAR T cells. The TCR α chain is commonly targeted and proof of principle studies have used a variety
of technologies to achieve this [129,130]. Transcription activator-like effector nucleases (TALEN) technology can be multiplexed to knock out both the TCR α chain and CD52, which allowed the transferred CAR T cells to be rendered resistant to lymphodepletion regimens [131]. In an elegant study using CRISPR/cas9 technology, the expression of the TCR α chain was simultaneously removed as the CAR was inserted into the genome. By targeting the CAR construct to the TCR α locus, not only was CAR expression simultaneously achieved and regulated as if it were a TCR chain but issues around safety due to insertional mutagenesis were avoided [132,133].

Genome editing of allogeneic T cells to eliminate TCR expression should inhibit GvHD, however the specificity of the CAR for a particular tumor antigen remains fixed which limits the capacity to treat multiple cancers. To overcome this, universal CAR (UniCAR) T cells are now being developed that target various tumors using ‘switch’ modules. The switch module contains a tumor-specific binding domain and an epitope that can be bound by the CAR, allowing multiple different tumors to be detected using the same CAR by simply changing the switch module. Not only does this allow broad clinical application of a single cell product but in addition the T cell response can be controlled by altering the switch module dose. Furthermore, if expression of the initial target is lost, a switch module specific for another antigen could be administered [134]. The first proof of principle study to demonstrate the effectiveness of the UniCAR technology targeted AML using CD33 and CD123. Targeting of each antigen alone led to cancer cell killing, however combining the two switch modules boosted cytoxicity. In vivo testing showed efficacy with a combination of switch modules highlighted by a shift in the bone marrow from AML blasts to CD3+ T cells [135]. In solid tumors, using PSCA- and PSMA-specific switch modules, cytoxicity was restricted to target cells and comparable to that of conventional CAR T cells [136]. Further development of this platform has been achieved using a split, universal and programable (SUPRA) CAR system. SUPRA CAR design involves fusion of the CAR intracellular signaling domains to an extracellular leucine zipper. Tumor cells are then detected when bound by a tumor antigen specific scFv fused to a cognate leucine zipper (zipFv). Targeting of mesothelin, Her2 and Axl was confirmed, showing the broad spectrum of solid tumors that could be targeted. In addition to alterations in adaptor molecule concentration, changing the affinity of the leucine zippers also correlated with cytotoxicity and IFNγ production. Cytokine release syndrome could also be treated using the zipFv technology by addition of a second zipFv that competed for the CAR. Furthermore, this inhibition could be fine-tuned by changing the affinity of the zipper domain of the scFv [137].

Finally, as previously discussed, γδ T cells are an attractive population for CAR T cell generation and due to their MHC independent target recognition there is a low risk of GvHD when used to develop allogeneic therapies. Allogeneic NK cells can also be genetically modified to express CARs. Adoptive transfer of CAR NK cells is safe and using a CD19-targeted CAR to treat chronic lymphocytic leukemia and lymphoma, 7 out of 11 patients achieved a complete response [138].

7. Conclusion

The potential of CAR T cells for treating aggressive cancers has been realized in recent years with extraordinary clinical results reported in hematological malignancies. However, solid tumors present a more complex problem due to the need for CAR T cells to locate tumor deposits and then penetrate and operate within a hostile TME. Therefore, further development of CAR T cell therapies to improve target selection, enhance T cell signaling and supply pro-inflammatory modulators are required. Combination therapies such as these that target both the cancer and its protective network are likely to give the best clinical benefit to patients.

8. Expert opinion

Recent success in hematological cancers has demonstrated the immense therapeutic potential of CAR T cell immunotherapy. While response rates in B cell and plasma cell malignancies are impressive, treatment of solid tumors has given disappointing results. Limitation of CAR T cell efficacy in solid tumors can be attributed to three areas of concern: lack of specific target antigens, poor T cell homing to tumors and T cell dysfunction within the TME. With regard to target antigen discovery, neoantigen specific CAR T cells have been explored. While neoantigen targeting may enhance the selectivity of CAR T cell therapy, such therapies would likely be patient specific so increase production costs. An alternative approach to improving CAR T cell selectivity is to co-express CCRs alongside a CAR. While this would allow a pattern of TAAs to induce CAR T cell activation, the risk of tumor escape increases as CAR T cell function would be lost with downregulation of either antigen. A simpler means to detect tumor cells may therefore be to use NKG2D to target CAR T cells to upregulated stress ligands present in many cancers, supporting NKG2D CAR use in an allogeneic, universal CAR T cell setting.

Lack of CAR T cell trafficking to the tumor also limits application to solid tumors. Improved CAR T cell trafficking has been achieved preclinically by co-expression of chemokine receptors alongside the CAR. However, expression of a specific chemokine receptor is unlikely to improve function for all solid tumors adding to the complexity of any therapy designed using this approach. How this improved homing is affected by the location of the tumor and whether metastatic lesions would share the same chemokine profile remains to be seen.

Finally, extensive studies have been performed to boost CAR T cell function within the harsh environment of the TME. Checkpoint blockade has produced promising results in solid tumors; however, some patients do not respond, and toxicities have been reported. Expression and secretion of checkpoint inhibitors by CAR T cells may avoid both these issues by localizing the drug to the TME and increasing its concentration. Further arming of CAR T cells will likely be required in the form of cytokines such as IL-12 or IL-18 or chimeric cytokine receptors to convert immunosuppressive signals into stimulatory ones. While each of these advances may improve CAR T cell efficacy, treating large cohorts of
patients remains a challenge due to the complexity of different tumors.

To simplify CAR T cell therapy for solid tumors, development of universal CARs that can recognize a variety of different cancers in large numbers of patients will be key. Targeting broadly applicable ligands such as NKG2DL or use of the UniCAR or SUPRA CAR systems will be integral to future success. Another key step in designing truly universal CAR T cell products will be the optimization of protocols to produce allogeneic CAR T cell therapies using CRISPR or TALEN technology to remove TCR expression. Allogeneic therapies will improve the speed at which patients can be treated and avoid issues with batch failures. Furthermore, should the expression profile of the tumor change and the target antigen be lost, technology such as the UniCAR and SUPRA CAR would allow additional chances for tumor detection and destruction. Combining a universal CAR T cell design with the expression of chemokine receptors and mediators that support CAR T cell function in the TME may improve the clinical efficacy seen to date.

Funding

Research in JM’s laboratory is supported by Leucid Bio, Breast Cancer Now, British Lung Foundation, the Wellcome Trust, the J P Moulton Charitable Foundation, the Medical Research Council, the King’s Health Partners Research and Development Fund, the Experimental Cancer Medicine Centre at King’s College London, the Cancer Research UK Centre at King’s Health Partners and by the National Institute for Health Research (NIHR) Biomedical Research Centre based at Guy’s and St Thomas’ NHS Foundation Trust and King’s College London. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health.

Declaration of interest

J Maher is Chief Scientific Officer of Leucid Bio while CM Hull is a senior scientist for Leucid Bio. 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.

Reviewer disclosures

One referee has received grant support from Novartis, Tmunity Therapeutics, Cabaletta and Carisma. They also have CAR-T related IP licensed by their institution to Novartis and have consulted for Rite/ Gilead Sciences. Peer reviewers on this manuscript have no other relevant financial relationships or otherwise to disclose.

References

Papers of special note have been highlighted as either of interest (+) or of considerable interest (++) to readers.

1. Finney HM, Lawson AD, Bebbington CR, et al. Chimeric receptors providing both primary and costimulatory signaling in T cells from a single gene product. J Immunol. 1998;161(6):2791–2797.
2. Kocshenderfer JN, Feldman SA, Zhao Y, et al. Construction and preclinical evaluation of an anti-CD19 chimeric antigen receptor. J Immunother. 2009;32(7):689–702.
3. Neeleup S, Locke FL, Bartlett NL, et al. Axicabtagene ciloleucel CAR T-cell therapy in refractory large B-cell lymphoma. N Engl J Med. 2017;377(26):2531–2544.

++ Illustrates true potential of CAR T cell therapy.

4. Maude SL, Laetsch TW, Buechner J, et al. Tisagenlecleucel in children and young adults with B-cell lymphoblastic leukemia. N Engl J Med. 2018;378(5):439–448.

++ Illustrates true potential of CAR T cell therapy.

5. Rafiq S, Hackett CS, Brentjens RJ. Engineering strategies to overcome the current roadblocks in CAR T cell therapy. Nat Rev Clin Oncol. 2020;17(3):147–167.
6. Hortobagyi GN. Trastuzumab in the treatment of breast cancer. N Engl J Med. 2005;353(16):1734–1736.
7. Sun M, Shi H, Liu C, et al. Construction and evaluation of a novel humanized HER2-specific chimeric receptor. Breast Cancer Res. 2014;16(3):R61.
8. Zhao Y, Wang QJ, Yang S, et al. A herceptin-based chimeric antigen receptor with modified signaling domains leads to enhanced survival of transduced T lymphocytes and antitumor activity. J Immunol. 2009;183(9):5563–5574.
9. Priceman SJ, Tlakawardane D, Jeang B, et al. Regional delivery of chimeric antigen receptor-engineered T Cells effectively targets HER2(+) breast cancer metastasis to the brain. Clin Cancer Res. 2018;24(1):95–105.
10. Morgan RA, Yang J, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18(4):843–851.

++ Highlights the potential risks of CAR T cell therapy.

11. Heslop HE. Safer CARS. Mol Ther. 2010;18(4):661–662.
12. Ahmed N, Brawley VS, Hegde M, et al. Human epidermal growth factor receptor 2 (HER2) specific chimeric antigen receptor-modified T cells for the immunotherapy of HER2-positive sarcoma. J Clin Oncol. 2015;33(15):1688–1696.
13. Johnson LA, Scholler J, Ohkuri T, et al. Rational development and characterization of humanized anti-EGFR variant III chimeric antigen receptor T cells for glioblastoma. Sci Transl Med. 2015;7(275):275ra22.
14. O’Rourke DM, Nasrallah MP, Desai A, et al. A single dose of peripherally infused EGFRVIII-directed CAR T cells mediates antigen loss and induces adaptive resistance in patients with recurrent glioblastoma. Sci Transl Med. 2017;9(399):eaao0984.
15. Davies DM, Foster J, Van Der Stegen SJ, et al. Flexible targeting of ErbB dimers that drive tumorigenesis by using genetically engineered T cells. Mol Med. 2012;18:565–576.
16. Van Schalkwyk MC, Papa SE, Jeannon JP, et al. Design of a phase I clinical trial to evaluate intratumoral delivery of ErbB-targeted chimeric antigen receptor T-cells in locally advanced or recurrent head and neck cancer. Hum Gene Ther Clin Dev. 2013;24(3):134–142.
17. Papa S, Adami A, Metoudi M, et al. A phase I trial of T4 CAR T-cell immunotherapy in head and neck squamous cancer (HNSCC). J Clin Oncol. 2018;36(15):3046.
18. Zhou R, Yazdanifar M, Roy LD, et al. CAR T Cells targeting the tumor MUC1 glycoprotein reduce triple-negative breast cancer growth. Front Immunol. 2019;10:1149.
19. Wilkie S, Picco G, Foster J, et al. Retargeting of human T cells to tumor-associated MUC1: the evolution of a chimeric antigen receptor. J Immunol. 2008;180(7):4901–4909.
20. Posey AD Jr., Schwab RD, Boesteauan AC, et al. Engineered CAR T cells targeting the cancer-associated tn-glycoform of the membrane mucin MUC1 control adenocarcinoma. Immunity. 2016;44(6):1444–1454.
21. Adusumilli PS, Zauderer MG, Rusch VW, et al. A phase I clinical trial of malignant pleural disease treated with regionally delivered autologous mesothelin-targeted CAR T cells: Safety and efficacy [abstract]. In: Proceedings of the American Association for Cancer Research Annual Meeting 2019; 2019 Mar 29-Apr 3; Atlanta, GA. Philadelphia (PA): AACR; Cancer Res 2019;79(13 Suppl):Abstract nr CT036.
22. Zhan XWB, Li Z, Li J, et al. Phase I trial of Claudin 18.2-specific chimeric antigen receptor T cells for advanced gastric and pancreatic adenocarcinoma. J Clin Oncol. 2019;37(15):2509.
23. Saltman DABJB, Poire X, Havelange V, et al. Results from the completed dose-escalation of the hematological arm of the phase I think study evaluating multiple infusions of NKG2D-based CAR T-cells as standalone therapy in relapse/refractory acute myeloid leukemia and myelodysplastic syndrome patients. Blood. 2019;134:83826.

24. Shaza LHA, Awada A, Canon J, et al. Results from the completed dose-escalation phase I SHRINK study evaluating the autologous NKG2D-based CAR T-cell therapy CYAD-01 in metastatic colorectal cancer patients. Louvain, Belgium: Institut Jules Bordet, Université Libre de Bruxelles, Grand Hôpital de Charleroi (GHAC), University Hospital Leuven (UZ Leuven), Celyad, Cliniques Universitaires Saint-Luc, Université Catholique de Louvain; 2019.

25. Obajin J, Davies DM, Maher J. Engineering of chimeric natural killer cell receptors to develop precision adaptive immunotherapies for cancer. Clin Exp Immunol. 2020;202(1):11–27.

26. Ali SA, Shi V, Maric I, et al. T Cells expressing an anti-B-cell maturation antigen chimeric antigen receptor cause remissions of multiple myeloma. Blood. 2016;128(13):1688–1700.

27. Lee L, Draper B, Chaplin N, et al. An APRIL-based chimeric antigen receptor for dual targeting of BCMA and TACI in multiple myeloma. Blood. 2018;131(7):746–758.

28. Hegde M, Mukherjee M, Grada Z, et al. Tandem CAR T cells targeting HER2 and IL13Ralpha2 mitigate tumor antigen escape. J Clin Invest. 2016;126(8):3026–3035.

29. Osborne SMM, Tholouli E, Ramakrishnan A, et al. Phase I Alexander study of AUTO3, the first CD19/22 dual targeting CAR T cell therapy, with pembrolizumab in patients with relapsed/refractory (r/r) DLBCL. Am Soc Clin Oncol. 2020;38:8001.

30. Wilkie S, Van Schalkwyk MC, Hobbs S, et al. Dual targeting of ErbB2 and MUC1 in breast cancer using chimeric antigen receptors engineered to provide complementary signaling. J Clin Immunol. 2012;32(5):1059–1070.

31. Lionitis E, Poussin M, Kattenhoff AW, et al. Chimeric antigen receptor T Cells with dissociated signaling domains exhibit focussed antitumor activity with reduced potential for toxicity in vivo. Cancer Immunol Res. 2013;1(1):43–53.

32. Emtage PC, Lo AS, Gomes EM, et al. Second-generation anti-carcinoembryonic antigen designer T Cells resist activation-induced cell death, proliferate on tumor contact, secrete cytokines, and exhibit superior antitumor activity in vivo: a preclinical evaluation. Clin Cancer Res. 2008;14(24):8112–8122.

33. Kloss CC, Condomines M, Cartellieri M, et al. Combinatorial antigen recognition with balanced signaling promotes selective tumor eradication by engineered T Cells. Nat Biotechnol. 2013;31(1):71–75.

34. Feucht J, Sun J, Eyquem J, et al. Calibration of CAR activation potential directs alternative T cell fates and therapeutic potency. Nat Med. 2019;25(1):82–88.

• Demonstrates how CAR design can impact T cell phenotype.

35. Ng YY, Tay JCK, Li Z, et al. T Cells expressing NKG2D CAR with a DAP12 Signaling domain stimulate lower cytokine production while effective in tumor eradication. Mol Ther. 2021 Jun 6;29(1):75-85.

36. Van Der Stegen SJ, Harrie M, Sadelain M. The pharmacology of second-generation chimeric antigen receptors. Nat Rev Drug Discov. 2015;14(7):499–509.

37. Weinkove R, George P, Dasyam N, et al. Selecting costimulatory domains for chimeric antigen receptors: functional and clinical considerations. Clin Trans Immunol. 2019;8(5):e1049.

38. Maher J, Brentjens RJ, Gunset G, et al. Human T-lymphocyte cytotoxicity and proliferation directed by a single chimeric TCRZeta/CD28 receptor. Nat Biotechnol. 2002;20(1):70–75.

39. Imai C, Mihara K, Andreansky M, et al. Chimeric receptors with 4–1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia. Leukemia. 2004;18(4):676–684.

40. Krause A, Guo HF, Latouche JB, et al. Antigen-dependent CD28 signaling selectively enhances survival and proliferation in genetically modified activated human primary T lymphocytes. J Exp Med. 1998;188(4):619–626.

41. Guedan S, Madar A, Casado-Medrano V, et al. Single residue in CD28-costimulated CAR-T cells limits long-term persistence and antitumor durability. J Clin Invest. 2020;130(6):3087–3097.

42. Fos C, Salles A, Lang V, et al. ICOS ligation recruits the p50alpha PKR regulatory subunit to the immunological synapse. J Immunol. 2008;181(3):1969–1977.

43. Wan Z, Shao X, Ji X, et al. Transmembrane domain-mediated Lck association underlies bystander and costimulatory ICOS signaling. Cell Mol Immunol. 2020;17(2):143–152.

44. Simpson TR, Quezada SA, Allison JP. Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Curr Opin Immunol. 2010;22(3):326–332.

45. Guedan S, Posey AD Jr, Shaw C, et al. Enhancing CAR T cell persistence through ICOS and 4–1BB costimulation. JCI Insight. 2018;3(1):e96976.

46. Croft M. The role of TNF superfamily members in T-cell function and diseases. Nat Rev Immunol. 2009;9(4):271–285.

47. Finney HM, Akbar AN, Lawson AD. Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCR zeta chain. J Immunol. 2004;172(1):104–113.

48. Hendriks J, Gravestein LA, Tesselaar K, et al. CD27 is required for generation and long-term maintenance of T cell immunity. Nat Immunol. 2000;1(5):433–440.

49. Song DG, Ye Q, Poussin M, et al. CD27 costimulation augments the survival and antitumor activity of redirected human T cells in vivo. Blood. 2012;119(3):696–706.

50. Desai P, Abboud G, Stanfield J, et al. HVEM imprints memory potential on effector CD8 T cells required for protective mucosal immunity. J Immunol. 2017;199(8):2968–2975.

51. Nunoya JI, Masuda M, Ye C, et al. Chimeric antigen receptor T Cell bearing herpes virus entry mediator co-stimulatory signal domain exhibits high functional potency. Mol Ther Oncolytics. 2019;14:27–37.

52. Zhao Z, Condomines M, Van Der Stegen SJC, et al. Structural design of engineered costimulation determines tumor rejection kinetics and persistence of CAR T cells. Cancer Cell. 2015;28(4):415–428.

53. Wingett DG, Vestal RE, Forcier K, et al. CD40 is functionally expressed on human breast carcinomas: variable inducibility by cytokines and enhancement of Fas-mediated apoptosis. Breast Cancer Res Treat. 1998;50(1):27–36.

54. Curran KJ, Seinstra BA, Nikhamin Y, et al. Enhancing antitumor efficacy of chimeric antigen receptor T Cells through constitutive CD40L expression. Mol Ther. 2015;23(4):769–778.

55. Kuhn NF, Purdon Tj, Van Leeuwen DG, et al. CD40 ligand-modified chimeric antigen receptor T Cells enhance antitumor function by eliciting an endogenous antitumor response. Cancer Cell. 2019;35(3):473–488.

56. Lai Y, Weng J, Wei X, et al. Toll-like receptor 2 costimulation potentiates the antitumor efficacy of CAR T Cells. Leukemia. 2018;32(3):801–808.

57. Foster AE, Mahendravada A, Shinners NP, et al. Regulated expansion and survival of chimeric antigen receptor-modified T cells using small molecule-dependent inducible MyD88/CD40. Mol Ther. 2017;25(9):2176–2188.

58. Prinzbing B, Schreiner P, Bell M, et al. MyD88/CD40 signaling retains CAR T cells in a less differentiated state. JCI Insight. 2020;5(21):e136093.

59. Collinson-Pautz MR, Chang WC, Lu A, et al. Constitutively active MyD88/CD40 costimulation enhances expansion and efficacy of chimeric antigen receptor T Cells targeting hematological malignancies. Leukemia. 2019;33(9):2195–2207.

60. Wang X, Jasinski DL, Medina JL, et al. Inducible MyD88/CD40 synergizes with IL-15 to enhance antitumor efficacy of CAR-NK cells. Blood Adv. 2020;4(9):1950–1964.

61. Kobold S, Grassmann S, Chaloupka M, et al. Impact of a new fusion receptor on PD-1-mediated immunosuppression in adoptive T cell therapy. J Natl Cancer Inst. 2015;107(8):djv146.
Liu X, Ranganathan R, Jiang S, et al. A chimeric switch-receptor targeting PD1 augments the efficacy of second-generation CAR T cells in advanced solid tumors. Cancer Res. 2016;76(6):1578–1590.

Prosser ME, Brown CE, Shami AF, et al. Tumor PD-L1 co-stimulates primary human CD8(+) cytotoxic T cells modified to express a PD1::CD28chimeric receptor. Mol Immunol. 2012;51(3–4):263–272.

Hoogi S, Eisenberg V, Mayer S, et al. A TIGIT-based chimeric co-stimulatory switch receptor improves T-cell anti-tumor function. J Immunother Cancer. 2019;7(1):243.

Shin JH, Park HB, Oh YM, et al. Positive conversion of negative signaling of CTLA4 potentiates antitumor efficacy of adoptive T-cell therapy in murine tumor models. Blood. 2012;119(24):5678–5687.

Whilding LM, Halim L, Draper B, et al. CAR T-cells targeting the integrin alphavbeta6 and co-expressing the chemokine receptor CXCR2 demonstrate enhanced homing and efficacy against several solid malignancies. Cancers (Basel). 2019;11(5):674.

- Demonstrates the potential benefit of co-expressing chemo-kine receptors alongside CARs.

Lo A, Wang LS, Scholler J, et al. Tumor-promoting desmoplasia is disrupted by depleting FAP-expressing stromal cells. Cancer Res. 2015;75(14):2800–2810.

Caruana I, Savoldo B, Hoyos V, et al. Heparanase promotes tumor infiltration and antitumor activity of CAR redirected T lymphocytes. Nat Med. 2015;21(5):524–529.

Ajina A, Maher J. Prospects for combined use of oncolytic viruses and CAR T-cells. J Immunother Cancer. 2017;5(1):90.

Li YQ, Liu FF, Zhang XM, et al. Tumor secretion of CCL22 activates intratumoral Treg infiltration and is independent prognostic predictor of breast cancer. PLoS One. 2013;8(10):e76379.

Mantovani A, Sozanni S, Locati M, et al. Infiltration of tumours by macrophages and dendritic cells: tumour-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. Novartis Found Symp. 2004;256:137–145. discussion 146–159.

Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–268.

Bronte V, Serafini P, Mazzoni A, et al. L-ariginine metabolism in myeloid cells controls T-lymphocyte functions. Trends Immunol. 2003;24(6):302–306.

Balkwill FR, Capasso M, Hagemann T. The tumor microenvironment at a glance. J Cell Sci. 2012;125(23):5591–5596.

Galleu A, Riffio-Vasquez Y, Trento C, et al. Apoptosis in mesenchymal stromal cells induces in vivo recipient-mediated immunomodulation. Sci Transl Med. 2017;9(416):eaam7828.

Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363(8):717–723.

Schachter J, Ribas A, Long GV, et al. Pembrolizumab versus ipilimumab for advanced melanoma: final overall survival results of a multicentre, randomised, open-label phase 3 study (KEYNOTE-006). Lancet. 2017;390(10055):1853–1862.

Robert C, Long GV, Brady B, et al. Nivolumab in previously untreated melanoma without BRAF mutation. N Engl J Med. 2015;372(4):320–330.

Weber JS, Kahler KC, Hauschild A. Management of immune-related adverse events and kinetics of response with ipilimumab. J Clin Oncol. 2012;30(21):2691–2697.

Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med. 2015;373(2):123–135.

Rafiq S, Yeku OO, Jackson HJ, et al. Targeted delivery of a PD-1-blocking scFv by CAR-T cells enhances anti-tumor efficacy in vivo. Nat Biotechnol. 2018;36(9):847–856.

Li S, Siriwon N, Zhang X, et al. Enhanced cancer immunotherapy by chimeric antigen receptor-modified T cells engineered to secrete checkpoint inhibitors. Clin Cancer Res. 2017;23(22):6982–6992.

Wilkie S, Burbridge SE, Chiapero-Stanke L, et al. Selective expansion of chimeric antigen receptor-targeted T-cells with potent effector function using interleukin-4. J Biol Chem. 2010;285(33):25538–25544.
105. Wang R, Dillon CP, Shi LZ, et al. The transcription factor Myc controls metabolic reprogramming upon T lymphocyte activation. Immunity. 2011;35(6):871–882.

106. Zheng W, O’Hear CE, Alli R, et al. PI3K orchestration of the in vivo persistence of chimeric antigen receptor-modified T cells. Leukemia. 2018;32(5):1157–1167.

107. Torres Chavez A, McKenna MK, Canestrari E, et al. Expanding CAR T cells in human platelet lysate renders T cells with in vivo longevity. J Immunother Cancer. 2019;7(1):130.

108. Foskolou IP, Barbieri L, Vernet A, et al. The S enantiomer of 2-hydroxylutarate increases central memory CD8+ T cells and improves CAR-T therapy outcome. Blood Adv. 2020;4(18):4483–4493.

109. Arcangeli S, Falcone L, Camisa B, et al. Next-generation manufacturing protocols enriching TSCM CAR T cells can overcome disease-specific T cell defects in cancer patients. Front Immunol. 2020;11:1217.

110. Alizadeh D, Wong RA, Yang X, et al. IL15 enhances CAR-T cell antitumor activity by reducing miR1251 activity and preserving their stem cell memory phenotype. Cancer Immunol Res. 2019;7(5):759–772.

111. Ninomiya S, Narala N, Huye L, et al. Tumor indoleamine 2,3-dioxigenase (IDO) inhibits CD19-CAR T cells and is downregulated by lymphodepleting drugs. Blood. 2015;125(25):3905–3916.

112. Huang Q, Xi J, Wang L, et al. Correction to: miR-153 suppresses IDO1 expression and enhances CAR T cell immunotherapy. J Hematol Oncol. 2018;11(1):90.

113. Yang Y, Kohler ME, Chien CD, et al. TCR engagement negatively affects CD8 but not CD4 CAR T cell expansion and leukemic clearance. Sci Transl Med. 2017;9(417):eaag1230.

114. Agarwal S, Hanauer JD, Frank AM, et al. In vivo generation of CAR T cells selectively in human CD4(+/-) lymphocytes. Mol Ther. 2020;28(8):1783–1794.

115. Hinrichs CS, Borman ZA, Gattinoni L, et al. Human effector CD8+ T cells derived from naive rather than memory subsets possess superior traits for adoptive immunotherapy. Blood. 2011;117(3):808–814.

116. Berger C, Jensen MC, Lansdorp PM, et al. Adoptive transfer of effector CD8+ T cells derived from central memory cells establishes persistent T cell memory in primates. J Clin Invest. 2008;118(1):294–305.

117. Gattinoni L, Lugli E, Ji Y, et al. A human memory T cell subset with stem cell-like properties. Nat Med. 2011;17(10):1290–1297.

118. Biasco L, Scala S, Basso Ricci L, et al. In vivo tracking of T cells in humans unveils decade-long survival and activity of genetically modified T memory stem cells. Sci Transl Med. 2015;7(273):273ra13.

Illustrates how memory phenotype of T cells may influence efficacy.

119. Khan MW, Curbishley SM, Chen HC, et al. Expanded human blood-derived gammadelta T cells display potent antigen-presentation functions. Front Immunol. 2014;5:344.

120. Fisher JP, Heujiervjans J, Yan M, et al. Gammadelta T cells for cancer immunotherapy: a systematic review of clinical trials. Oncoimmunology. 2014;3(1):e27572.

121. Wu Y, Kyle-Cezar F, Woolf RT, et al. An innate-like Vδ1+γδ T cell compartment in the human breast is associated with remission in triple-negative breast cancer. Sci Transl Med. 2019;11(513):eaax9364.

122. Peng G, Wang HY, Peng W, et al. Tumor-infiltrating gammadelta T cells suppress T and dendritic cell function via mechanisms controlled by a unique toll-like receptor signaling pathway. Immunity. 2007;27(2):334–348.

123.capsomidis A, Benthal G, Van Acker HH, et al. Chimeric antigen receptor-engineered human gamma delta T cells: enhanced cytotoxicity with retention of cross presentation. Mol Ther. 2018;26(2):354–365.

124. Rozenbaum M, Meir A, Aharony Y, et al. Gamma-Delta CAR-T cells show CAR-directed and independent activity against leukemia. Front Immunol. 2020;11:1347.

Illustrates versatility of CAR γδ T cells.

125. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. Nat Rev Immunol. 2007;7(12):942–953.

126. Cruz CR, Micklethwate KP, Savoldo B, et al. Infusion of donor-derived CD19-redirected virus-specific T cells for B-cell malignancies relapsed after allogeneic stem cell transplant: a phase 1 study. Blood. 2013;122(17):2965–2973.

127. Quach DH, Becerra-Dominguez L, Rouce RH, et al. A strategy to protect off-the-shelf cell therapy products using virus-specific T-cells engineered to eliminate alloreactive T-cells. J Transl Med. 2019;17(1):240.

128. Mo F, Watanabe N, McKenna MK, et al. Engineered off-the-shelf therapeutic T cells resist host immune rejection. Nat Biotechnol. 2020;39:56–63.

129. Torikai H, Reik A, Liu PQ, et al. A foundation for universal T-cell based immunotherapy: t cells engineered to express a CD19-specific chimeric-antigen-receptor and eliminate expression of endogenous TCR. Blood. 2012;119(24):5697–5705.

130. Qasim W, Zhan H, Samarasinghe S, et al. Molecular remission of infant B-ALL after infusion of universal TALEN gene-edited CAR T cells. Sci Transl Med. 2017;9(374):eaaj2013.

131. Poiriot L, Philip B, Schiffer-Mannioui C, et al. Multiplex genome-edited T-cell manufacturing platform for “off-the-shelf” adoptive T-cell immunotherapies. Cancer Res. 2015;75(18):3853–3864.

132. Eyquem J, Mansilla-Soto J, Giavridis T, et al. Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. Nature. 2017;543(7643):113–117.

133. Depil S, Duchateau P, Grupp SA, et al. ‘Off-the-shelf’ allogeneic CAR T cells: development and challenges. Nat Rev Drug Discov. 2020;19(1):185–199.

134. Liu D, Zhao J, Song Y. Engineering switchable and programmable universal CARs for CAR T therapy. J Hematol Oncol. 2019;12(1):69.

135. Cartellieri M, Feldmann A, Koritka S, et al. Switching CAR T cells on and off: a novel modular platform for retargeting of T cells to AML blasts. Blood Cancer J. 2016;6(8):e458.

136. Feldmann A, Arndt C, Bergmann R, et al. Retargeting of T lymphocytes to FSCA− or PSMA positive prostate cancer cells using the novel modular chimeric antigen receptor platform technology “UniCAR”. Oncotarget. 2017;8(19):31368–31385.

137. Cho JH, Collins JJ, Wong WW. Universal Chimeric antigen receptors for multiplexed and logical control of T cell responses. Cell. 2018;173(6):1426–1438 e11.

138. Liu E, Marin D, Banerjee P, et al. Use of CAR-transduced natural killer cells in CD19-positive lymphoid tumors. N Engl J Med. 2020;382(6):545–553.