Biologics: Targets and Therapy

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REVIEW

How Can We Engineer CAR T Cells to Overcome Resistance?

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Abstract: Chimeric antigen receptor (CAR) T cell therapy has achieved unrivalled success in the treatment of B cell and plasma cell malignancies, with five CAR T cell products now approved by the US Food and Drug Administration (FDA). However, CAR T cell therapies for solid tumours have not been nearly as successful, owing to several additional challenges. Here, we discuss mechanisms of tumour resistance in CAR T cell therapy and the emerging strategies that are under development to engineer CAR T cells to overcome resistance.

Keywords: chimeric antigen receptor, cancer, immunotherapy, T-cell, resistance

Introduction

Chimeric antigen receptors (CARs) were first described over 30 years ago in which the variable regions of an immunoglobulin were fused into the constant regions of an αβ T cell receptor (TCR).[1] Eshhar simplified this design through the use of a single-chain antibody fragment to direct target specificity, while an activating moiety such as CD3ξ was employed to deliver a cytotoxic signal.[2] A key advance that resulted in clinical impact was the incorporation of co-stimulatory modules such as CD28 or 4–1BB within this basic framework.[3] These so-called second generation (2G) CAR T cells achieved dramatic efficacy in relapsed/refractory patients with selected haematological malignancies. As a result, the US Food and Drug Administration (FDA) has approved four CD19-specific and one B cell maturation antigen (BCMA)-specific CAR T cell products for the treatment of B cell and plasma cell malignancies, respectively.[4–8] Despite this success and continued research, the development of CAR T cell therapies for solid tumours continues to prove challenging with only sub-optimal efficacy achieved to date. Solid tumours deploy a unique set of obstacles that enable them to resist CAR T cell function and anti-tumour response. To address this, it is necessary to consider how we can engineer CAR T cells to overcome the hostile tumour microenvironment (TME) whilst avoiding immune pressure induced resistance, summarised in Table 1. In the sections that follow and are illustrated in Figure 1, we will consider a variety of strategies that have demonstrated promise in the quest for more resistant CAR T-cells.

Refinement of CAR Binding and Spacer Domain

Many CARs bind to antigen using single-chain antibody fragments (scFvs), comprising a variable heavy and variable light chain joined by a flexible linker. Selection of scFvs with appropriate affinity is important to avoid on-target off-

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Table 1 Summary of Mechanisms of Tumour Resistance and How CAR T Cells Can Be Engineered to Overcome Resistance

Tumour Resistance Mechanism	Description and Challenge to CAR T Cells	Mechanism to Overcome Resistance
Tumour microenvironment provides a physical barrier	Barrier prevents efficient homing and infiltration to tumour	• Expression of chemokines[125–128] • Targeting tumour stroma[133,134] • Target extracellular matrix[136]
Immune checkpoints	Immune checkpoints eg PD-1/PDL-1 expressed on tumour cells and suppressive immune cells inhibit CAR T cell function	• Secretion of anti-checkpoint antibodies[143–147] • Dominant negative checkpoint receptor expression[148] • Checkpoint receptor knockout/ downregulation[149–155] • Checkpoint switch receptors[157–159]
Immunosuppressive cytokines	Secretion of immunosuppressive cytokines by tumour and suppressive immune cells prevents optimal T cell activation and proliferation	• Dominant negative cytokine receptors[163–166] • Cytokine switch receptors[167] • Knock out of cytokine receptors[169]
Suppressive molecules in TME	Suppressive molecules released in to the TME inhibit CAR T cell function eg adenosine, prostaglandin E2	• Receptor inhibition/knockout eg Adenosine receptor[172–175] • Manipulation of inhibitory pathways in CAR T cells[176]
Low availability of nutrients in TME	Malignant cells accelerate metabolism reducing the availability of nutrients for CAR T cells and increasing harmful by products	• Small molecule inhibitors against harmful metabolites eg IDO[185] • Expression of receptors to enhance uptake of metabolites[187] • Utilisation of alternative metabolites[188]
Loss of target antigen	Immune pressure can result in antigen loss by tumour cells rendering them undetectable by CAR T cells	• Target multiple antigens: ○ Dual targeting CARs[209–213] ○ Trivalent CARs[214,215] ○ Tandem CARs[216,217] ○ Universal CARs/BiTEs[219–222] • Expression of artificial ligand/receptors[223,224] • Promotion of epitope spread[226]

tumour toxicities. Low-affinity ErbB2 (HER2) or epidermal growth factor receptor (EGFR) targeting CAR T cells can distinguish between healthy cells expressing low level of antigen and tumour cells expressing high level of antigen without compromising T cell activation.[9–11] Similarly, a humanised folate receptor-alpha (αFR) specific scFv with reduced affinity for target antigen compared to the parental murine scFv could distinguish between high and low-density tumour antigen in vitro and in vivo.[12] Selection of low-affinity scFvs has also improved CAR T cell function in some cases.[13,14] Lower affinity was associated with a faster off-rate and increased serial killing of tumour cells, leading to enhanced function in vitro and in vivo.[13] In addition to optimisation of affinity, the selection of humanised or fully human scFvs (rather than traditionally used murine variants) is preferred in order to prevent anti-CAR immune responses, as discussed in later sections.[15] One concern with the use of scFvs as CAR binding domains is their frequent propensity to oligomerise. As a result, CAR clustering may occur, leading to tonic signalling and early exhaustion.[16] Nanobodies represent an alternative to scFvs which may reduce the risk of unwanted oligomerisation, as they consist of a single heavy chain only.[17] Furthermore, due to their small size, nanobodies can engage with some epitopes that are inaccessible to scFvs.[18] Alternative binding domains include naturally occurring tumour-sensing receptors such as natural killer (NK) cell receptors,[19] modified cytokines such as IL-13 mutein[20] or the T1E panErbB ligand,[21] and integrin-targeting peptides such as A20[22]

The spacer/hinge domain connects the binding domain and the transmembrane domain within a chimeric antigen receptor. Many CAR constructs utilise spacer/hinge regions derived from one of the four human IgG

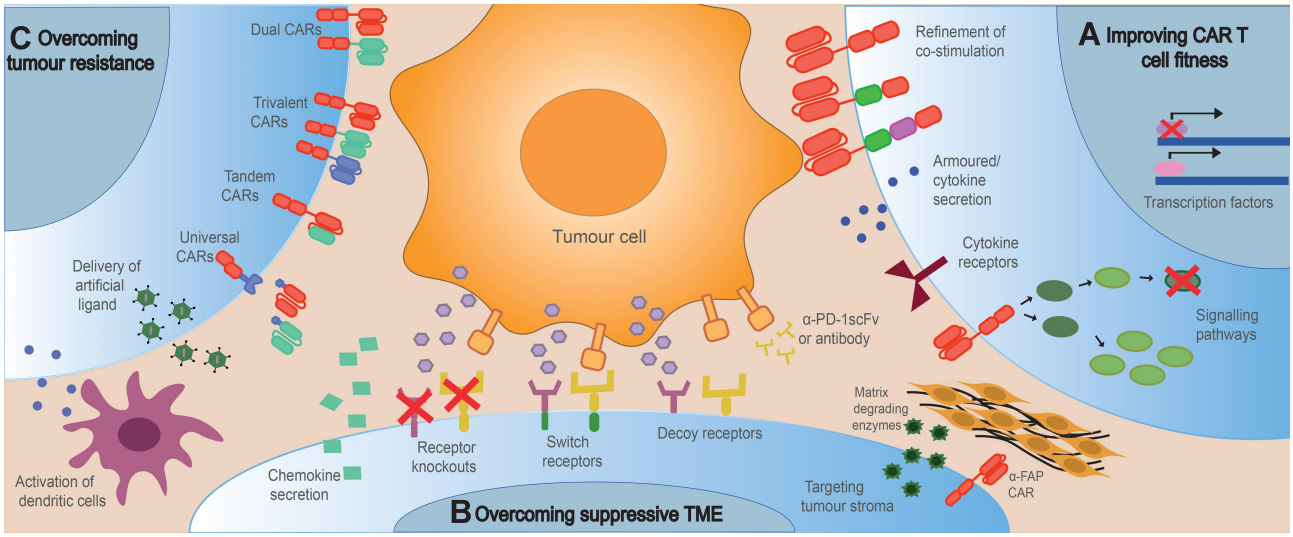

Figure 1 High level overview of strategies to engineer resistance in CAR T-cells. Tumour resistance to CAR T cell therapy can be overcome by (**A**) improving CAR T cell fitness to enhance proliferation, persistence and cytotoxicity; (**B**) engineering CAR T cells to resist the suppressive TME including checkpoint inhibitors, immunosuppressive cells and cytokines and the tumour stroma, whilst enhancing tumour homing and infiltration; (**C**) Engineering CAR T cells to overcome tumour resistance caused by antigen loss or downregulation by promoting epitope spreading, targeting multiple antigens or introducing artificial ligands to the tumour.

subclasses, or from CD8α and CD28. In the case of IgG-derived sequences, mutation of residues involved in Fc receptor engagement may be desirable to mitigate unwanted interactions.[23] The selection of an appropriate spacer domain of optimal length is important for efficient binding of ligand, and is dependent on the position of the target epitope on the tumour surface.[24] For example, incorporation of the highly flexible, elongated IgD hinge improved CAR-mediated recognition of the aberrantly glycosylated tumour target, Muc1.[25] The use of different spacer domains may also affect the intensity of CAR T cell activation. Illustrating this, CD19 CARs containing the spacer and transmembrane domain from CD28 secreted lower cytokine levels and underwent reduced activation-induced cell death (AICD) compared with those containing the spacer and transmembrane domain of CD8α,[26] although the influence of changing the spacer domain only was not evaluated in this study.[26]

Refinement of Co-Stimulation

Approved 2G CAR T cells therapies contain the co-stimulatory domains CD28 (Yescarta, Tecartus) or 41BB (Kymriah, Breyanzi, Abecma).[4,5] Although these therapies provide comparable efficacy, the cell products demonstrate quite different kinetics of anti-tumour activity.[27,28] When compared to 41BB-containing counterparts, CD28 CAR T cells undergo early expansion and mediate rapid initial tumour cell killing, exemplified by in vivo stress test models.[27] Faster activation kinetics of CD28 containing CAR T cells was associated with increased phosphorylation of CAR CD3ζ, Lck, ZAP-70 and LAT following in vitro activation.[29] Early phosphorylation events ultimately lead to higher Ca^{2+} influx, CD69 expression and increased IL-2 and interferon (IFN)-γ secretion, resulting in faster cytotoxic responses.[27,29] Rapid activation following antigen exposure of CD28 CAR T cells may be dependent on basal phosphorylation of Lck in these T cells.[29,30] A consequence of the superior initial activation of CD28 CARs is early exhaustion leading to poor persistence and diminished long-term anti-tumour function.[31] Guedan et al[31] reversed excessive activation and improved the persistence of CD28 CAR T cells by mutating a single amino acid within the CD28 endodomain. The YMNM motif of CD28 binds to SH2 containing proteins, including Grb2 and phospholipase C (PLC)γ1, activating downstream signalling pathways. Mutation of the asparagine (N) in this motif to phenylalanine (F) disrupts this interaction, reducing Ca^{2+} influx and NFAT activation, protecting cells from premature exhaustion, yet maintaining lytic capacity.[31] These authors confirmed improved function following a single acid mutation by using mesothelin targeting CARs which demonstrated superior efficacy in two in vivo pancreatic cancer models.[31] CD28 also contains a PRRP motif, which binds ITK, activating PLC-γ and ERK signalling pathways, and a PYAP motif, which binds Lck.[32] Boucher et al[32] also investigated the effect of

mutating these signalling motifs in order to reduce CAR T cell exhaustion. CD19 targeting CAR T cells in which both the CD28 YMNM and PRRP motifs were mutated while the PYAP motif remained intact demonstrated superior persistence and reduced exhaustion in a B-ALL mouse model, demonstrating that reduction of CD28 signalling was beneficial in this model.[32] In an alternative approach, Sun et al[29] proposed that recruitment of phosphatases to the CAR would decrease early phosphorylation events, preventing excessive activation and the resulting early exhaustion. To achieve this, a FK506 binding protein (FKBP) rapamycin binding (FRB) domain was introduced into a CD28 CAR and was expressed alongside SHP1 phosphatase containing a FKBP domain. Following the addition of an inert rapalog, SHP1 was recruited to the CAR via FRB-FKBP binding, reducing CAR phosphorylation upon activation without compromising anti-tumour activity of a CD19 targeting CAR against an in vivo lymphoma xenograft model.[29] Reduced exhaustion of CD28 CAR T cells was also achieved by integrating the CAR cDNA into the T-cell receptor α constant (*TRAC*) locus via CRISPR/Cas9 editing. This allowed *TRAC* promoter-regulated CAR expression, internalisation and re-expression following antigen encounter and resulted in CAR T cells with a less differentiated phenotype and reduced exhaustion.[33]

In contrast to CD28 variants, 41BB-containing CAR T cells demonstrate slower anti-tumour kinetics, yet improved persistence.[27,28,34] Furthermore, 41BB CAR T cells are generally more resistant to exhaustion due to reduced tonic signalling, and exhibit a more favourable metabolic profile and less differentiated state.[34] Modification of 41BB CAR T cells has been attempted to improve their initial activation kinetics but still maintain their longevity. Overexpression of Lck in 41BB CAR T cells counteracts the recruitment of SHP1 phosphatase, leading to increased CAR CD3ζ phosphorylation and Ca^{2+} influx.[29] Lck overexpression improved initial expansion following antigen exposure without inducing an exhausted phenotype and improved the efficacy of GD2 targeting CAR T cells against an in vivo neuroblastoma model.[29] Recently, it has been shown that 41BB-containing CARs continue to signal in endosomes following antigen encounter and internalisation.[35] Mutation of intracellular lysine residues within the CAR prevents ubiquitination and 41BB signalling is thereby enhanced via colocalization with TRAF2 in endosomes, increasing signalling by mammalian target of rapamycin (mTOR), mitochondrial oxidative phosphorylation and memory cell differentiation.[35]

Harnessing the signalling capacities of both CD28 and 41BB has been attempted by placing both costimulatory domains upstream of CD3ζ in third generation (3G) CAR T cells. Some studies have demonstrated the superior efficacy of CD28 and 41BB containing 3G CAR T cells compared to their 2G counterparts with improved expansion, cytokine production and anti-tumour function.[36–39] However some studies only show borderline superiority and even inferior activity compared to 2G CARs.[28,40,41] Furthermore, clinical success of 3G CAR T cells has been limited. Despite this, the addition of a further costimulatory domain has been attempted with 4G CAR T cells consisting of 41BB, CD28 and CD27.[42] Combining CD28 and 41BB signalling in other configurations has also been attempted. For example, the expression of 41BB or 41BBL alongside a CD28 2G CAR improves proliferation, persistence and anti-tumour efficacy compared to their 2G and 3G counterparts in both in vitro and in vivo models.[27,43] Alternatively, in an attempt to improve CAR safety and specificity, a construct containing a first generation (1G) CAR expressed alongside a chimeric co-stimulatory receptor (CCR) containing CD28 and 41BB was engineered.[44] Although this CAR construct provides a safety mechanism, as both CAR and CCR engagements are necessary for T cell activation, signalling by both CD28 and 41BB elements within the fused CCR was not demonstrated.

Second-generation CARs utilising co-stimulatory molecules other than CD28 and 41BB have also been investigated. Incorporation of inducible T cell co-stimulator (ICOS) resulted in Th1/Th17 polarisation of CAR T cells with increased IFN-γ, IL-17 and IL-22 secretion compared to both 41BB and CD28 CAR T cells.[45] Furthermore, ICOS CAR T cells demonstrated enhanced persistence in an in vivo model of non-small cell lung cancer.[45] Unlike CD28 CAR T cells, CAR T cells containing OX40 co-stimulation do not secrete IL-10 and yet secrete comparable levels of pro-inflammatory cytokines.[46] Functional studies demonstrating enhanced function of 2G OX40 CAR T cells are lacking and studies investigating 3G OX40/CD28 CAR T cells have shown contrasting results of either enhanced or diminished cytokine secretion.[47,48] Similar to 41BB, incorporation of CD27 in CAR designs led to enhanced persistence and survival in vivo compared to CD28 CAR T cells.[49] The addition of either inducible or constitutively active MyD88 and CD40 has also resulted in enhanced

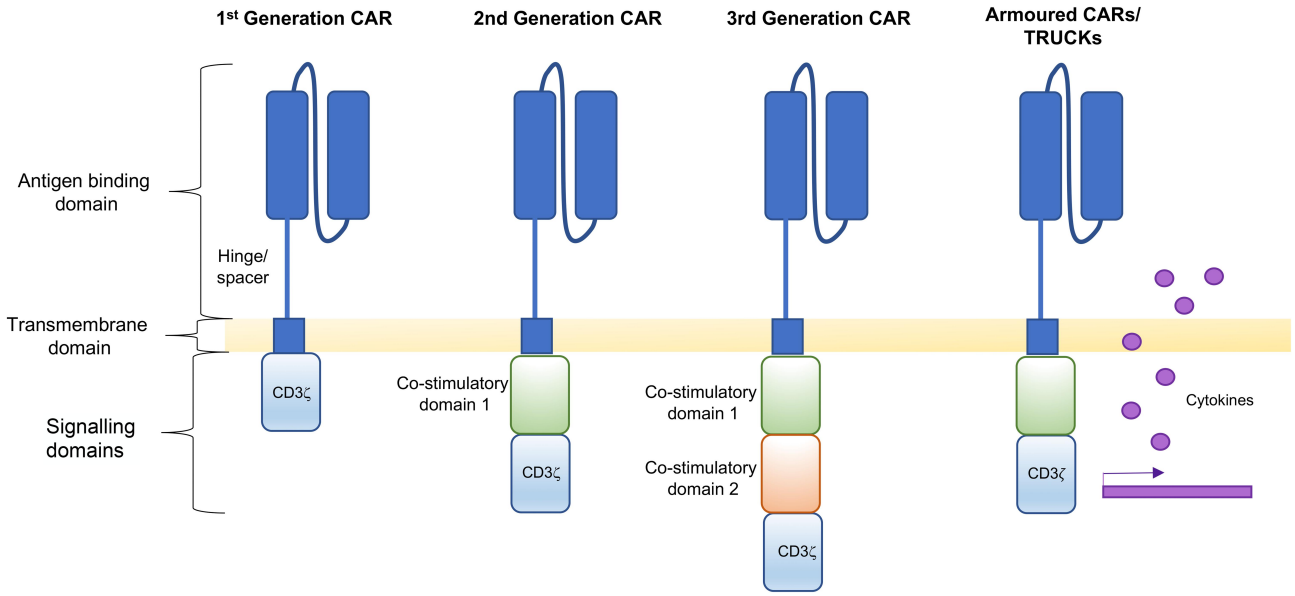

Figure 2 Structural components of CAR T cells. The structure of a first generation CAR consists of an antigen binding domain attached to a transmembrane domain via a hinge/spacer, followed by a CD3ξ signalling domain. Second generation CARs have an additional co-stimulatory domain and third generation CARs contain two co-stimulatory domains. Fourth generation CARs are armoured to secrete cytokines. All structural components of CAR T cells illustrated can be refined to enhance function.

performance of CAR T cells.[50–52] More recently, Prinzing et al[53] found that MyD88/CD40 CAR T cells demonstrated improved in vitro proliferation and restimulation ability while remaining in a less differentiated state compared to both CD28 and 41BB containing CAR T cells.[53] The structural components of CAR T cells that can be refined to enhance function are illustrated in Figure 2.

Armoured (Cytokine-Expressing) CAR T Cells

Engineering CAR T cells to secrete cytokines that enhance proliferation and effector function (known variously as "armoured CARs" or "TRUCKs") represents another strategy that may be used to boost resistance of these cells (Figure 2). Several cytokines have been used in this manner as summarised below and in Table 2. Interleukin (IL)-12 was one of the first cytokines to be constitutively expressed alongside a CAR, in order to promote resistance to the immunosuppressive TME.[54–56] Concerns over toxicity related to constitutive cytokine production led to the engineering of CAR T cells in which IL-12 transcription was placed under the control of the NFAT promoter. By this means, IL-12 production was rendered dependent on CAR T cell activation, meaning that this toxic cytokine should remain localised to the TME.[57,58] Although studies on both immunodeficient and immunocompetent mouse models demonstrated control of IL-12 secretion, toxicity was seen in a clinical trial in which ex vivo expanded tumour-infiltrating lymphocytes (TILs) were armoured with inducible IL-12, suggesting possible leakiness of NFAT promoter activity.[59] In an alternate approach, exemplified by Sachdeva et al,[60] the IL-12 gene was placed under the IL-2Rα or PDCD1 regulatory elements in order to couple cytokine secretion to CAR activation.[60] Armouring CAR T cells with IL-12 can also induce accumulation of activated macrophages, as demonstrated in an immune competent mouse model, resulting in the ability to target antigen-negative tumour cells.[57,61]

Armouring CARs with constitutively expressed IL-15 can also augment effector functions and enhance fitness. When tested in vitro and in immunocompetent mouse models, IL-15 maintained naïve and central memory T-cell phenotype, with increased expression of the anti-apoptotic protein Bcl-2 and reduced PD-1 expression.[62,63] Similar results were observed when IL-15 is tethered to the CAR T cell membrane, an approach that also prevents unwanted bystander cell activation.[64] Adachi et al[65] engineered CAR T cells to co-express both IL-7 and CCL19 to promote T cell and dendritic cell (DC) recruitment and tumour infiltration in an immunocompetent mouse model. Secretion of IL-7 and CCL19 from CAR T cells improved anti-tumour responses in solid tumour mouse models with increased overall survival. Furthermore, mice treated with IL-7 and CCL19 secreting CAR T cells could eradicate both antigen positive and antigen-negative tumour

Table 2 Summary of Armoured CAR T Cells

Cytokine	Benefit in CAR T Cells	Reference
IL-12	• Enhanced IFN-γ secretion • Increased survival/persistence • Decreased apoptosis • Enhanced proliferation • Reduction of tumour associated macrophages • Enhanced resistance to PDL-1 inhibition • Recruitment of immune cells	Koneru et al[54] Pegram et al[55] Yeku et al[56] Chmielewski et al[57] Liu et al[58] Sachdeva et al[60] Kueberuwa et al[61]
IL-15	• Promotes naïve and central memory phenotypes • Increased expression of anti-apoptotic proteins • Reduced expression of exhaustion markers • Enhanced natural killer cell activation • Reduced tumour associated macrophage accumulation • Enhanced expansion, persistence and increased stem cell memory populations when expressed in combination with IL-21	Lanitis et al[62] Markley et al[63] Batra et al[66]
IL-7 and CCL19	• Enhanced proliferation • Enhanced T cell and DC recruitment • Enhanced tumour infiltration	Adachi et al[65]
IL-7	• Enhanced proliferation	Markley et al[63]
IL-21	• Enhanced proliferation • Enhanced expansion, persistence and increased stem cell memory populations when expressed in combination with IL-15	Markley et al[63] Batra et al[66]
IL-9	• Enhanced central memory cells • Reduced exhaustion markers • Enhanced persistence	Liu et al[67]
IL-18	• Enhanced proliferation • Enhanced IFN-γ secretion • Reduced exhaustion markers • Increased accumulation of immune cells in TME	Hu et al[68] Chmielewski et al[69]
IL-23	• Enhanced proliferation • Increased granzyme B secretion • Reduced expression of exhaustion markers • Reduced toxicity compared to IL-15 and IL-18 armoured CARs	Ma et al[70]
IL-24	• Enhanced expansion • Enhanced survival • Increase naïve cells and decreased effector cell populations • Tumour suppressor	Hu et al[71]
IL-36-γ	• Enhanced expansion and persistence • Increased dendritic cell secretion of IL-6 • Increased endogenous T cell secretion of IFN-γ and TNF-α	Li et al[72]
Cytokine receptor		
IL-6R (gp130 expression)	• Enhanced proliferation and expansion • Enhanced survival	Jiang et al[73]

(*Continued*)

Table 2 (Continued).

Cytokine	Benefit in CAR T Cells	Reference
IL-7R	• Enhanced proliferation • Enhanced survival	Shum et al[74] Zhao et al[75] Perna et al[76]
IL-4αβ switch receptor	• Switch from anti-inflammatory to pro-inflammatory signal • Selective expansion • Enhanced proliferation	Wilkie et al[77]
IL-4/7 switch receptor	• Switch from anti-inflammatory to pro-inflammatory signal • Selective expansion • Enhanced proliferation	Leen et al[78] Mohammed et al[79]
IL-4/21 switch receptor	• Switch from anti-inflammatory to pro-inflammatory signal • Selective expansion • Enhanced proliferation	Wang et al[80]
c-fms	• Enhanced proliferation • Promoted chemotaxis to tumour	Lo et al[83]

rechallenge, indicating the development of a robust memory response and epitope spread.[65] Markley et al[63] set out to identify the optimal common gamma cytokine support for CD19 targeting CAR T cells by the constitutive co-expression of IL-2, IL-7, IL-15 or IL-21 in these cells. Evaluation was performed in a systemic B-cell lymphoma model in immunodeficient mice. They demonstrated that IL-7 and IL-21 armoured CAR T cells achieved superior anti-tumour responses compared to those producing IL-15 and IL-2, a finding that was suggested to depend on enhanced proliferation but not enhanced lytic activity of CAR T cells. Furthermore, no long-lived cells could be identified following IL-7 armoured CAR T cell treatment and only T effector memory cells could be identified in IL-21 armoured CARs in the mouse models with complete responses.[63] When tested on a hepatocellular carcinoma xenograft model in immunodeficient mice, the combined constitutive co-expression of both IL-15 and IL-21 in CAR T cells resulted in superior expansion, persistence and anti-tumour efficacy, associated with increased stem cell memory and central memory populations, when compared to single cytokine expression.[66] Similarly, IL-9 expression in CAR T cells also enhances the pool of central memory cells, with lower expression of exhaustion markers and enhanced in vivo persistence.[67]

The addition of constitutively active or inducible IL-18 to CAR T cells can also improve anti-tumour function with enhanced proliferation, IFN-γ production and a reduction in markers of exhaustion compared to IL-12 secreting CAR T cells.[68,69] Furthermore, IL-18 secretion from CAR T cells engaged the local immune response in immunocompetent tumor-bearing mice by inducing an increase in M1-polarised macrophages and NK cells and a decrease in T regulatory cells (Tregs), suppressive DCs and M2-polarised macrophages within the TME.[69] Alternatively, engineering CAR T cells to secrete IL-23 demonstrated reduced toxicity compared to constitutive expression of IL-15 and IL-18, with enhanced effector function demonstrated in both xenograft and syngeneic immunocompetent solid tumour mouse models.[70] To induce CAR T cells to secrete IL-23, cells were engineered to express the IL-23 p40 subunit, which unlike the IL-23α p19 subunit and the IL-23R, is not upregulated upon T cell activation. The secretion of IL-23 was therefore rendered dependent on CAR T cell activation.[70] In another approach, armouring CAR T cells with IL-24 has recently been shown to improve cytotoxicity, expansion and survival, with increased naïve cell and reduced effector cell populations in vitro with no signs of toxicity in immunodeficient mice.[71] Furthermore, IL-24 acts as a tumour suppressor in many cancer types, promoting the therapeutic activity of IL-24 armoured CAR T cells.[71] IL-36-γ armoured CARs have also been shown to mediate enhanced anti-tumour activity by engaging endogenous immune responses. IL-36-γ CAR T cells increase IL-6 production by DCs and IFN-γ and tumour necrosis factor (TNF)-α release by endogenous T cells, which could then eradicate a secondary challenge with antigen-negative tumour cells.[72]

In summary, many cytokines have now been tested for their ability to enhance CAR T cell anti-tumour responses.

Further studies are necessary to compare and identify the best cytokine or combination of cytokines to armour CAR T cells.

Cytokine Receptor Expression in CAR T Cells

An alternative strategy to improve CAR T cell expansion and function involves the introduction of cytokine receptors into these cells. Jiang et al[73] engineered CAR T cells to express a constitutively active form of gp130, a membrane protein which induces IL-6 trans signalling following the binding of IL-6/soluble IL-6 receptor (sIL-6R). Compared to a synthetic IL-6/sIL-6R complex, termed hyper IL-6, the constitutively active gp130 similarly enhanced CAR T cell function but without induction of GvHD in immunodeficient mouse models.[73] Expression of constitutively active IL-7R enhanced CAR T cell proliferation, survival and anti-tumour response in immunodeficient mouse models, even when Tregs were co-infused.[74–76] The concern of autonomous cell expansion with constitutively active cytokine receptor expression can in principle be circumvented by using switch chimeric cytokine receptors.[77,78] CAR T cells have been engineered to respond to the immunosuppressive cytokine IL-4 by fusing the IL-4Rα ectodomain to the β_c subunit of IL-2 and IL-15 receptor,[77] the IL-7 receptor endodomain,[78,79] or IL-21 receptor endodomain.[80] Using these chimeric receptors, tumour derived IL-4 is harnessed to deliver a positive signal, increasing proliferation and anti-tumour response. Chimeric cytokine receptors are also useful for enhancing the expansion and selective proliferation of CAR T cells during the manufacture of cell products.[77,81,82] Similarly, CAR T cells have been engineered to respond to tumour derived colony-stimulating factor (CSF)-1.[83] Expression of c-*fms*, the CSF-1 receptor, in CAR T cells not only enhanced proliferation but also promoted chemotaxis in response to CSF-1.[83]

Manipulation of Signalling Pathways in CAR T Cells

Targeting distinct pathways in T cell signalling can influence effector function and differentiation of CAR T-cells. Cytokine signalling pathways can be enhanced by incorporating signalling domains directly into the CAR structure. Kagoya et al[84] incorporated the IL-2Rβ domain which mediates STAT5 activation, alongside the YXXQ STAT-3 motif into 2G CARs.[84] The addition of these domains resulted in superior persistence, proliferation and anti-tumour efficacy compared to matched 2G counterparts due to enhanced JAK/STAT signalling, in both liquid and solid tumour models in NSG mice.[84] In a related approach, the expression of constitutively active STAT5 improved anti-tumour activity of CD19 CAR T cells, accompanied by enhanced persistence demonstrated in a mouse B cell lymphoma model.[85] The phosphatase PTPN2 dephosphorylates multiple components of the JAK/STAT pathway inhibiting cytokine signalling. Engineering of PTPN2-deficient CAR T cells targeting ErbB2 resulted in enhanced cytotoxicity, tumour homing and tumour eradication when infused into lymphodepleted mice bearing ErbB2+ mammary tumours.[86]

The phosphoinositide 3-kinase (PI3K)/Akt signalling pathway is activated following engagement of the TCR and its coreceptors and promotes cell survival, glycolysis and cytokine synthesis.[87] Within the TME, however, suppressive cytokines and molecules activate phosphatases which counteract Akt signalling.[88] To overcome this, Akt has been overexpressed in CAR T cells which increased tumour cell killing both in vitro and in vivo models.[89] Similar results were observed when a constitutively active Akt protein was expressed in CAR T cells.[90] However, the sustained activation of Akt in CD8 T cells promotes terminal differentiation.[91] Inhibiting Akt during ex vivo expansion of CAR T cells improved anti-tumour responses of CD19 CAR T cells in tumour-bearing immunodeficient mice, without reducing cell yield.[91–93] Furthermore, Akt inhibition increases memory cell populations by promoting localisation of the transcriptional regulator of T cell memory, FOXO1, to the nucleus.[93] Similarly, manipulating the PI3K/Akt signalling pathway further upstream by pre-treatment of CD33 targeting CAR T cells with a PI3K inhibitor prevented differentiation ex vivo, which enabled enhanced in vivo persistence in immunodeficient mice bearing CD33+ tumour cells.[94] Careful manipulation of the PI3K/Akt pathway is necessary to engineer CAR T cells with favourable characteristics. Inhibition of Akt during manufacturing helps to prevent terminal differentiation. However, Akt expression by CAR T-cells is important when they are located within the TME to enhance effector responses.

The complexity of T cell signalling makes it challenging to identify targets to engineer CAR T cells with enhanced fitness and anti-tumour immunity. More recently CRISPR-Cas9 technology has been used to thoroughly screen potential therapeutic targets to improve T cell therapy. Gurusamy et al[95] genetically edited 25 T cell receptor-driven kinases and monitored phenotypes related to T cell

fitness, including cell expansion, differentiation, oxidative stress and genomic stress.[95] The MAP kinase p38α was identified as a target which, when inhibited in CAR T cells, led to improved anti-tumour response in immune competent mouse tumour models.[95] In an independent study, a CRISPR-Cas9 screen was combined with a screen of >500 small molecule drugs to identify mechanisms to improve CAR T cell cytotoxicity.[96] FADD and TRAIL-R2 were identified as important components for the induction of cancer cell apoptosis which could be sensitised through treatment with SMAC mimetics.[96] Using these methods to screen for important components of CAR T cell signalling to improve fitness and cytotoxicity will allow us to identify ways to engineer more efficacious CAR T cells to overcome resistance.

Manipulation of Transcription Factors in CAR T Cells

Targeting transcription factors in CAR T cells has proven a useful tool to improve fitness. Lynn et al[97] demonstrated that tonic signalling from a GD2 targeting CAR caused dysregulation of AP-1 transcription factor family members with increased expression of immunoregulatory components, BATF, JunB and IRF4.[97] Engineering CAR T cells to overexpress c-Jun shifts the balance in favour of the formation of proinflammatory c-Jun/c-Fos heterodimers, resulting in reduced terminal differentiation, increased expansion and improved anti-tumour responses against both solid tumour and leukemic xenograft models.[97] NFAT is a transcription factor associated with T cell activation and effector responses, but also contributes to T cell exhaustion and tolerance.[98] Only when NFAT is bound to AP-1 can it promote the expression of activating genes, which is likely the mechanism of improved function when CAR T cells are engineered to overexpress c-Jun.[97,98] Furthermore, exhaustion of CD19 targeting CAR T cells has been associated with the activity of three NR4A family transcription factors (NR4A1, NR4A2, NR4A3) which are regulated by NFAT.[99] Specifically, NR4A1 promotes T cell exhaustion by not only inducing tolerance-related genes but also by competing for AP-1 binding sites, thereby preventing transcription of activating genes.[100] Triple knockout of the NR4A transcription factors in CAR T cells eliminated exhausted phenotypes and enhanced anti-tumour responses in a solid tumour xenograft model.[99] Another NFAT driven transcription factor family, TOX, has similar functions to NR4A transcription factors in promoting T cell exhaustion, and these two transcription factor families can positively regulate the expression of each other.[101,102] CAR TILs engineered to no longer express two TOX transcription factors (TOX and TOX2) also achieved improved in vivo anti-tumour responses with reduced expression of inhibitory receptors.[101]

Improving T Cell Fitness During Manufacturing of CAR T Cell Therapy

Most therapeutic CAR T cells are manufactured as autologous products using patient-derived leukapheresis. Despite the success of autologous CAR T cell therapy in the clinic, the manufacture of these products has encountered many difficulties.[103] An adequate number of T cells must be harvested to ensure sufficient starting material for manufacture. However, the quantity and quality of patient-derived cells are both often compromised due to disease burden and/or prior treatment.[103–105] To ensure sufficient CAR T cell expansion, cytokines are used during the manufacturing process, most commonly IL-2. However, supplementation with IL-7/IL-15 has been shown to induce superior activation and expansion with increased populations of CAR T cells with naïve and stem cell memory phenotypes.[106–108] Alternatively, CAR T cells have been engineered to secrete cytokines to promote their expansion and survival, as discussed above. Similarly, CAR T cells can be engineered to co-express switch receptors that also promote selective expansion during manufacture in response to cognate ligand.[58,62,63]

Manufacture of autologous CAR T cells is often lengthy, costly and can be subject to compromise by patient T cell dysfunction. Moreover, products are not immediately available at the time of clinical need. To address these shortcomings, allogeneic "off the shelf" CAR T cell products are under active development, whereby a single cryopreserved batch of drug contains multiple dosing units.[103] Indeed, allogeneic CD19 CAR T cells have been tested in clinical trials in patients with B cell malignancies with some success.[109,110] However, the development of graft-versus-host disease (GVHD) remains of concern when using allogeneic cell products. CAR constructs containing 41BB co-stimulatory modules have elicited increased GVHD compared to CD28-containing counterparts in lymphoma mouse models, possibly due to excessive activation of CD28 CAR T cells resulting in cell death, whereas 41BB CAR T cells

have superior persistence.[111] To prevent GVHD, allogeneic CAR T cells can be edited to knock out αβ TCR chains without compromising CAR T cell efficacy.[33,112–115] Alternatively, allogeneic CAR engineered cell products can be derived from virus-specific T-cells (www.atarabio.com, accessed April 2nd, 2021), NK cells, γδ T cells, invariant NK T cells, macrophages or induced pluripotent stem cells.[116–122]

Improving CAR T Cell Homing and Infiltration of Solid Tumours

The success of CAR T cell therapy in liquid tumours can partially be attributed to the high probability of tumour cell-CAR T cell interaction in circulation. By contrast, CAR T cells must migrate to and infiltrate solid tumours in order to achieve therapeutic success in that setting. In selected cases, CAR T cell therapy can be delivered intratumorally or using a regional delivery approach.[123,124] However, many solid tumours are inaccessible to intratumoral injection, owing to location, proximity to vital structures and/or metastatic spread. To direct homing of CAR T cells to specific tumour types, a suitable chemokine receptor may be co-expressed in these cells. The chemokine CXCL8 is produced by tumour cells and chemoattracts neutrophils and myeloid-derived suppressor cells which naturally express the receptors, CXCR1 and 2.[22,100,125] CAR T cells engineered to express these chemokine receptors show improved homing to tumours and enhanced tumour regression in solid tumour models in immunodeficient mice.[22,125,126] CAR T cells engineered to express CCR2b or CCR4 also demonstrate improved trafficking to CCL2-expressing or CCL17- and CCL22-expressing tumour cells, respectively.[127,128] However, it should be noted that excessive secretion of some chemokines (eg CXCL12) can inhibit T cell migration.[129–131] Pharmacological inhibition of the chemokine receptor CXCR4 in combination with checkpoint inhibition led to improved T cell homing to CXCL12 secreting tumours in a mouse model of pancreatic ductal adenocarcinoma.[132] Furthermore, in a proof-of-concept study, patients who previously resisted immunotherapy were treated with CXCR4 inhibitor and showed increased immune cell infiltration into tumours.[131] Engineering CAR T cells to be deficient in CXCR4 may also enhance trafficking to tumours.

The tumour stroma consists of fibroblasts, immune cells, extracellular matrix and vasculature and forms a physical, chemical and biological barrier to immune cell infiltration whilst supporting tumour growth. Fibroblast activation protein (FAP) is expressed on cancer-associated fibroblasts (CAFs) and has been targeted in an attempt to improve CAR T cell infiltration. FAP targeting CAR T cells inhibit the growth of subcutaneous solid tumours in immunocompetent mouse models by targeting FAP+ stromal cells, whilst increasing endogenous CD8 T cell infiltration.[133] Administration of anti-FAP CAR T cells in combination with CAR T cells that target tumour-associated EphA2 achieved improved therapeutic efficacy in immunodeficient mice bearing a lung tumour xenograft, when compared to treatment with either CAR T cell population alone.[134] Production of a dual antigen targeted CAR that incorporates specificity for FAP and an additional target could circumvent the need for multiple infusions. One concern with CARs targeting FAP is the potential for on-target/off-tumour toxicity as FAP is widely expressed in healthy tissues and has important functions in wound healing. In keeping with this, some but not all studies have shown that anti-FAP CAR T cells can cause bone marrow toxicity in mouse models.[135]

The tumour extracellular matrix (ECM) has also been targeted to improve CAR T cell penetration in solid tumours. Illustrating this, CAR T cells that have been engineered to secrete heparanase induces degradation of heparan sulfate proteoglycans (HSPG) in the ECM. Improved infiltration and anti-tumour response by heparinase-expressing CAR T cells was demonstrated in immunodeficient mice implanted with xenograft models of neuroblastoma.[136]

Overcoming Immune Checkpoints in CAR T Cells

Tumours and associated stromal cells express immune checkpoints that decrease CAR T cell function.[137,138] The checkpoint inhibitors, ipilimumab (anti-CTLA4), nivolumab and pembrolizumab (anti-PD-1) are frequently used to treat cancer patients and are under investigation for use in combination with CAR T cell therapy.[139–142] To avoid the need for separate dosing, CAR T cells can be engineered to circumvent immune checkpoints as summarised in Table 3. Secretion of anti-PD-L1 antibodies or scFvs by CAR T cells improved anti-tumour responses with reduced expression of exhaustion markers in multiple mouse models including a humanised mouse model of renal cell carcinoma and a patient-derived xenograft (PDX) model of gastric cancer.[143–147] In an alternative strategy, a dominant negative

Table 3 Summary of Techniques to Overcome Checkpoint Blockade by CAR T Cells

Checkpoint Blockade in CAR T Cells	Mechanism	Reference
Anti-checkpoint antibody secretion	Blockade of PD-L1 on tumour cells	Suarez et al[143]
Anti-checkpoint scFv secretion	Blockade of PD-L1 on tumour cells	Zhou et al[144] Li et al[145] Rafiq et al[146] Ping et al[147]
Dominant negative receptor	Expression of PD-1 receptor lacking intracellular domain outcompetes binding to PD-L1	Cherkassky et al[148]
Knockout of checkpoint receptors	Knock out of PD-1 by CRISPR/Cas9	Ren et al[149] Rupp et al[150] Choi et al[151]
Checkpoint receptor downregulation	Incorporation of shRNAs/siRNAs in CAR T cells for downregulation of PD-1/Tim-3/Lag-3/CTLA-4 expression	Cherkassky et al[148] Liu et al[152] Zou et al[153] Simon et al[154] Condomines et al[155]
Secretion of minibodies	Blockade of CTLA-4 on tumours	Yin et al[156]
Switch receptors	Conversion of PD-1 inhibitory signal to costimulatory or activating signal as in PD-1/CD28 switch receptor or PD-1 CARs	Liu et al[157] Liu et al[158] Parriott et al[159]

PD-1 receptor has been designed, consisting of PD-1 lacking the intracellular domain, and expressed in CAR T cells. The dominant negative receptor can outcompete endogenous receptors for PD-L1 on tumours.[148] Genetic elimination of PD-1 has been achieved by CRISPR/Cas9 technology in CAR T cells, which were engineered alongside endogenous TCR and β2 microglobulin (B2M) knockdown to produce checkpoint resistant, allogeneic CAR T cells.[149–151] PD-1 has also been downregulated using small hairpin (sh)RNA in CAR T cells to improve anti-tumour responses.[148,152] Combined knockdown of immune checkpoints has been investigated by Zou et al,[153] where PD-1 as well as Tim-3 and Lag-3 were downregulated by shRNA in CAR T cells. This triple knockdown approach led to enhanced tumour infiltration and superior efficacy in a xenograft model that was dependent on increased CD56 expression.[153] PD-1 knockdown combined with CTLA-4 knockdown using small interfering (si)RNAs however, has not shown much improvement compared to PD-1 knockdown alone.[154] Additionally, shRNA knockdown of CTLA-4 alone has shown no significant effect in CD28 2G CAR T cells.[155] CAR T cells secreting anti-CTLA-4 minibodies, however, improved control of tumour growth in a glioma xenograft model compared to control CAR T cells, whereas CAR T cells secreting anti-PD-1 or anti-Tim-3 minibodies showed no improvement.[156]

An alternative approach to overcome immune checkpoints involves the construction of a switch receptor. Illustrating this, the extracellular domain of PD-1 was fused to the intracellular domain of CD28, thereby converting a potentially inhibitory signal to an activating signal. Such a PD-1 switch receptor was co-expressed with 1G and 2G CARs, leading to improved cytokine secretion and tumour killing of solid tumour xenograft model and in a Phase 1 clinical trial treating B cell lymphoma.[157,158] Similarly, sources of either signal one (eg CD3ξ) or signal two (eg DAP10) have been fused into the extracellular domain of PD-1 producing an anti-PD-1 CAR which can target a range of tumour types.[159]

Overcoming Immunosuppressive Cells and Cytokines Within the TME

The tumour microenvironment includes a complex population of immunoinhibitory cells including Tregs, tumour

associated macrophages (TAMs), myeloid-derived suppressor cells (MDSC), and DCs. These cells inhibit optimal CAR T cell function by producing inhibitory cytokines, enzymes and immunoinhibitory proteins. Patients are generally lymphodepleted before CAR T cell treatment to support expansion of the infused cells by removing inhibitory cell populations and increasing the pool of available activating cytokines.[160] Without lymphodepletion, CAR T cell production of IL-2 can enhance Treg function and expansion. Suryadevara et al[161] produced a CAR T cell with decreased IL-2 secretion potential by mutating the PYAP motif in CD28, preventing Lck binding. These CAR T cells were able to resist the inhibitory effects of Tregs in the TME of non-lymphodepleted mice; however, anti-tumour function was dependent on additional 41BB signalling.[161]

The inhibitory cytokine transforming growth factor (TGF)-β is not only produced by Tregs in the tumour microenvironment but also by tumour cells and stromal cells. 2G CARs containing CD28 rather than 41BB are more resistant to the inhibitory effects of TGF-β in a manner that is dependent on increased IL-2 signalling, in contrast to Treg resistance.[162] The introduction of cytokine autocrine loops into CAR T cells can confer resistance to TGF-β. This was elegantly demonstrated in CD28 2G CAR T-cells that lacked an Lck binding motif and therefore did not produce IL-2, but in which a hybrid IL-7 receptor delivered IL-2 signalling.[162] The functionality of a dominant negative TGF-β receptor, consisting of a receptor lacking intracellular signalling domains, has also been demonstrated to enhance anti-tumour responses of both tumour-specific CTLs and CAR T cells[163–165] Introduction of the dominant negative receptor in prostate-specific membrane antigen targeting CAR T cells can enhance proliferation, cytokine secretion, persistence and tumour eradication in a metastatic prostate cancer mouse model.[166] In a proof-of-principle study demonstrating CAR activation by soluble antigen, a second generation anti-TGF-β CAR was produced using scFvs with specificity for this cytokine.[167] The CAR not only functions as a switch receptor by converting an inhibitory signal into an activating signal but also works as a dominant negative receptor by inhibiting endogenous TGF-β signalling.[167] Subsequently, it was demonstrated that CD4+ T cells transduced with the TGF-β CAR enhanced in vitro anti-tumour responses of anti-CD20 CAR T cells in the presence of TGF-β.[168] The investigators demonstrate that the expression of the anti-TGF-β CAR in Tregs did not enhance their suppressive effects or inhibit CD19-CAR T cell effector response. However, a concern is that enrichment of transduced Tregs in a CAR T cell product may occur via the induction of a TGF-β autocrine loop.[168] More recently, CRISPR/Cas9 technology has been used to knockout the TGF-β receptor II in CAR T cells, resulting in enhanced anti-tumour responses in cell line-derived and PDX solid tumour models in immunodeficient mice.[169] Furthermore, knockdown of TGF-β receptor II reduced exhaustion marker expression and not only decreased regulatory cell differentiation but also increased the presence of memory cell populations.[169] Mechanisms of conferring resistance to TGF-β could in principle be translated to other inhibitory cytokines in order to improve CAR T cell function within the TME.

Overcoming Immunosuppressive Molecules Within the TME

Adenosine is produced in the TME from ATP by ectonucleases expressed on tumour cells.[170] Adenosine binds to adenosine receptors on T cells, leading to the activation of protein kinase A (PKA) and the initiation of immunosuppressive effects. Illustrating this, PKA interferes with T cell signalling pathways, resulting in impaired T cell proliferation and effector function.[171] Pharmacological inhibition of the adenosine receptor A_{2A} in CAR T cells has been achieved by incorporating small molecule inhibitors into nanoparticles.[172] Nanoparticle loaded CD19 CAR T cells accumulated within the TME and elicited anti-tumour responses in CD19 positive solid tumour xenograft models.[172] Alternatively, CAR T cells deficient in the adenosine receptor A_{2A} showed improved in vivo anti-tumour responses associated with increased cytokine production.[173–175]

Prostaglandin E_2 (PGE_2) is also produced within the TME by tumour-derived cyclooxygenase 2 (COX2)-mediated conversion of arachidonic acid. Similar to adenosine, PGE_2 binds receptors that are found on T cells, resulting in PKA activation. Manipulation of the PKA pathway in CAR T cells can circumvent the negative effects of adenosine and PGE_2 in the TME. PKA activation is dependent on its recruitment to the immune synapse by the anchor protein ezrin.[176] Disruption of this binding by the introduction of small peptides in CAR T cells improved tumour infiltration and anti-tumour responses

against both human and mouse mesothelioma cell lines in vivo and rendered CAR T cells resistant to tumour derived PGE_2 and adenosine.[176]

Overcoming Disruptive Effects of Tumour Metabolism

Activation of oncogenes in tumour cells increases their metabolic capacity leading to increased tumour growth, deprivation of microenvironmental nutrients, accumulation of harmful metabolites and increased tumour resistance to immune cell attack.

Tumour cells outcompete immune cells for glucose, causing insufficient glycolysis and reduced effector functions of TIL cells. High expression of glycolysis-related genes in tumours is associated with reduced infiltration and sensitivity to cytotoxicity mediated by adoptively infused T cells. Furthermore, highly glycolytic tumour cells render themselves resistant to immunotherapy by downregulating the IFN-γ pathway and expression of chemokines.[177] Incorporating mechanisms to enable CAR T cells to resist these effects may help to overcome tumour resistance to immunotherapy. Checkpoint blockade can disrupt mTOR signalling in tumour cells, resulting in increased glucose availability and T cell function, as demonstrated in an in vivo mouse sarcoma model.[178] Tumour cells increase glucose metabolism by upregulating glycolytic enzymes including lactate dehydrogenase A (LDHA). Lactate dehydrogenase A converts pyruvate to lactic acid, which is exported out into the TME, reducing pH. Glycolytically active immune cells rely on a gradient for lactic acid export. Increased lactic acid in the TME compromises this export process and can lead to net lactic acid uptake by immune cells. Inefficient lactic acid export prevents sufficient glycolysis and causes poor proliferation and cytokine production associated with reduced NFAT upregulation and translocation to the nucleus.[179,180] Levels of LDHA and lactic acid accumulation in the TME are both associated with poor prognosis and tumour metastasis.[180] Inhibition of LDHA reduced tumour cell growth when undertaken in combination with tumour reactive T cell immunotherapy in a mouse model of melanoma; an approach that could be translated to CAR T cell therapy.[177] Neutralisation of the acidic TME can also improve T cell infiltration into solid tumours and improves tumour regression in response to checkpoint inhibitors, as demonstrated in an in vivo model of melanoma.[181]

Similar to glucose, tumour cells outcompete T cells for tryptophan in the TME, leading to reduced T cell proliferation. Tumour cells produce indoleamine 2,3-dioxygenase (IDO), an enzyme that metabolises tryptophan to generate harmful metabolites. Tryptophan metabolites including kynurenine and 3-hydroxyanthranilic acid (3-HAA) directly inhibit CAR T cell expansion, cytotoxicity, cytokine secretion and promote apoptosis.[182] One potential beneficial effect of lymphodepletion prior to adoptive cell therapy entails downregulated IDO expression in malignant cells.[183] Furthermore, the combination of CAR T cell therapy with small molecule inhibitors of IDO can improve in vivo anti-tumour activity.[184,185]

Tumour cells and tumour-associated myeloid cells produce arginase which catabolises arginine, an amino acid that supports T cell proliferation and function. Depletion of arginine in the TME is detrimental to the function of CAR T cells, reducing proliferation and cytotoxicity.[186] Expression of two arginine resynthesis enzymes, argininosuccinate synthase (ASS) and ornithine transcarbamylase (OTC) in CAR T cells enables cells to utilise low levels of arginine in the tumour microenvironment maintaining CAR T cell function and proliferation, exemplified in both leukaemia and solid tumour mouse models.[187]

Finding alternative nutrients for T cell metabolism is one method which has been explored to avoid nutrient competition in the TME to support T cell proliferation and function. Inosine has been identified as an alternative T cell substrate which many tumour cell lines are unable to utilise. Supplementation of inosine in both in vitro and in vivo models enhances the ability of CAR T cells to control tumour growth.[188]

Manipulating CAR T cells to utilise specific metabolism pathways may foster resistance within the nutrient depleted TME. Naïve T cells rely upon oxidation of fatty acids.[189,190] However, effector T cells upregulate glycolytic pathways after antigen exposure. Conversely, memory populations increase oxidative phosphorylation as a source of energy.[189,190] Incorporation of alternative costimulatory domains within CARs can promote different metabolic fates.[191] 41BB containing CAR T cells demonstrate improved persistence due to central memory differentiation, associated with enhanced mitochondrial biogenesis and oxidative metabolism.[191] In contrast, CD28 containing CARs promote enhanced glycolysis associated with differentiation of effector phenotypes.[191] Manipulation of CAR T cells during expansion can also improve metabolic potential. Sukumar et al[192] demonstrated that highly

glycolytic T cells have reduced capacity to develop into memory T cells.[192] Inhibiting glycolysis during expansion increased differentiation of central memory and stem cell memory phenotypes, improving anti-tumour function in a in vivo melanoma model.[192] Methods to isolate T cells with a desired metabolic profile for adoptive cell therapy (ACT) have also been investigated. Selecting T cells based on their mitochondrial membrane potential (using a lipophilic catatonic dye) selects memory T cell precursors that have superior persistence and anti-tumour ability in the pmel-1 mouse model system.[193] Applying this to CAR T cells may improve their function, although the required incorporation of additional manufacturing steps is not ideal.

Overcoming Antigen Negative Tumour Resistance

Relapse due to antigen loss following CAR T cell treatment has been observed with multiple targets including CD19,[194] BCMA,[195] CD22,[196] EGFRvIII,[197] IL-13Rα2[124,198] and ErbB2.[199] Antigen loss can occur due to frameshift and missense mutations resulting in deletion of the target antigen and outgrowth of antigen-negative cancer cells that are resistant to CAR T cell therapy.[194,200] Lineage switch following CD19 CAR T cell therapy has also been observed due to immune pressure inducing a drastic reprogramming of malignant cells, switching from lymphoid to myeloid phenotypes.[201,202] In the case of CD19, alternative splicing can lead to the production of a mutant protein lacking the transmembrane domain, preventing cell surface expression. Skipping of exon 2 results in the expression of a truncated CD19 lacking the extracellular epitope recognised by the commonly used FMC63 or SJ25C1 scFvs, rendering cancer cells undetectable by derived CARs.[194] Fischer et al[203] demonstrated that these spliced variants exist pre-CAR T cell treatment in B-ALL patients and therefore could be used as predictors for antigen escape.[203] More recently it has been shown that CAR T cells can induce reversible tumour antigen loss via trogocytosis whereby CAR T cells actively remove antigen from the tumour cell surface. Not only does this result in antigen escape but also induces fratricide and exhaustion of the CAR T cells.[204]

Resistance to CAR T cell therapy can also occur due to downregulation of tumour antigen and low target density on tumour cells.[196,205] The threshold for CAR T cell activation is dependent on target density and therefore antigen downregulation prevents successful CAR T cell activation and anti-tumour responses.[206–208] Majzner et al[208] investigated how CAR T cell structure affects the threshold level for activation. Comparison of the two FDA approved CD19 targeting CAR T cell products revealed that CD28 co-stimulation provided a lower activation threshold as compared to 41BB. The activation threshold of 41BB containing CARs could be reduced by the addition of a further CD3ζ domain, enabling improved response to low antigen density tumour cells in vitro and in vivo.[208] Activation threshold is also dependent on hinge and transmembrane domain of CD19 targeting CAR T cells.[208] Both 1G and 2G CAR T cells containing the hinge and transmembrane domains from CD28 have reduced activation threshold compared to CARs containing hinge and transmembrane domains from CD8. This was shown to be dependent on improved receptor clustering within the immunological synapse and recruitment of ZAP70.[208]

To avoid relapse due to antigen escape, targeting of multiple antigens has been attempted. This has been demonstrated following multiple infusions of distinctly targeted CAR T cells, dual targeting CARs, trivalent CARs, tandem CARs and universal CARs (Figure 3). Dual targeting CARs, where two distinct 2G CARs are expressed on a single cell, has been exemplified for many target combinations.[204,209–213] Dual targeting CD19 and CD22 CAR T cells have demonstrated the ability to overcome antigen escape induced by trogocytosis.[204] Using mathematical models, Hedge et al[199] analysed the expression pattern of antigens on glioblastoma cells and proposed that targeting of both ErbB2 and IL-13Rα2 would be the most efficient way to prevent therapeutic failure of CAR T cell immunotherapy due to antigen loss and/or heterogeneity. The benefit of targeting two cancer-associated antigens was demonstrated using pooled ErbB2 and IL-13Rα2 CAR T cells and dual targeting CAR T cells, expressing the two distinct second-generation CARs on a single cell.[199] In this study, the authors showed that targeting three antigens did not enhance the probability of recognising the majority of tumour cells compared to targeting two antigens in their cohort of primary tumours. Nonetheless, trivalent CAR T cells have been developed for glioblastoma in which three 2G CARs targeting ErbB2, IL-13Rα2 and EphA2 are expressed on a single cell. Trivalent CARs in this setting were able to overcome tumour antigenic heterogeneity, variability between patients and demonstrated increased cytotoxicity and cytokine release compared to

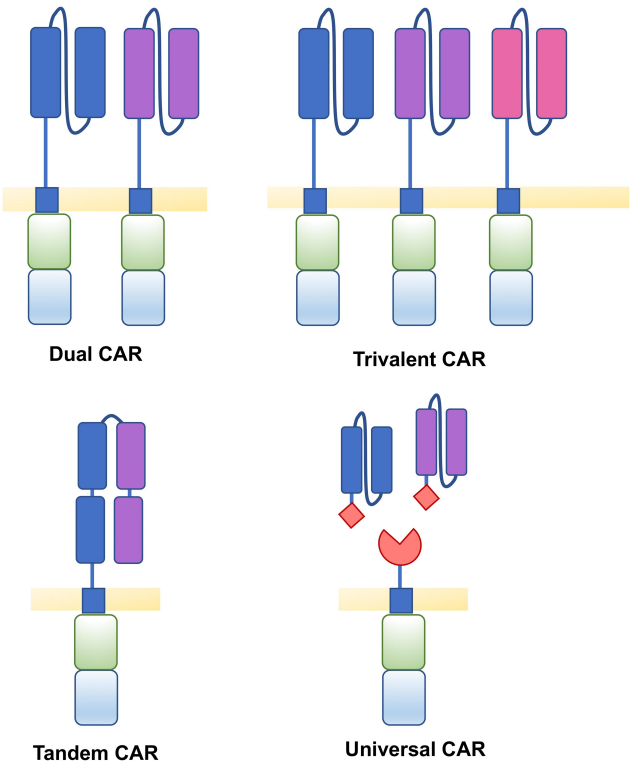

Figure 3 Structure of CARs to target multiple antigens. Tumour antigen loss can be overcome by targeting multiple antigens by various CAR T cell structures. Dual CAR T cells contain two distinct 2G CARs expressed on a single cell which recognise two different target antigens. Trivalent CAR T cells contain three distinct CARs able to recognise three different target antigens. Tandem CARs contain two distinct scFvs fused together by a flexible linker. Universal CARs consist of universal receptor and an antigen binding adaptor molecule, of which different target binders can be utilised.

dual targeting CAR T cells.[214] Additionally, trispecific CAR T cells targeting CD19, CD20 and CD22 have been developed to overcome CD19 antigen loss and demonstrated the ability to target and kill CD19 negative blasts from relapsed patients that received CD19 CAR T cell therapy.[215] Tandem CARs are an alternative strategy to target multiple antigens, whereby two distinct scFvs are fused together by a flexible linker.[216,217] Tandem CARs can be activated via one antigen but activation is enhanced when both targets are engaged, as demonstrated in a proof-of-concept study targeting CD19 and ErbB2[216] and CD20 and ErbB2.[218]

Universal CARs consist of a universal receptor and an antigen-binding adaptor molecule. Multiple antigens can be targeted by infusing multiple adaptor molecules with specificity for different targets.[219] Importantly, in the case of antigen escape, a different antigen can be targeted without the need for additional CAR T cell production and infusion. In a related concept, CAR T cell immunotherapy can be combined with the infusion of bispecific antibodies (BiTEs) which bind a target antigen and CD3, thereby inducing an immunological synapse and T cell activation.[220] Co-treatment with BiTEs and CAR T cells allow for dual-antigen targeting, avoiding the outgrowth of antigen-negative tumours. Combining αFR- targeting CAR T cells with an oncolytic virus secreting an EGFR-targeting BiTE enabled activation of CAR T cells in response to αFR positive as well as αFR negative but EGFR positive solid tumour mouse models.[221] Furthermore, CAR negative T cells found in the CAR T cell product could also be activated by the EGFR-BiTEs, enhancing the anti-tumour response.[221] CAR T cells have also been engineered to secrete BiTEs themselves. For example, secretion of an EGFR-targeting BiTEs by EGFRvIII targeting CAR T cells enhanced anti-tumour immunity against heterogenous glioblastoma tumour xenografts in mouse models.[222]

An alternative strategy to avoid antigen escape is to induce expression of artificial antigens which CAR T cells can recognise. Glycometabolic labelling of tumour cells with unnatural sugar residues can be targeted by CARs in which specificity is directed by the artificial ligand – so-called Click CAR T cells. Incorporation of artificial receptor and ligand into CD19 CAR T cells and target tumour cells improved selectivity, infiltration and homing.[223] The introduction of artificial targets also has the potential to avoid off-tumour/on-target toxicity. Park et al[224] used an oncolytic virus to deliver a target antigen, specifically truncated CD19, into solid tumours and demonstrated successful tumour eradication in both human xenograft models and a mouse immunocompetent model. Rechallenge of mice previously treated with oncolytic viruses and CAR T cells inhibited the growth of new tumours, suggesting induction of endogenous immune response and epitope spreading.[224] Epitope spreading following CAR T cell therapy has been demonstrated with a 3G EGFRvIII targeting CAR which was able to control antigen-negative tumour rechallenge in immunocompetent mouse models.[225] Promoting epitope spreading can maintain tumour control even when the CAR T cell target is lost. The cross-presenting DC growth factor FMS-like tyrosine kinase 3 ligand (Flt3L) has recently been expressed by CAR T cells leading to the facilitation of an endogenous anti-tumour immune response, with increased epitope spreading.[226] In vivo experiments with Flt3L CAR T cells demonstrated increased DC and T cell activation which led to control of both antigen positive and negative

tumour when used in combination with an additional adjuvant.[226]

Overcoming Resistance in Tumours That Retain Target Antigen

Acquired resistance to CAR T cell therapy can also occur without antigen loss. An in vitro model of CAR T cell resistance suggests that one mechanism by which such resistance can be acquired is disruption of TRAIL signalling.[227] Sensitising resistant tumour cells with histone deacetylase inhibitors can partially reverse resistance by restoring the TRAIL apoptotic pathway.[227] Kearney et al[228] performed a CRISPR screen to identify additional mechanisms of acquired tumour immune resistance. Following immune pressure, tumour cells can lose their sensitivity to TNF-mediated killing, which is associated with caspase-8 downregulation.[228] SMAC mimetics can sensitise cells to TNF-mediated killing and could be used in combination with CAR T cells to avoid acquired resistance.[229]

Alternatively, it is notable that certain oncogenic pathways can render tumour cells intrinsically resistant to adoptive T cell therapy. For example, loss of phosphatase and tensin homolog (PTEN) in tumour cells is associated with reduced T cell trafficking, increased production of inhibitory cytokines and resistance to apoptosis, as demonstrated in in vitro and in vivo melanoma models.[230] Furthermore, PTEN loss is associated with increased vascular endothelial growth factor expression, resulting in increased trafficking of immunosuppressive cells into the TME.[230]

Enhancing CAR T Cell Efficacy by Refining Immunogenicity of Target Binders

Anti-CAR T cell immune responses may be pre-existent or may develop following CAR T cell immunotherapy. Clinical evaluation of the anti-CD19 CAR T cell therapy, Tisagenlecleucel, demonstrated that the majority of patients had detectable anti-CAR antibodies before treatment, which increased in a small group of patients post-CAR T cell therapy.[231,232] The existence of anti-CAR antibodies, however, did not appear to affect expansion or efficacy in this study.[231,232] In contrast, patients who develop cellular immune responses to CAR T cells did not respond to initial or subsequent doses of CD19 CAR T cells.[233] Unlike the situation in CD19 CAR T cell immunotherapy, CAR-specific antibody responses appear to disrupt the efficacy of CAR T cells targeting different antigens. A study investigating bispecific CAR T cells targeted against two epitopes of BCMA correlated the presence of anti-CAR antibodies to disease relapse and reduced number of circulating CAR T cells.[234] Furthermore, in a Phase I clinical trial, CAR T cells targeting αFR in patients with ovarian cancer developed anti-CAR antibody responses, reducing CAR T cell efficacy and persistence.[235] CAR T cell therapy has even induced anaphylaxis, an event thought to reflect the induction of an IgE response against murine scFv epitopes.[236] Methods to reduce CAR immunogenicity have been extensively reviewed by Wagner et al[237] and encompasses the removal of immunogenic CAR components, exchange of mouse-derived scFvs with humanised/human scFvs,[238] eliminating immunogenic linkers through the use of heavy chain only CARs including nanobodies[239] and replacement of scFvs with receptor-ligand binding moieties.[240–242]

Conclusions

Despite intensive efforts, no successful CAR T cell therapy for solid tumours has been developed as yet. It is apparent that engineering CAR T cells to overcome just one aspect of tumour resistance may not be enough. The emerging picture of a highly successful CAR T cell seems to be one that is less differentiated from the stem/central memory cell phenotype, allowing long-term persistence in patients. Furthermore, CAR T cells must be able to successfully home to and infiltrate highly complex tumours and, once within the hostile TME, they must resist immunosuppression to maintain full functionality. It remains to be seen whether the use of one or more of the strategies described in this review can enable CAR T-cells to resist the panoply of defence mechanisms commonly deployed in solid tumours.

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JM is founder, chief scientific officer and shareholder in Leucid Bio. MG is an employee and shareholder in Leucid Bio. The authors report no other conflicts of interest in this work.

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