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Journal
Nature reviews. Drug discovery, 20(7)

ISSN
1474-1776

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Publication Date
2021-07-01

DOI
10.1038/s41573-021-00189-2

Peer reviewed

TITLE
Navigating CAR-T cells through the solid-tumour microenvironment

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ABSTRACT

The adoptive transfer of T cells that are engineered to express chimeric antigen receptors (CARs) has shown remarkable success in treating B-cell malignancies but only limited efficacy against other cancer types, especially solid tumours. Compared with haematological diseases, solid tumours present a unique set of challenges, including a lack of robustly expressed, tumour exclusive antigen targets as well as highly immunosuppressive and metabolically challenging tumour microenvironments, that limit treatment safety and efficacy. Here, we review protein- and cell-engineering strategies that seek to overcome these obstacles and produce next-generation T cells with enhanced tumour-specificity and sustained effector function for the treatment of solid malignancies.

INTRODUCTION

Adoptive T-cell therapy, or the infusion of disease-targeting T cells as the therapeutic agent, has demonstrated remarkable potential to treat advanced-stage cancers. In this novel treatment paradigm, primary human T cells are genetically modified to express tumour-specific receptors — typically either a chimeric antigen receptor (CAR) or T-cell receptor (TCR) (**Box 1**) — which enable the engineered T cells to mount a tumour-specific immune response when infused into the patient.

CARs are synthetic receptors that are comprised of extracellular ligand-binding domains fused to intracellular co-stimulatory and activation domains (**Figure 1a**). First-generation CARs lack co-stimulatory signaling domains and have limited efficacy owing to insufficient signaling strength and durability; thus, second-generation and subsequent CAR designs have incorporated one or more co-stimulatory domains to enhance and sustain T-cell activation signaling[1–4]. CARs can be built to target a variety of tumour antigens owing to their structural modularity, providing a readily adaptable platform for treatment of many types of cancers. In the case of CAR T cells targeting the CD19 antigen expressed in B cells — which became the first gene-therapy product to be approved by the U.S. Food and Drug Administration — patients with relapsed or refractory B-cell malignancies achieved complete remission rates of up to 90%[5]. However, despite promising outcomes against haematological tumours, adoptive T-cell therapy has been much less effective against solid tumours, which comprise the vast majority of cancers. Compared with liquid tumours, solid tumours pose unique challenges to treatment with CAR T cells. First, high antigen heterogeneity in solid tumours provides them with an effective mechanism of escape from CAR T cells, which typically encode specificity towards a single antigen target and are thus unable to recognize all cancer cells in the tumour. However, broadening T-cell specificity towards multiple antigens increases the risk of on-target, off-tumour toxicity. Second, solid tumours are often surrounded by physical barriers — such as collagen-rich stroma — that effectively prevent T-cell infiltration. In addition to physical barriers, T cells must also confront highly immunosuppressive tumour microenvironments (TME) whose cellular, molecular, and metabolic profiles ultimately lead to T-cell exhaustion **[G]** and dysfunction. So far, CAR T cells have been inadequately equipped to surmount these additional obstacles posed by solid tumours.

Here, we review both T-cell–intrinsic and extrinsic factors that blunt the therapeutic efficacy of CAR-T cells in solid tumour settings, along with current methods that seek to overcome these

hurdles. We begin with a discussion on the choice of target antigens, which is a major determinant of both safety and efficacy in adoptive T-cell therapy. We next discuss challenges posed by the immunosuppressive TME and engineering strategies aimed at overcoming this barrier. Lastly, we provide a brief overview of known toxic effects associated with CAR-T cell therapy based on experience in the clinic, and present recent engineering approaches that may serve to mitigate treatment-associated toxicity and enhance the safety of CAR-T cell therapy.

[H1] TARGET ANTIGEN CHOICE

Target antigen choice is a major determinant of safety and efficacy for CAR-T cell therapy. The CAR redirects T-cell cytotoxicity toward its cognate antigen, irrespective of the identity of the cell that presents the cognate antigen. Consequently, healthy cells that share target-antigen expression are at risk of 'on-target, off-tumour' bystander killing, whereas cancer cells that dynamically regulate target antigen expression can escape CAR-T cell surveillance. In this section, we review challenges associated with antigen selection and discuss potential solutions.

[H2] Ensuring tumour specificity

In principle, the ideal tumour antigen should be highly and uniformly expressed on tumour cells but absent on healthy tissue. However, the identification of suitable tumour antigens has been a longstanding challenge, and the vast majority of tumour antigen targets to-date, for both haematological and solid malignancies, have shared antigen expression in subsets of healthy cells (**Table 1**)[6–8]. Consequently, targeting of tumour-associated antigens (TAAs) but not tumour-specific antigens with adoptively transferred T cells carries the risk of on-target, off-tumour toxic effects.

Clinical reports over the years have shown the severities of on-target, off-tumour toxic effects, which range from predictable and clinically manageable to unanticipated and fatal (**Table 2**). CAR-T cell therapies targeting the pan–B-cell marker CD19 have demonstrated impressive clinical responses for the treatment of haematological malignancies[9,10], but successful treatment by CD19 CAR-T cells also invariably results in B-cell aplasia **[G]**, which is a predictable consequence of targeting CD19 that can be clinically managed by immunoglobulin transfusion[11].

T-cell targeting of other TAAs has similarly led to undesired but clinically tractable adverse events. Melanoma antigen recognized by T cells 1 (MART-1) and glycoprotein 100 (gp100) are TAAs expressed not only in melanomas but also in healthy melanocytes in the skin, eyes, and ears[12]. Patients with metastatic melanoma who received T cells engineered to express TCRs specific for MART-1 or gp100 experienced transient melanocyte toxicity, resulting in damages to skin, eyes, or ears that were treatable with steroid applications[12]. Notably, a subset of patients experienced melanocyte toxic effects without appreciable tumour clearance[12], indicating that on-target, off-tumour toxicities can happen even in the absence of robust anti-tumour response. Patients with metastatic colorectal cancer who received T cells expressing carcinoembryonic antigen (CEA)-targeted TCRs experienced severe transient colitis due to CEA expression on healthy epithelial cells in the gastrointestinal tract, with limited anti-tumour responses[13]. Similarly, treatment with carboxy-anhydrase-IX (CAIX) CAR-T cells in patients with metastatic renal carcinoma resulted in dose-limiting toxicity to the liver and bile-duct epithelial cells despite being a first-generation CAR, which is expected to provide limited tumour-killing efficacy[14]. These cases highlight the delicate balance between eliciting potent anti-tumour activities with preventing severe off-target toxic effects.

In certain clinical studies, unanticipated off-target toxic effects have resulted in life-threatening complications. Melanoma associated antigens (MAGE) are cancer-testes antigens (CTAs) that are absent from healthy adult tissue but overexpressed in a variety of cancers[15]. However, three out of nine patients treated with MAGE-A3–targeted TCR-T cells experienced severe neurotoxicity, resulting in two fatalities[15]. This was attributed to cross-reactivity of the MAGE-A3 TCR to unanticipated MAGE-A12 expression in the brain[15]. In a separate MAGE-A3 TCR study, two patients experienced lethal cardiac toxicity due to myocardial damage induced by TCR cross-reactivity with the protein titin, which is found in myocardium[16,17]. It should be noted that the tested MAGE-A3 TCRs were avidity- and affinity-enhanced with the intention of boosting anti-tumour efficacy, which came at the unfortunate cost of lethal cross-reactivity. Similar unanticipated reactivity to healthy tissue has also been reported in the context of CAR-T cell therapy. A patient receiving human epidermal growth factor receptor 2 (HER2)-targeted CAR T cells experienced fatal pulmonary toxicity. Histological analysis attributed the toxicity to low HER2 expression in lung cells, which triggered HER2 CAR-T cell activation and led to pulmonary oedema and rapid elevation of serum cytokine levels that triggered cytokine release syndrome (CRS, also known as ‘cytokine storm’), ultimately leading to multiorgan failure[18].

Mesothelin has emerged as a promising TAA for solid tumours given its overexpression in various solid tumours and its limited expression in healthy mesothelial cells **[G]** [19,20]. Clinical trials conducted at multiple institutions have demonstrated minimal on-target, off-tumour toxic effects[21–25]. Despite a favourable safety profile, mesothelin-targeted CAR T cells have shown limited efficacy in clinical trials as a monotherapy[21–23,25]. Recent clinical data from a Phase I trial combining monoclonal antibodies (mAbs) targeting programmed cell death protein 1 (PD1) with intrapleural delivery of mesothelin-targeted CAR T cells presents encouraging efficacy data with 2 of 14 patients demonstrating complete metabolic response and 5 of 14 patients demonstrating partial response[24]. With more room for improvement, strategies that can augment the infiltration, persistence, and functionality of CAR T cells in the TME are critical and will be discussed throughout this review.

Given the risk of targeting antigens that are associated with, but not exclusive to, tumour cells, several engineering strategies have been developed to improve the tumour-targeting specificity of CAR T cells. One strategy is to fine-tune the affinity of CARs to their cognate antigens, such that only tumour cells overexpressing the target antigen are killed while healthy tissue with normal expression levels are spared[26–28]. However, such tuning strategies require a large differential in antigen expression levels between healthy and diseased cell types, or risk a compromise in anti-tumour efficacy. Furthermore, low-affinity single-chain variable fragment (scFv) sequences may not be readily available for a TAA of interest. Another strategy is to engineer CARs that target tumour-associated glycopeptide epitopes which stem from mutations that cause aberrant glycosylation[29–32]. Notably, CARs targeting antigens modified with tumour-associated glycan Tn (GalNAcα1-*O*-Ser/Thr) have an innate capacity to recognize other Tn-modified antigens[31]. ScFv protein engineering can further broaden CAR reactivity to various tumour-associated, Tn-modified epitopes[32]. Ultimately, extensive testing of the tolerability of off-tumour toxicity is still necessary to ensure that low TAA or glycopeptide epitope expression by cells essential for survival do not trigger T-cell responses to the detriment of healthy tissue.

Multi-input receptors that activate T cells only in the presence of a specific combination of antigens have been developed to increase tumour-targeting specificity of CAR molecules. As effective CAR-T cell activation requires both a T-cell activation signal and a co-stimulatory signal, splitting the two signals into two receptors that each target a different antigen could enable a higher level of specification, requiring both antigens to be present before triggering robust T-cell response. To do so, second-generation CARs can be split into a first-generation CAR (without co-stimulatory

domains) paired with a second, chimeric co-stimulatory receptor (CCR) that comprises an scFv fused to one or more co-stimulatory domains but no CD3ζ chain (**Figure 1b**). The first-generation CAR provides only the T-cell activation signal, and the CCR provides only the co-stimulatory signal. The antigen for each receptor (such as CD19 and PSMA) must both be present to trigger robust CAR-T cell response, thus yielding Boolean AND-gate logic **[G]**[33].

Another AND-gate strategy can be implemented by the use of the synthetic Notch (synNotch) receptor system, which requires lentiviral integration of two transgenes — a synNotch receptor expressed from a constitutive promoter, and a CAR expressed from an inducible promoter. The synNotch receptor consists of an extracellular ligand-binding domain (such as a CD19-binding scFv), a transmembrane domain derived from the Notch receptor, and an orthogonal transcription factor (such as the transcriptional activator fusion protein Gal4-VP64), that is released via proteolytic cleavage upon ligand binding[34] (**Figure 1b**). When bound to its cognate ligand (such as CD19), the synNotch receptor releases its transcription factor to induce transcription of the CAR. The CAR protein can subsequently trigger T-cell activation upon binding its own, separate cognate antigen (such as inactive tyrosine kinase transmembrane receptor 1 (ROR1))[34,35]. Unlike the CAR–CCR combination, which requires simultaneous recognition of antigens A and B, the synNotch system is a sequential AND-gate in which the synNotch receptor recognizes antigen A prior to CAR expression and recognition of antigen B. The synNotch system has been shown to reduce toxicity when the off-tumour target is spatially segregated from the intended tumour cells, but remains vulnerable to off-tumour toxicity when healthy cells expressing antigen B are co-localized with target cells expressing antigen A[35]. A recent variation of the synNotch platform takes advantage of this collateral-damage effect to enable a “priming” mechanism that overcomes antigen heterogeneity in glioblastoma (GBM). T cells are engineered to express an anti-epidermal growth factor variant III (EGFRvIII) synNotch receptor, which drives the expression of a bispecific CAR targeting ephrin-A2 (EphA2) and interleukin-13 receptor subunit alpha-2 (IL13Rα2). EGFRvIII is GBM-specific but not uniformly expressed on GBM cells, thus susceptible to antigen escape. EphA2 and IL13Rα2 are expressed on the vast majority of GBM cells but are also found on healthy tissue, thus susceptible to off-tumour toxicity. In this system, presence of EGFRvIII in a subset of GBM cells can prime the expression of the EphA2/IL13Rα2 CAR, and the CARs then direct killing of all GBM cells (both $EGFRvIII^{+}$ and $EGFRvIII^{-}$) that are colocalized with the T cells. These synNotch/CAR-T cells were shown to eliminate GBM patient-derived xenografts (PDXs) that are heterogeneous in EGFRvIII expression, while restricting the activity of the EphA2/IL13Rα2 CAR to the brain to minimize potential off-tumour toxicity.

In addition to AND-gate logic, CARs can increase targeting specificity by triggering T-cell activation only in the presence of a TAA and not in the presence of an antigen expressed by healthy cells (Boolean AND-NOT logic **[G]**)[36]. One method to achieve AND-NOT logic is through the split, universal, and programmable (SUPRA) CAR system[37], in which T cells are engineered to express a 'zipCAR' comprising a leucine-zipper ectodomain fused to transmembrane and intracellular signaling domains. The zipCARs, which lack ligand-binding domains, must be reconstituted with exogenous 'zipFv' proteins — scFvs fused to a matching leucine zipper — to enable T-cell activation in the presence of a TAA. One could simultaneously administer a second class of zipFv molecules designed to compete against the zipCAR for binding to the first zipFv, to prevent the reconstitution of functional CARs in the presence of self-antigens, thus achieving AND-NOT Boolean logic (**Figure 1c**)[37].

The AND or AND-NOT gate designs described above require that both input signals be present on the target-cell surface, which limits the repertoire of targetable antigens. Cytoplasmic oncoprotein verifier and response trigger (COVERT) molecules are engineered granzyme B molecules fused to an N-terminal inhibitory peptide sequence that is proteolytically removed by tumour-associated intracellular proteases. CAR-T cells equipped with COVERT molecules recognize a surface antigen and initiate the delivery of COVERT proteins into the target cell. Once inside the target cell, COVERT proteins are converted into active granzyme B if, and only if, the cognate tumour-associated protease is present. Active granzyme B triggers target-cell apoptosis through the proteolytic activation of caspases or through the cleavage of substrates that activate mitochondrial and DNA damage pathways[38]. Therefore, only target cells that express both a surface antigen recognizable to the CAR and an intracellular protease recognizable to the COVERT would be subject to killing[39]. Importantly, T cells equipped with COVERT molecules are able to target intracellular proteases without the need for antigen presentation by major histocompatibility complex (MHC).

Although AND or AND-NOT–based Boolean logic strategies can increase targeting specificity, they must also contend with a number of limitations. These limitations include an increased risk of tumour escape since the elimination of just one of the two or more inputs required for T-cell recognition would be sufficient to protect tumour cells from detection, as well as the necessity for multi-component expression, which reduces transduction efficiency and genetic stability. Emerging strategies have enabled incorporation of Boolean AND-gate logic into single CAR

molecules. For example, Boolean AND-gate logic can be achieved with a CAR that targets the Tn glycoform of Mucin 1 (Tn-MUC1)[30]. Tumour cells need to both express TAA MUC1 and harbour mutations that lead to aberrant Tn glycosylation in order to be recognized by Tn-MUC1 CAR T cells. Another strategy incorporates Boolean AND-gate logic by expressing CARs under hypoxia, a prevalent characteristic of the TME[40]. Hypoxia-induced CAR expression can be accomplished through CAR transcription from a hypoxia-inducible promoter[41] or through the C-terminal fusion of oxygen-dependent degradation domain to the CAR[42].

[H2] Overcoming antigen heterogeneity

The ideal way to avoid on-target, off-tumour toxicity without incurring the need for complex genetic circuitry design is to select antigens that are truly unique to tumour cells. A rare example of a tumour-specific antigen is the EGFRvIII, an oncogenic mutant form of EGFR commonly found in GBM[43,44]. However, EGFRvIII expression is dynamically modulated by tumour cells in response to treatment[45], and GBM is typically highly heterogeneous[46], such that only a fraction of tumour cells would be killed if only a single antigen such as EGFRvIII were targeted. Indeed, many other solid-tumour types are also highly heterogeneous in antigen expression, presenting a major challenge for targeted treatments such as CAR-T cell therapy. Furthermore, antigen escape — a phenomenon whereby tumours that have downregulated or lost antigen expression evade detection — poses a similar challenge that limits therapeutic efficacy[47]. One strategy to target heterogeneous tumours more effectively involves engineering of single-chain bispecific CARs containing two ligand-binding domains that can recognize two different tumour antigens, and either antigen is sufficient to trigger T-cell activation (**Figure 1d**). Such bispecific CARs have been demonstrated in the contexts of leukaemia and lymphoma treatment, by targeting a combination of either CD19 and CD20[48], or CD19 and CD22[49]. Bispecific CAR-T cells targeting B-cell maturation antigen (BCMA) and SLAM family member 7 (SLAMF7, also known as CS1) were also able to overcome antigen escape in mouse models of multiple myeloma[50]. One can also encode specificity against multiple tumour antigens at once by co-expressing different CARs on the same T cell (**Figure 1d**). This was demonstrated by Bielamowicz and colleagues, who showed that trivalent CAR T cells targeting a combination HER2, EphA2, and IL13Rα2 effectively target heterogeneous GBM tumour samples from multiple patients[51]. However, the expression of multiple CARs in the same T cell requires careful optimization of gene-delivery protocols to overcome decreases in transduction efficiency that accompany increases in transgene payload size[50,52]. A strategy to target heterogeneous tumours with a single-input receptor can be achieved

by using CARs that recognize a broad range of Tn-modified glycopeptide epitopes[31,32]. Many tumours also upregulate natural killer group 2 member D (NKG2D) ligands, which are recognized by the endogenous NKG2D receptor expressed by natural killer (NK) cells. NKG2D CARs consisting of the extracellular domain of the endogenous NKG2D receptor fused to the CD3ζ cytoplasmic domain have been used to broadly target heterogeneous tumours[53]. Another strategy was recently demonstrated in the form of a CAR whose ligand-binding domain consists of chlorotoxin (CLTX) — a 36–amino-acid peptide that can bind a broad range of GBM and neuroectodermal tumours but not react with healthy tissue[54]. CLTX CAR T cells recognized and killed brain tumour neurospheres derived from multiple patients, without any observed toxicity as evidenced by lack of off-target cytotoxicity against healthy human tissue *in vitro* and lack of adverse reactions and morphological abnormalities in various tissues of NSG mice when administered at doses up to 50 x 10^6 CAR-T cells[55]. The authors demonstrated that membrane-bound matrix metalloproteinase-2 (MMP2) is essential for CLTX CAR-T cell activation[55], but the precise target of CLTX remains to be elucidated, and it is unknown whether such broadly, yet specific, tumour-reactive peptides exist for other cancer types. Nevertheless, these CARs demonstrate the intriguing possibility of effectively overcoming intratumoural heterogeneity with minimal complexity.

It is worth noting that strategies to broaden tumour recognition could simultaneously increase the risk of on-target, off-tumour toxicity. One strategy to overcome this limitation is a layered approach to multi-antigen targeting. For example, EGFR is widely expressed on normal tissue and it is therefore a poor antigen target for CAR-T cell therapy. However, safe targeting of EGFR has been achieved by engineering oncolytic adenoviruses (OAd) — which specifically infect malignant cells — to secrete a bispecific T-cell engager (BiTE) **[G]** targeting EGFR. Given that EGFR and folate receptor-α (FR-α) are commonly co-expressed in many solid tumours, combination with FR-α–targeting CAR T cells resulted in efficient eradication of heterogeneous tumour xenografts, with less *in vitro* killing of healthy fibroblasts and keratinocytes compared to EGFR-targeted CAR T cells[56]. Similarly, in EGFRvIII CAR-T cells that secrete a BiTE targeting EGFR, limiting the BiTE molecule to the environ of EGFRvIII CAR-T cells circumvented toxicity and improved anti-tumour efficacy against heterogeneous GBM[57]. An alternate strategy to overcoming tumour antigen heterogeneity without compromising safety relies on administering oncolytic viruses engineered to express truncated CD19 (CD19t)[58]. These oncolytic viruses infect tumour cells to express CD19t, which does not signal in infected cells but can subsequently be targeted by CD19 CAR T cells. While the safety of this approach is dependent on the tolerability of on-target, off-tumour

toxic effects associated with CD19 CAR-T cell therapy, it circumvents the major challenge of identifying antigens that can be safely targeted while also enabling broad recognition of heterogenous tumours.

[H2] Intracellular targets and neoantigens

Intracellular antigens presented by MHCs can expand the repertoire of targetable antigens beyond the surface proteome. Wilms' tumour antigen 1 (WT1) is an intracellular oncoprotein overexpressed in acute myeloid leukemia (AML), and it has been successfully targeted by T cells expressing WT1-specific TCRs[59,60]. Endogenous T cells undergo thymic self-selection, a process that selects against autoimmunity by depleting T cells bearing TCRs that bind strongly to self-antigens derived from endogenous proteins. Since WT1 is an endogenous protein, most of the WT1-specific TCRs isolated from patients had low-binding affinity to MHC-presented WT1[59]. Screening of multiple donors enabled the identification of a high-affinity WT1-specific TCR, which specifically recognizes WT1 peptide fragments presented by the human leukocyte antigen A*201$^+$ (HLA-A2) MHC subtype. In one clinical trial, AML patients who had undergone allogeneic haematopoietic cell transplantation received prophylactic treatment with donor-derived CD8$^+$ T cells expressing the WT1-specific TCR, with the aim of increasing graft-versus-leukaemia effect through WT1 recognition. The treatment resulted in relapse-free survival of all patients during the evaluation period of the trial[60]. MAGE family members are another example of intracellular proteins presented by MHC molecules. T cells expressing a MAGE-A4–specific TCR, isolated from a cytotoxic lymphocyte clone, were well tolerated by patients, although 7 of the 10 patients who received adoptively transferred T cells developed progressive disease during the study period, suggesting limited efficacy[61]. Although increasing TCR-binding affinity can potentially increase the anti-tumour response, it can come at the cost of unanticipated cross-reactivity against healthy cells that share low expression of the peptide-MHC target[15–17].

Peptide-MHC (pMHC) complexes presenting intracellular antigens can also be targeted by antibody-derived moieties, bypassing the need to isolate TCR sequences from endogenous pMHC-reactive T cells. For example, pMHC-targeting antibodies or scFvs can be obtained by screening phage display libraries, and the identified ligand-binding sequence can be incorporated into a CAR[62,63], with the caveat that this grafting process can sometimes alter the scFv's ligand-binding property. For example, a high-affinity antibody isolated for the New York Esophageal Squamous Cell Carcinoma-1 (NY-ESO-1) antigen through phage display was found to lose its

specificity in a CAR format, a result attributed to excessive CAR binding to HLA-A2[62]. Rational engineering of the antigen-binding fragment (Fab) resulted in minimized interactions with HLA-A2, which improved CAR specificity but reduced its binding affinity to NY-ESO-1. Ultimately, the modified CAR T cells did not effectively eradicate tumour owing to insufficient signal strength provided by the low-affinity CAR[62]. Such experiences underscore the need to closely couple screening methods with the final implementation format to ensure translatability of the screening results to the application of interest.

Neoantigens, which are novel epitopes generated through patient-specific tumour mutations, can be a source of tumour-specific targets for T-cell therapy. Neoantigens can be computationally predicted following whole-exome sequencing of tumour biopsies[64]. Although bioinformatics algorithms can robustly identify somatic mutations, predictions for processing and display of neoantigen epitopes by MHC molecules remains an active area of research[64]. For instance, putative neoantigens are often ranked by the predicted binding affinity between the neoantigenic peptide and the MHC molecule[65,66], but neoantigen-reactive T-cell profiling from patients responding to anti-PD1 therapy showed that neoantigen reactivity only loosely correlates with the predicted binding affinity between the neoantigenic peptide and the MHC[67]. Despite challenges associated with neoantigen prediction, personalized neoantigen vaccines have been successful at expanding diverse neoantigen-reactive T cells from patients with melanoma [68,69]. Administration of synthetic peptide-based neoantigen vaccines resulted in tumour regression without severe autoimmune toxicity in patients with melanoma, highlighting the appeal of targeting neoantigens in the form of vaccines[69].

Neoantigen-specific T cells can be isolated from tumour-infiltrating lymphocytes (TILs) or generated through transgenic expression of neoantigen-reactive TCRs. Because TILs are often found to possess differentiated and exhausted phenotypes[70], they may be less effective at exerting anti-tumour control in light of emerging evidence that show that less-differentiated T cells have a higher capacity for mediating tumour control[71]. However, neoantigen-reactive TCRs can be transgenically expressed in less differentiated T-cell subsets to potentiate stronger anti-tumour responses, but the isolation and characterization of neoantigen-reactive TCRs is challenging, given the rarity of naturally occurring tumour-reactive T cells. As such, the identification of neoantigen-reactive TCRs is an active area of research, and has been reviewed by Yamamoto and colleagues[72]. Recent work by Peng *et al.* addressed this challenge by establishing a sensitive and streamlined approach for capturing, characterizing, and sequencing the TCR of neoantigen-

specific patient T cells[67]. Neoantigen-based therapies are a promising approach for cancers with high tumour mutational burden[73], but remain challenging for cancers with low mutational burden given that only a small subset of somatic mutations generate T-cell–reactive neoepitopes[74,75].

[H1] THE SOLID-TUMOUR MICROENVIRONMENT

[H2] Boosting CAR-T–cell infiltration

Once infused into the cancer patient, CAR-T cells are confronted with diverse challenges presented by the TME. Indeed, evasion of anti-tumour immune responses can begin even before adoptively transferred T cells encounter tumour cells. In contrast to disseminated, circulating tumours, solid tumours present a physical barrier that excludes tumour-infiltrating T cells through an interactive network of molecular and cellular mechanisms. These include expression of chemokines and other chemical signals that preferentially recruit suppressive immune cells[76]; stromal cells such as cancer-associated fibroblasts (CAFs) that promote a dense, fibrotic environment limiting T-cell trafficking through aberrant extracellular matrix (ECM) deposition[77]; and dysregulated vasculature with downregulated expression of adhesion molecules necessary for T-cell infiltration[78,79] (**Figure 2**).

Chemokines are a major molecular determinant governing the degree of cytotoxic T-cell infiltration in a solid tumour. In many solid tumours, the chemokine expression profile is skewed to preferentially recruit suppressive cell types while avoiding inflammatory, anti-tumour immune cells[76]. To circumvent the aberrant chemokine signature of the solid TME and promote infiltration of adoptively transferred T cells, multiple studies have engineered CAR T cells to express chemokine receptors that recognize chemokines upregulated in the TME. For instance, CAR T cells that co-express the C-C chemokine receptor CCR2 — which binds CCL2, a chemokine upregulated in several different cancer types — exhibit enhanced infiltration and anti-tumour efficacy in mouse models of neuroblastoma and pleural mesothelioma[80,81]. CAR T cells engineered to express IL-7 and CCL19 — factors known to be essential for the formation of T-cell zones in lymphoid organs — promote infiltration of both T cells and dendritic cells (DC) in multiple syngeneic solid tumour models, resulting in greater anti-tumour immunity[82].

Transforming growth factor-beta (TGF-β) is another chemical signal that plays a prominent part in excluding cytotoxic T cells from solid tumours. Besides acting directly on T cells to limit tumour

infiltration by downregulating chemokine receptors such as CXCR3[83], TGF-β also signals in stromal cells to promote a phenotype that shields tumours from immune surveillance[84–86]. Stromal cells activated by TGF-β upregulate production of ECM proteins such as collagen, forming a dense physical network that limits T-cell motility. Among the stromal cells in the TME, CAFs have drawn attention as a promising therapeutic target owing to their well-studied role in promoting tumour progression and barring immune-cell trafficking to tumours[77]. CAFs express high levels of fibroblast activation protein-alpha (FAP), and multiple groups have demonstrated that FAP-targeting CAR-T cells can be combined with tumour-targeting CAR-T cells or cancer vaccines to enhance anti-tumour immunity[87,88]. However, one study reported that FAP-targeted CAR T cells had on-target, off-tumour toxicity against bone-marrow stromal cells, resulting in lethal bone toxic effects and cachexia in tumour-bearing mice[89].

To circumvent concerns of toxicity associated with CAF-depletion strategies, other studies have geared towards reprogramming CAFs. Given its role in promoting CAF differentiation, TGF-β has been identified as an attractive target for overcoming immune exclusion mediated by the tumour stroma[84–86]. However, systemic inhibition of TGF-β, which is ubiquitously expressed throughout the body, can result in cardiac toxic effects[90]. Conversely, pharmacological inhibition of NADPH Oxidase 4 (NOX4), a downstream target of TGF-β signaling, offers greater specificity towards CAFs and therefore poses less risk of toxicity[91]. Furthermore, while TGF-β inhibition can prevent CAF differentiation, it fails to reverse the phenotype of already-differentiated CAFs[92]. In contrast, NOX4 inhibition can both prevent CAF differentiation and reverse the CAF phenotype to a quiescent state[92]. In mice, NOX4 inhibition resulted in greater $CD8^+$ T-cell infiltration of CAF-rich tumours and sensitized these tumours to anti–PD-1 checkpoint blockade. Strategies to modulate the tumour stroma have not yet been combined with CAR-T cell therapy, but may be a promising avenue for future investigation.

In addition to targeting the cellular drivers of immune exclusion from solid tumours, an alternate approach is to target the molecular drivers of immune exclusion. Since ECM proteins comprise a large part of the physical barrier presented by the TME, one strategy involves engineering CAR T cells capable of degrading ECM proteins. Specifically, GD2 CAR-T cells secreting heparanase, which degrades heparan sulfate proteoglycans, were capable of degrading ECM *in vitro* and infiltrated tumour xenografts more efficiently *in vivo*, resulting in increased survival of treated mice[93]. The inhibitory molecule prostaglandin E_2 (PGE_2), which is abundant in many solid TMEs, can also be targeted to enhance T-cell infiltration. CAR T cells expressing a small peptide that

disrupts proper localization of protein kinase A (PKA), a downstream target of PGE_2 signaling, not only kill tumour cells more potently, but also infiltrate tumours more efficiently through increased expression of CXCR3 and superior adhesion to molecules associated with tumour cells and endothelium, such as fibronectin and vascular cell adhesion protein 1 (VCAM-1)[94]. Another major physical constraint that limits immune infiltration is the dysregulated vasculature surrounding solid tumours. The tumour vasculature is characterized by highly tortuous vessels with aberrant flow characteristics, which contribute to tumour hypoxia, and can limit T-cell extravasation into tumour tissue by downregulation of adhesion molecules such as VCAM1 and intercellular adhesion molecule 1 (ICAM1)[95]. Strategies to normalize the tumour vasculature and promote immune infiltration include targeting the vascular endothelial growth factor (VEGF) and angiopoietin signaling axes — to name a few — and have been reviewed extensively elsewhere[78,79].

In some cancer types, inadequate T-cell infiltration can be circumvented by more direct delivery methods, as opposed to conventional intravenous infusion. In an orthotopic xenograft model of malignant pleural mesothelioma, intrapleural delivery of mesothelin-targeted CAR T cells induced a superior therapeutic response compared with intravenous delivery[96]. Furthermore, despite being regionally administered, CAR T cells were able to traffic to and clear tumours outside of the pleural cavity. Intrapleural delivery of mesothelin-targeted CAR T cells was also found to be well-tolerated in a phase I clinical trial, with no evidence of on-target, off-tumour toxicity[24]. In patients with liver metastases, results from phase I clinical studies suggest that intrahepatic delivery of CEA-targeted CAR T cells is both safe and effective[97–99], in contrast to the on-target, off-tumour toxic effects that occur when CEA-targeted CAR-T cells are infused systemically[13]. Similarly, in brain tumours, multiple studies have demonstrated that direct administration of T cells into the brain results in enhanced therapeutic efficacy compared to intravenous infusion[100,101]. Of note, these studies demonstrated that intracerebroventricular delivery of CAR T cells elicited the greatest therapeutic response in tumour-bearing mice, outperforming both systemic delivery via tail-vein and direct injection into the tumour site. The finding that locoregional delivery of CAR T cells results in improved therapeutic outcomes compared with both intravenous and intratumoural delivery suggests that a semi-systemic route of administration, in which the therapeutic cell population can circulate within a confined spatial region where tumour cells are likely to be found, may provide the optimal balance between coverage and concentration of effector functions.

As a complementary approach to optimizing the route of T-cell administration, T-cell infiltration into solid tumours can be further enhanced by engineering the cell-delivery platform. Specifically,

CAR T cells can be embedded in functionalized biopolymer scaffolds, which promote robust T-cell expansion and, when implanted at the site of tumour resection, release cells in a sustained manner[102]. To unleash a more potent anti-tumour immune response, biopolymer scaffolds can also be engineered to co-deliver CAR T cells with other immunotherapeutic agents. To demonstrate this, CAR T cells were co-embedded in a biopolymer scaffold with a stimulator of interferon genes (STING) agonist, which recruits and activates antigen-presenting cells, thereby stimulating tumour recognition by endogenous T cells[103]. In multiple syngeneic tumour models, biopolymer-mediated delivery conferred a superior therapeutic response *in vivo* compared with intratumoural delivery of both CAR-T cells and STING agonists, indicating that the delivery platform produces optimal treatment outcomes by not only enhancing CAR-T cell infiltration, but also providing high local concentrations of STING agonist at the tumour site[103]. More recently, Coon *et al.* demonstrated that nitinol thin films can serve as an implantable scaffold for CAR-T cell delivery, enabling robust expansion in tumour tissue[104]. In a xenograft model of unresectable ovarian carcinoma, implantation of films seeded with CAR T cells controlled tumour growth more effectively than both intravenous and local CAR-T cell injection. Nitinol films claim an additional advantage to biopolymer scaffolds because they are less bulky and can accommodate more precise and reproducible loading of T-cell cargoes, which may be of particular interest when manufacturing cells at larger scale for clinical applications[104]. These studies highlight the potential of interdisciplinary approaches to better treat solid tumours.

[H2] Resisting immune suppression in the TME

Beyond physical barriers to T-cell infiltration, the tumour microenvironment is populated with suppressive cell types — such as regulatory T cells (Treg cells), myeloid-derived suppressor cells (MDSCs), and tumour-associated macrophages (TAMs) — that promote immune tolerance. The chemokine and cytokine expression profile of the TME not only acts to exclude cytotoxic T cells, but also preferentially recruits immune suppressor cells from the periphery, and can polarize existing immune cells at the tumour site towards an immunosuppressive phenotype. Cytokines typically overexpressed in the solid TME include TGF-β, VEGF, interleukin 14 (IL-4), and IL-10, which can both directly inhibit T-cell function and promote accumulation of suppressive immune cells. The recruited suppressor cells can also produce immunosuppressive cytokines themselves, reinforcing the tolerogenic state of the TME[105–107].

One approach to augmenting anti-tumour immunity in the suppressive TME is to equip T cells with synthetic receptors that inhibit or rewire the endogenous response to these soluble factors. Most notably, multiple variants of synthetic receptors targeting TGF-β have been reported in the literature. These include a TGF-β dominant-negative receptor (DNR)[108], a TGF-β receptor (TGF-βR)/4-1BB chimera[109], and a TGF-β–responsive CAR[110–112], with each receptor encoding different responses to TGF-β. For instance, the TGF-β DNR — which is a truncated form of TGF-β receptor II that lacks the cytoplasmic signaling domain — inhibits endogenous TGF-β signaling by forming signaling-incompetent ligand/receptor complexes. The TGF-β CAR can similarly inhibit endogenous TGF-β signaling by outcompeting endogenous TGF-β receptors for ligand binding, but it can additionally transduce T-cell activating signals upon TGF-β binding, thereby converting TGF-β into a potent T-cell stimulant. Studies with both the TGF-β DNR and TGF-β CAR have shown that T cells expressing these synthetic receptors can inhibit polarization of T cells into Treg cells[111,113]. Synthetic receptors have also been engineered to target other suppressive cytokines, such as IL-4[114,115]. Chimeric IL-4 receptors have been constructed through fusion of the extracellular domain of the IL-4 receptor (IL-4R) to either the intracellular domain of the IL-2 receptor beta chain (IL-2Rβ)[114] or the intracellular domain of the IL-7 receptor (IL-7R)[115]. When co-expressed with CARs that target tumour antigens such as prostate stem cell antigen (PSCA), both IL-4R/IL-2Rβ and IL-4R/IL-7R chimeric receptors can potently enhance both T-cell expansion and tumour-cell killing *in vitro*[114,116]. To target multiple immunosuppressive cytokines, a first-generation PSCA CAR, an IL-4R/IL-7R chimera, and a TGF-βR/4-1BB chimera have been simultaneously expressed in T cells, conferring potent and specific tumour-cell killing at sites characterized by high expression of IL-4 and TGF-β[117].

CAR T cells have also been equipped with synthetic receptors that redirect the inhibitory PD-1/PD-L1 axis and the tumour necrosis factor receptor superfamily member TNFRSF6 (also known as FAS) and its ligand TNFL6 (also known as FASL) signaling axis[118–121]. In a xenograft tumour model of pleural mesothelioma, in which high levels of PD-L1 expressed on tumour cells dampened anti-tumour function of mesothelin-targeted CAR T cells, co-expression of a PD-1 DNR enhanced CAR-T cell function both *in vitro* and *in vivo*[118]. Expression of a PD-1 DNR provided an additional advantage over systemic co-administration of a PD-1 inhibitor, which required repeated doses in order to achieve a favourable therapeutic outcome[118]. Alternatively, switch receptors consisting of a fusion between the PD-1 ectodomain and CD28 endodomain can enhance anti-tumour function of CAR T cells in multiple solid tumour xenograft models[119,120]. CD28 signaling through the PD-1/CD28 receptor was necessary to achieve optimal control over tumour burden[120].

Similarly, in syngeneic tumour models, expression of a FAS DNR in either CAR- or TCR-engineered T cells protected T cells from FASL-induced apoptosis, leading to increased *in vivo* persistence and enhanced tumour eradication[121].

Alternatively to targeting immunosuppressive molecules in the TME, 'armoured' CAR T cells engineered to secrete pro-inflammatory cytokines — such as IL-12[122,123], IL-18[124,125], and IL-23[126] — can favourably shape the TME for enhanced anti-tumour immunity. CAR T cells secreting either IL-12 or IL-18 can recruit inflammatory M1 macrophages to the TME, and autocrine IL-12 or IL-18 signaling in CAR T cells enhances interferon-gamma (IFN-γ) secretion, which can inhibit Treg proliferation, thereby protecting T cells from Treg cell-mediated suppression[122–125]. IL-23, which promotes T-cell proliferation, consists of a p19 and p40 subunit, in which only the p19 subunit is upregulated by T cells upon activation. Engineering T cells to only express the p40 subunit results in reconstitution of functional IL-23 only upon T-cell activation, which can minimize potential toxicity that might otherwise be observed with constitutive IL-23 expression[126]. Additionally, this strategy enhanced the anti-tumour function of CAR T cells in multiple syngeneic and xenograft tumour models, with superior efficacy compared to CAR T cells expressing IL-18[126].

Beyond a molecular approach to overcoming the TME, several therapies take aim at inhibiting the suppressor cell types found in the TME. In particular, there is a growing appreciation for the role of suppressive myeloid cells — most notably TAMs and MDSCs, which consist of a heterogenous mix of different cell states — in the solid-tumour milieu[105]. Specific therapeutic interventions targeting the myeloid compartment include inhibition of colony stimulating factor 1 receptor (CSF1R), which depletes TAMs, and has primarily been studied in combination with either chemotherapy or checkpoint blockade[127]. A previously unappreciated role of myeloid cells in shaping responses to immunotherapy was highlighted by a recent study that demonstrated that targeted genetic ablation of PD-1 on myeloid cells inhibited tumour growth more effectively than ablation of PD-1 expression on T cells, owing to decreased differentiation of myeloid progenitors into MDSCs, unleashing a more potent anti-tumour T-cell response[128].

The therapeutic benefit of combining adoptive T-cell transfer with targeting of tumour-associated myeloid cells has been less well-characterized. In a murine pancreatic ductal adenocarcinoma (PDAC) model, administering an agonistic CD40-targeting mAb, which activates myeloid cells, can reprogram tumour-associated myeloid cells towards a more inflammatory, M1-like phenotype. When combined with adoptive T-cell therapy, this approach yielded greater anti-tumour efficacy

than myeloid-cell depletion by administering a CSF1R-targeting mAb[129]. Another novel cell-based approach leveraged the overexpression of NKG2D ligands on MDSCs in the TME. Although endogenous NK cells, which express NKG2D receptors, are capable of eliminating MDSCs that express NKG2D ligands, suppressive factors in the TME such as TGF-β downregulate endogenous NKG2D receptor expression in NK cells. NK cells were therefore engineered to express a NKG2D CAR, which maintains high surface expression levels even in the presence of TGF-β. NK cells expressing NKG2D CARs selectively targeted MDSCs, and when administered in combination with disialoganglioside (GD2)-targeted CAR T cells, resulted in enhanced tumour control in a neuroblastoma xenograft model[130].

Besides myeloid cells, Treg cells are also a major contributor to the immunosuppressive TME, and are of particular concern in applications in which cytokine boosting of anti-tumour T-cell function may be desired. Specifically, IL-2 can potently stimulate effector T-cell function, but because Treg cells overexpress the IL-2 receptor alpha chain (IL-2Rα), IL-2 administration can lead to the counterproductive consequence of preferentially expanding suppressive Treg cells. To more selectively stimulate effector T cells, recent engineering efforts have produced IL-2 variants that preferentially bind IL-2Rβ over IL-2Rα[131,132]. A similar approach to selectively stimulate anti-tumour T cells over Treg cells relies on an engineered orthogonal IL-2 (orthoIL-2) and IL-2Rβ (orthoIL-2Rβ) ligand/receptor pair[133]. T cells expressing the orthogonal IL-2Rβ are selectively stimulated by orthogonal IL-2, which does not interact with either endogenous T cells or Treg cells. This orthogonal ligand/receptor system enables highly selective stimulation of adoptively transferred engineered T cells, minimizing both the risk of toxicity by excessive stimulation of host T cells and the risk of potential outgrowth of Treg cells.

Most studies of the immune-cell composition of the TME have been done in the absence of adoptively transferred T cells. However, adoptive cell transfer itself can shape the immune landscape, although this change can differ depending on the type of tumour. Factors that can influence the impact of CAR-T cells on immune cells in the TME include the degree of pre-existing inflammation in tumours (for example ‘hot’ versus ‘cold’), responses to cytokines produced by T cells, and the degree of immunogenic cell death triggered by CAR T cells upon tumour-cell recognition. IFN-γ produced by $CD8^+$ T cells can trigger CSF1 production by melanoma cells, which results in the development of TAMs and subsequent resistance to PD-1 blockade[134]. In a clinical trial of EGFRvIII-targeting CAR-T cells against GBM, an increased prevalence of

intratumoural Treg cells was found following T-cell treatment, hinting at a potential mechanism of resistance to therapy[135].

In syngeneic mouse models, treatment with CAR T cells alone can be sufficient to stimulate an endogenous anti-tumour immune response. In a glioma model, mice treated with EGFRvIII CAR T cells were resistant to re-challenge with antigen-negative tumours[136]. Although specific mechanisms of such resistance were not explored, the authors suggest that treatment with CAR T cells might promote host immunity against tumour antigens other than EGFRvIII. Consistent with these findings, treatment with IL13Rα2 CAR-T cells in a different murine glioma model also induced an endogenous anti-tumour immune response[137]. In this study, glioma cells were engineered to express ovalbumin (OVA) as a surrogate antigen. Following treatment with IL13Rα2 CAR T cells — which do not target OVA — a greater number of host OVA-reactive intratumoural T cells was detected by tetramer staining compared with treatment with control T cells expressing a truncated, non-signaling CAR. It is worth noting that OVA itself is highly immunogenic and may not accurately reflect a treatment-induced immune response. Nevertheless, treatment with IL13Rα2 CAR T cells resulted in a decrease in intratumoural MDSCs and increased recruitment of DCs, hinting at a potential cellular mechanism by which CAR-T cell treatment might induce epitope spreading. Besides DCs, adoptively transferred T cells can also recruit other myeloid cells that support anti-tumour function. In a murine PDAC model, treatment with T cells engineered to express a mesothelin TCR increased accumulation of M1-like Ly6C^{high} TAMs which can produce pro-inflammatory cytokines, present antigens, and express co-stimulatory molecules. This, in turn, correlated with increased T-cell infiltration and persistence in tumours[129]. The anti-tumour effect of Ly6C^{high} TAMs may also explain why indiscriminate depletion of the intratumoural myeloid compartment via CSF1R blockade may be less beneficial than reprogramming with a CD40 agonist[129].

Together, results from these studies suggest that, at least in some instances, CAR-T cell therapy itself can remodel the TME to more favourably support anti-tumour function. However, given the generally poor responses to CAR-T cell therapy observed in solid tumours, further engineering approaches are likely necessary to fully unleash an endogenous anti-tumour immune response. One avenue is the recruitment of antigen-presenting cells (APCs). For instance, co-delivery of CAR T cells with a STING agonist results in synergistic activation of host DCs, which can then prime endogenous T-cell responses against tumours[103]. A novel strategy that mimics vaccine boosting involves intratumoural inoculation of amphiphile (amph)-ligands that selectively traffic to

lymph nodes for presentation by host APCs, resulting in the activation and expansion of amph-ligand–specific CAR T cells[138]. Although amph-ligand 'vaccination' is designed to selectively boost the function of antigen-specific T cells, the study demonstrated that mice treated with the combination of amph-ligand CAR-T cells and amph-ligand vaccine could also reject re-challenge with antigen-negative tumour cells. Furthermore, splenocytes from these mice exhibited strong IFN-γ production *ex vivo* when challenged with antigen-negative tumour cells, suggesting that amph-ligand vaccination can also stimulate endogenous immune recognition of tumours. Notably, CAR T cells themselves can be engineered to promote robust host immunity against tumours. Specifically, CAR-T cells engineered to secrete Fms-like tyrosine kinase 3 ligand (FLT3L) increased expansion and maturation of intratumoural DCs, resulting in epitope spreading as demonstrated by clearance of mixed antigen-positive and antigen-negative tumours, followed by elimination of antigen-negative tumours upon re-challenge[139].

CAR T cells are also capable of triggering pyroptosis, a form of immunogenic cell death, in tumour cells[140]. Although T-cell mediated pyroptosis has been linked to CRS, pyroptosis may be suitable for settings such as solid tumours, in which a more potent immune response may be warranted. In such instances, the degree of T-cell mediated pyroptosis may be tuned by altering levels of gasdermin expression in tumour cells. Gasdermins are a class of pore-forming proteins, whose cleavage by granzymes results in pyroptosis[141]. Potential methods to tune gasdermin expression include treatment with decitabine, a chemotherapy drug that reverses silencing of gasdermin E expression in tumours[142], and nanoparticle-mediated delivery[143].

[H2] Maintaining T-cell metabolic fueling

Aside from cellular mediators of immune suppression, the metabolic profile of the TME is highly unconducive to anti-tumour immunity. Effective CAR-T cell responses involve the proliferation of CAR T cells, secretion of cytokines, and killing of tumour cells — all of which are metabolically demanding tasks. Therefore, the metabolic fueling of CAR T cells in the TME is imperative to sustaining the energetic requirements for an effective anti-tumour response. Both intrinsic and extrinsic factors impact CAR-T cell metabolism in the TME and consequently the therapeutic capacity of CAR-T cells (**Figure 3**).

Competition for metabolic resources in an already nutrient-poor niche is a challenge for CAR T cells in the TME. Cancer cells often have dysregulated cellular metabolism to support oncogenic

growth[144]. One well-characterized feature is 'the Warburg effect', in which cancer cells primarily rely on aerobic glycolysis over the more energetically efficient mitochondrial oxidative phosphorylation to sustain biomass production[145]. As such, tumour cells are able to outcompete T cells for glucose in the TME[146]. Because T-cell activation involves rapid induction of aerobic glycolysis[147], and a glycolytic T-cell metabolic signature is linked to increased effector T-cell function[148,149], glucose-limited CAR T cells are unable to function as effectively in the TME. Glucose deficiency in T cells leads to phosphoenolpyruvate (PEP) insufficiency, resulting in a dampening of TCR signaling and effector responses, which can be remedied by PEP supplementation[149]. Acetate supplementation can also reinvigorate IFN-γ expression in glucose-restricted T cells[150]. Small-molecule glycolytic inhibitors have also been able to improve responses to checkpoint inhibitor immunotherapy[151,152].

Exhausted T cells have metabolic profiles characterized by suppressed mitochondrial respiration, decreased glucose uptake and glycolytic flux, and impaired mitochondrial function[147,153,154]. Overexpression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) can improve the metabolic fitness of T cells, thereby resisting T-cell exhaustion[147,153,155]. Treatment with mitochondria-targeted antioxidants can restore $CD8^+$ effector T-cell function by counteracting the disruption of mitochondrial activity[154,156,157]. Another T-cell intrinsic metabolic constraint is the post-translational impairment of enolase 1 through mechanisms that have yet to be ascertained, which results in the inability to generate PEP and the downstream glycolytic metabolite pyruvate, and a subsequent inhibition of effector T-cell functions[158]. Glycolytic activity required for effector T-cell function can be rescued through the overexpression of phosphoenolpyruvate carboxykinase 1 (PCK1)[149], which converts oxaloacetate to PEP, or through exogenous supplementation of PEP or pyruvate[158]. Another metabolic feature that represses T-cell anti-tumour capacity is the upregulation of sphingosine kinase 1 (*SPHK1*) in TILs, which polarizes T cells towards the immunosuppressive Treg cell phenotype by acting through the *SPHK1*/sphingosine 1-phosphate (S1P)/peroxisome proliferator-activated receptor gamma (PPARγ) axis[159]. Furthermore, lipolysis is important for memory T-cell development[160] but is suppressed by *PPARγ* transcriptional activity, which is activated by *SphK1*-generated *S1P*, in TILs[159]. Suppression of the *SphK1/S1P/PPARγ* axis through the genetic ablation of *SphK1* or *PPARγ* improved *in vivo* anti-tumour control in pre-clinical melanoma mouse models[159].

Extrinsic metabolic factors that limit CAR-T–cell efficacy include the presence of immunosuppressive metabolites in the TME. One example is the indoleamine 2,3-dioxygenase

(IDO)/Tryptophan/Kynurenine axis that suppresses T-cell effector function through multiple mechanisms[161]. On the one hand, tryptophan depletion in the TME due to nutrient competition prevents effective mammalian target of rapamycin (mTOR) function and consequently T-cell activation; at the same time, tryptophan conversion to kynurenine by IDO directly suppresses effector T and NK cells while recruiting and activating MDSCs[161]. On the other hand, increased kynurenine/tryptophan ratio in patients receiving anti–PD-1 therapy correlated with poor patient survival[162]. However, combination therapy using an IDO1 inhibitor (epacadostat) and an PD-1 inhibitor (pembrolizumab) did not improve upon pembrolizumab monotherapy in a phase-III clinical trial[163]. As an alternative approach, infusion of PEGylated kynureninase, which can directly degrade kynurenin, synergized with checkpoint inhibitor therapy in a syngeneic mouse model[164]. Direct modulation of the IDO/tryptophan/kynurenine axis by engineered T cells remains an intriguing possibility to be explored.

Adenosine is another immunosuppressive metabolite present in the TME that can be generated through the catalysis of extracellular adenosine triphosphate (ATP) by ectoenzymes CD39 and CD73, which are expressed by tumour cells, immunosuppressive immune cells, and various stromal cell types[165–167]. Genetic ablation of adenosine receptors A_1 and A_{2A} in HER2 CAR T cells and systemic administration of pharmacological A_{2A} receptor antagonists enhanced anti-tumour responses in fibrosarcoma and breast cancer syngeneic mouse models [168]. Moreover, systemic administration of pharmacological antagonists targeting adenosine receptors A_1 and A_{2A} synergized with anti–PD-1 therapy, leading to striking improvements in anti-tumour efficacy of CAR T cells compared with CART cells paired with adenosine receptor inhibition alone[168].

Lactate, a metabolite present at elevated levels in the TME owing to increased secretion by metabolically hyperactive tumour cells, inhibits lactate export by effector T cells[169], dampens T-cell signaling mediated by nuclear factor of activated T cells (NFAT)[170], and diminishes lactate dehydrogenase-mediated NAD recycling[171], whereas it preferentially activated Treg cell expansion, which does not depend on heightened glycolysis[171]. Tumour-cell–secreted lactic acid can also polarize macrophages towards an immunosuppressive M2 phenotype[172]. Furthermore, the acidic TME caused by lactic-acid secretion also directly contributes to the blunting of effector T-cell functions by reducing the production of cytokines, perforin, and to a lesser extent granzyme B, which can be partially reversed by neutralizing intratumoural acidity through treatment with proton-pump inhibitors[173] or bicarbonate therapy[174]. The myriad of ways in which metabolism directly impacts T-cell fitness suggest that reprogramming T cells to calibrate their metabolic

fluxes — which can be accomplished via both genetic and pharmaceutical means — may be a fruitful approach to enhancing T-cell function in the TME[175,176].

[H2] Preventing CAR-T cell exhaustion

CAR T cells are prone to exhaustion upon chronic stimulation in the TME, which compromises effective anti-tumour immunity. T-cell exhaustion can be broadly characterized by dysfunction of effector responses, sustained co-inhibitory receptor expression, and reprogrammed transcriptional and epigenetic states[177]. In particular, T-cell exhaustion has more recently been defined by the remodeling and plasticity of the epigenetic landscape[178,179]. The development of assay for transposase-accessible chromatin (ATAC)-seq has enabled tracking of the epigenomic remodeling trajectory of the progression from naïve to exhausted T cells at single-cell resolution. For instance, ATAC-seq analysis revealed that early progression toward exhaustion is associated with increased accessibility of nuclear receptor subfamily 4 group A member 1 (*NR4A1)* motifs, and further progression toward terminal exhaustion is associated with increased accessibility of *cis*-elements proximal to T-cell dysfunction gene *TOX*[179]. Several transcription factors have also been identified as drivers of T-cell exhaustion, such as thymocyte selection-associated high mobility group box protein (TOX), which is now recognized as a critical transcription factor driving the epigenetic remodeling associated with exhausted T cells[180–183] (**Box 2**). The NR4A transcription factor family as well as protein tyrosine phosphatase non-receptor type 2 (PTPN2) have also been identified as transcriptional drivers of $CD8^+$ T-cell exhaustion[184–186]. Transcription factor T cell factor 1 (TCF-1) has also emerged as a marker of stemness among 'progenitor exhausted' $CD8^+$ T cells that dictate the fate of T cells into terminal effectors or exhausted T cells (**Box 2**)[187–189].

In light of the increasing understanding of T-cell exhaustion biology, genetic engineering strategies have been developed to reinvigorate and potentiate CAR-T cell responses. For instance, recent studies reported that CAR-T cells with triple-knockout of *Nr4a1*, *Nr4a2*, and *Nra4a3* show improved tumour control and reduced exhaustion in mice bearing melanoma tumours[184]; genetic ablation of *Ptpn2* improves anti-tumour immunity in murine colon adenocarcinoma, mammary carcinoma, and melanoma tumour models[185,186]; and overexpression of transcription factor AP-1 (c-Jun) enhances CAR-T–cell resistance to exhaustion, thereby improving anti-tumour function[190]. Tempering CAR signaling strength through the rational modulation of CD3ζ immunoreceptor tyrosine-based activation motifs (ITAM) **[G]** has also been

shown to enhance CAR T cell potency by reducing T-cell exhaustion[191]. Finally, therapies combining CAR T cells with immune checkpoint inhibitors have also been successful at counteracting T-cell exhaustion in mouse models of pleural mesothelioma, leukaemia, melanoma and ovarian cancer[192,193]. A comprehensive discussion on CAR-T cell combination therapies have been reviewed elsewhere[194].

[H1] CLINICAL TOXICITIES AND MITIGATION

Although CAR-T cell therapies are predominantly limited by their lack of efficacy in the solid-tumour setting, safety considerations are also critical in the development of CAR T cell therapy. As observed in the clinic, CAR T cells that eradicate tumours effectively also carry the risk of potentially severe toxic effects. In addition to on-target, off-tumour toxic effects discussed previously, patients receiving CAR T cells are also at risk of sometimes-lethal side effects in the form of CRS and neurotoxicity. In light of the toxic effects commonly associated with CAR T cell therapy, mitigation strategies and engineered safety controls are active areas of research.

[H2] Cytokine release syndrome

The majority of clinical experience comes from CD19 CAR-T cell trials with emerging data from BCMA CAR-T–cell trials[5,9,195–201]. CRS is triggered by a high-level immune reaction in which activated CAR T cells induce cytokine production by other immune cells, triggering a positive feedback loop that leads to dangerous levels of serum cytokine concentrations[202]. CRS is conventionally treated with an anti-IL–6 receptor alpha (IL6R) antibody (such as tocilizumab), TNF inhibitor (such as etanercept), or corticosteroids[202]. Standard treatment of CRS is initiated upon detection of clinical symptoms, at which point CRS has already begun and, in some cases, has passed the point of effective therapeutic control. Therefore, strategies that prevent, instead of remedy, CRS could be highly advantageous. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been implicated as a driver of CRS, and antibody inhibition and genetic knockout of the gene encoding GM-CSF, *CSF2,* in CAR T cells were able to prevent CRS in an acute lymphoblastic leukemia (ALL) xenograft model in the presence of human peripheral blood mononuclear cells (PBMCs)[203]. Blockade of another CRS-inducing cytokine, IL-1, through treatment with an IL-1 receptor agonist (anakinra) was also successful at protecting mice from CRS mortality[204,205]. Importantly, these strategies did not compromise the anti-tumour capacity of CAR T cells. Given the efficacy of tocilizumab in the treatment of CRS in most patients, CAR-T

cells have also been engineered to target IL-6 signaling, although CRS control was not demonstrated in the preclinical *in vivo* study[206].

[H2] Neurotoxicity

Along with CRS, neurotoxicity is another potentially serious side effect of CAR-T cell therapy in the clinic. As with CRS, much of the clinical experience with neurotoxicity comes from treatment of haematological malignancies, and it is unclear whether the same will occur in solid-tumour settings. Interestingly, no neurotoxicity was observed in a clinical trial of IL13Rα2 CAR T cells against GBM, despite direct and repeated delivery into the brain[207]. There are, however, data from preclinical studies that found evidence of neurotoxicity in the treatment of solid tumours with other CAR T cells, as in the case of GD2 CAR-T cell therapy against diffuse midline glioma[208]. GD2, unlike IL13Rα2, is expressed on healthy brain tissue, and incorporation of a high-affinity GD2 scFv in this study may have promoted CAR-T cell accumulation in the brain, resulting in encephalitis[208].

The mechanism by which CAR T cells induce neurotoxicity has yet to be elucidated, but one hypothesis posits that endothelial cell activation triggered by high cytokine levels increases permeability of the blood-brain barrier and leads to the accumulation of inflammatory T cells in cerebrospinal fluid[209]. Given the presumed role of inflammation in neurotoxicity, clinical management is typically through the administration of corticosteroids. However, optimal treatment strategies for CRS and neurotoxicity are not perfectly aligned, as there is speculation that tocilizumab — which effectively alleviates CRS by binding the receptor for IL-6 — may prove to be detrimental when treating neurotoxicity due to a resultant build-up of free IL-6 that can passively diffuse into the central nervous system[210]. Interestingly, recent single-cell sequencing analysis revealed a subset of healthy mural cells that express CD19[211], suggesting that the neurotoxicity observed in clinical trials of CD19 CAR-T cell therapy may actually be a result of on-target, off-tumour toxicity specific to CD19 CAR-T cells. In light of these findings, the observed neurotoxicity may best be addressed through engineering efforts to enhance tumour-targeting specificity.

[H2] CAR-T cell safety and control

Given the inherent risks associated with CAR-T cell therapy, inducible safety controls that can be built into or applied in conjunction with CAR-T cells are desirable. Suicide genes can be implemented into CAR T cells that lead to cell death upon small-molecule–mediated induction of the suicide gene[212]. Herpes simplex virus thymidine kinase (HSV-TK) is a suicide gene that depletes HSV-TK-expressing cells in the presence of ganciclovir, an FDA-approved small-molecule drug[213–215]. However, the clinical utility of HSV-TK is limited by its immunogenicity and relatively slow mechanism of action[214]. Inducible caspase 9 (iCasp9) is a suicide gene of human origin that is expressed as a fusion protein comprising a FK506-binding protein (FKBP) and truncated Caspase 9, which is inactive in its monomeric form. Administration of the bioinert small-molecule AP1903 induces dimerization of iCasp9 through FKBP, thereby activating Caspase 9-mediated apoptosis (**Figure 4a**)[212]. CAR-T cell co-expression of CD20 or truncated human epidermal growth factor (tEGFR) present alternative avenues to deplete adoptively transferred CAR-T cells; administration of CD20- and EGFR-targeting antibodies (such as rituximab and cetuximab, respectively) can induce antibody-dependent cellular cytotoxicity that depletes the CAR-T cell population *in vivo*[216,217]. However, suicide switches generally result in the permanent depletion of administered CAR T cells, thus forcing the termination of therapy.

Novel strategies that enable drug-controlled activation or pausing of CAR signaling can allow physicians to temporally modulate CAR-T cell activity after infusion into patients — be it to prevent exhaustion or serious adverse events. A transient pharmacological CAR-T cell OFF switch has been reported using a tyrosine kinase inhibitor (dasatinib) by temporarily interfering with the CAR-T cell signaling cascade[218]. Most conventional CARs propagate T-cell activation signaling through CD3ζ, which relies on lymphocyte-specific protein tyrosine kinase (LCK) phosphorylation of CD3ζ and ζ-chain TCR-associated protein kinase 70 kDa (ZAP70). Therefore, dasatinib can partially or completely inhibit CAR signaling depending on the dosing regimen (**Figure 4b**)[218]. Furthermore, protein-degradation tags whose activities can be controlled by small-molecule ligands have been used to regulate CAR protein expression, thus enabling temporary disablement of CAR-T cell signaling without requiring elimination of the CAR-T cell population[219,220]. For example, a switch-off (SWIFF) CAR was engineered by fusing a protease target site, protease, and degron to the C-terminal end of a CAR[219]. In the absence of small-molecule protease inhibitor, the protease target site is cleaved**,** protecting the CAR from degron-mediated degradation. In the presence of small-molecule protease inhibitor, the degron remains fused and degrades the CAR (**Figure 4c**)[219]. Using these strategies, physicians can administer small-molecule drugs to dampen CAR-T cell activity until symptoms of toxicity or the toxicity itself subsides.

The STOP-CAR system is another strategy which incorporates chemically disreputable heterodimer (CDH) domains that enables drug-mediated pausing of CAR-T cell activity. STOP-CARs consist of a recognition chain, comprising of an scFv and a co-stimulatory domain, and a signaling chain, comprising of the CD3ζ T-cell signaling domain[221]. Importantly, both recognition and signaling chains contain CDH domains that spontaneously dimerize to produce a functional second-generation CAR. Administration of a small-molecule drug disrupts functional dimerization of the CAR, thereby ablating CAR-T cell activity[221]. Conversely, the GoCAR-T ® system enables drug-mediated activation of CAR-T cell activity. GoCAR-T ® cells co-express a first-generation CAR and a drug-inducible co-stimulatory domain, termed inducible MyD88/CD40 (iMC)[222]. The iMC molecule consists of truncated TLR signaling domains MyD88 and CD40 — which require dimerization for functional signaling — and FKBP dimerization domains[223]. Here, dimerization of iMC induced by the small molecule rimiducid provides the co-stimulatory signal for the first-generation CAR, thereby activating potent CAR-T cell functionality; conversely, stopping rimiducid administration may dampen CAR-T cell activity by depriving CAR-T cells of co-stimulation in case of toxicity [222]. Temporal control of CAR-T cell activity by clinically approved drugs can significantly

temper safety concerns that often accompany engineering strategies aimed at enhancing the efficacy, and thus potential toxicity, of CAR-T cell therapies.

FUTURE OUTLOOK

Several studies in recent years have shed light on how CAR T cells interact with solid tumours, pointing to both T-cell–intrinsic and extrinsic factors that contribute to underwhelming therapeutic outcomes in non-haematological cancer settings. Advances in the field point to promising new routes to the generation of CAR T cells better equipped to treat solid tumours. For instance, a growing body of mechanistic studies of non-cellular cancer immunotherapeutics, such as PD-1 inhibitors, can guide rational design of optimal and synergistic combinations with CAR-T cell therapy. In light of the antigen heterogeneity and immunosuppressive environment of solid tumours, it is likely that effective CAR-T–cell treatment regimens will need to incorporate strategies that recruit the host immune response to stimulate recognition of tumour antigens not targeted by CAR T cells and reshape the immune-cell composition of the TME to favour anti-tumour function. Strategies that can better sustain the metabolic demands of CAR-T cell killing in the TME will also enhance the potency and durability of CAR-T cell therapy.

In addition, novel T-cell engineering approaches and sophisticated gene-editing techniques — the latter of which enables functional library screens **(Box 3)** — will also be important drivers of breakthrough in combatting the TME. Besides library screening, advances in gene-editing enable integration of larger transgenic payloads that can program CAR T cells with more complex functionality. At the same time, sophisticated CAR designs that enhance CAR T cell performance while minimizing transgene size will be crucial given the challenges of multi-component gene expression. Lastly, the ability to efficiently knock out gene expression in T cells has led to the development of allogeneic, ‘off-the-shelf’ CAR-T cell products that promise to not only streamline the cumbersome manufacturing process, but also minimize interpatient variability in T-cell fitness. Whereas CAR-T cell production trends towards a more universal ‘one-size-fits-all’ methodology, a more personalized approach — made possible by advances in bioinformatics and the growing body of patient datasets — will guide target-antigen selection and combination therapies catered to each individual’s needs. Altogether, a multi-faceted approach incorporating cutting-edge biotechnology will likely be required to effectively navigate the many barriers to treatment presented by solid tumours.

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Author contributions

The authors contributed equally to all aspects of the article.

ACKNOWLEDGMENTS

A.J.H. was supported by the Mark Foundation (grant to Y.Y.C.) and L.C.C. was supported by the National Science Foundation (grant to Y.Y.C.).

COMPETING INTERESTS

Y.Y.C. and A.J.H. declare competing financial interests in the form of patent applications whose value may be affected by the publication of this work.

Table 1. Ongoing clinical trials of CAR-T cells against solid tumours

Antigen target	Number of unique CARs in active clinical trials	CAR generation		Clinical trial phases		Clinical trial identifiers
AXL	2	**3rd Generation**	1	**I**	1	NCT03198052, NCT03393936
		Unspecified	1	**I/II**	1	
B7-H3	7	**2nd Generation**	3	**I**	5	NCT03198052, NCT04385173, NCT04185038, NCT04077866, NCT04483778, NCT04483778, NCT04432649
		3rd Generation	1			
		4th Generation	1	**I/II**	2	
		Unspecified	2			
CD147	2	**Unspecified**	2	**I**	2	NCT03993743, NCT04045847
CD171	3	**2nd Generation**	2	**I**	3	NCT02311621, NCT02311621, NCT02311621
		3rd Generation	1			
CD20	1	**Unspecified**	1	**I**	1	NCT03893019
CD44v6	2	**4th Generation**	2	**I/II**	2	NCT04430595, NCT04427449
CD70	2	**Unspecified**	2	**I**	1	NCT02830724, NCT04438083
				I/II	1	
CEA	6	**Unspecified**	6	**I**	4	NCT03818165, NCT04348643, NCT03682744, NCT02850536, NCT04513431, NCT04037241
				I/II	1	
				II/III	1	
CLDN18.2	3	**Unspecified**	3	**I**	3	NCT04404595, NCT04467853, NCT03874897
CLDN6	1	**Unspecified**	1	**I/II**	1	NCT04503278
DLL3	1	**Unspecified**	1	**I**	1	NCT03392064
DR5	2	**Unspecified**	2	**I/II**	2	NCT03638206, NCT03941626
EGFR	6	**2nd Generation**	3	**I**	6	NCT03198052, NCT03638167, NCT03542799, NCT03618381, NCT03618381, NCT04153799
		3rd Generation	1			
		4th Generation	2			
EGFRvIII	2	**Unspecified**	2	**I/II**	2	NCT03638206, NCT03941626
EpCAM	4	**2nd Generation**	1	**I**	2	NCT03563326, NCT03013712, NCT02915445, NCT04151186
		3rd Generation	1	**I/II**	1	
		Unspecified	2	**N/A**	1	
ErbB	1	**2nd Generation**	1	**I/II**	1	NCT01818323

FRα	2	**2nd Generation**	1	**I**	1	NCT03585764, NCT03185468
		4th Generation	1	**I/II**	1	
GD2	13	**2nd Generation**	2	**I**	9	NCT03356795, NCT04196413, NCT04539366, NCT02761915, NCT03373097, NCT02765243, NCT04099797, NCT03635632, NCT04430595, NCT03721068, NCT02992210, NCT01953900, NCT01822652
		3rd Generation	2			
		4th Generation	6	**I/II**	4	
		Unspecified	3			
gp100 (MHC-1)	1	**2nd Generation**	1	**I**	1	NCT03649529
GPC3	10	**2nd Generation**	1	**I**	10	NCT03198052, NCT04506983, NCT03198546, NCT03198546, NCT04121273, NCT04377932, NCT02905188, NCT02932956, NCT03980288, NCT03884751
		3rd Generation	2			
		4th Generation	3			
		Unspecified	4			
HER2	7	**2nd Generation**	3	**I**	6	NCT03198052, NCT03500991, NCT03696030, NCT04430595, NCT02442297, NCT04511871, NCT00902044
		3rd Generation	1			
		4th Generation	1	**I/II**	1	
		Unspecified	2			
IL13Rα2	2	**2nd Generation**	2	**I**	2	NCT04510051, NCT02208362
LeY	2	**2nd Generation**	1	**I**	2	NCT03851146, NCT03198052
		3rd Generation	1			
LFA-I	1	**3rd Generation**	1	**I**	1	NCT04420754
MMP-2	1	**2nd Generation**	1	**I**	1	NCT04214392
MSLN	18	**2nd Generation**	3	**I**	11	NCT03198052, NCT03638206, NCT03356795, NCT03941626, NCT04503980, NCT04489862, NCT03747965, NCT03814447, NCT03916679, NCT03638193, NCT03799913, NCT03545815, NCT03497819, NCT03323944, NCT03615313, NCT03054298, NCT02414269, NCT02792114
		3rd Generation	1	**I/II**	6	
		4th Generation	4	**N/A**	1	
		Unspecified	10			
MUC1	5	**3rd Generation**	1	**I**	1	NCT03198052, NCT03356795, NCT03633773, NCT03706326, NCT03525782
		Unspecified	4	**I/II**	4	

MUC1*	1	**2nd Generation**	1	**I**	1	NCT04020575
MUC16	1	**4th Generation**	1	**I**	1	NCT03907527
MUC16ecto	1	**4th Generation**	1	**I**	1	NCT02498912
NECTIN4	1	**4th Generation**	1	**I**	1	NCT03932565
NKG2D	1	**2nd Generation**	1	**I**	1	NCT03692429
NKG2DL	2	**2nd Generation**	1	**I**	2	NCT04270461, NCT04107142
		Unspecified	1			
PSCA	3	**1st Generation**	1	**I**	2	NCT03198052, NCT03873805, NCT02744287
		2nd Generation	1	**I/II**	1	
		3rd Generation	1			
PSMA	6	**4th Generation**	4	**I**	3	NCT03356795, NCT04053062, NCT04227275, NCT03089203, NCT03185468, NCT04429451
		Unspecified	2	**I/II**	3	
ROR1	1	**Unspecified**	1	**I**	1	NCT02706392
ROR2	2	**Unspecified**	2	**I**	1	NCT03960060, NCT03393936
				I/II	1	
TM4SF1	1	**Unspecified**	1	**N/A**	1	NCT04151186
TnMUC1	1	**Unspecified**	1	**I**	1	NCT04025216
Unspecified	6	**4th Generation**	2	**I**	1	NCT03356782, NCT04085159, NCT04433221, NCT03184753, NCT03170141, NCT03356808
		Unspecified	4	**I/II**	5	

Abbreviations: AXL, AXL Receptor Tyrosine Kinase; CAR, chimeric antigen receptor; CEA, carcinoembryonic antigen; CLDN18.2, Claudin-18 isoform 2; CLDN6, Claudin 6; DLL3, delta like canonical notch ligand 3; DR5, death receptor 5; EGFR, epidermal growth factor receptor; EGFRvIII, epidermal growth factor receptor variant III, EpCAM, epithelial cell adhesion molecule; ErbB, Erb-b2 receptor tyrosine kinases; FRα, folate receptor alpha; GD2, disialoganglioside; gp100, glycoprotein 100; GPC3, glypican 3; HER2, human epidermal growth factor receptor 2; IL13Rα2, interleukin-13 receptor α2; LeY, Lewis Y; LFA-I, lymphocyte function-associated antigen 1; MHC-1, major histocompatibility complex I; MMP-2, matrix metalloproteinase-2; MSLN, mesothelin; MUC1, mucin-1; MUC1*, extracellular domain of cleaved mucin-1; MUC16, mucin -16; MUC16ecto, mucin-16 ectodomain; NECTIN4, nectin cell adhesion molecule 4; NKG2D, natural killer group 2D; NKG2DL, natural killer group 2D ligand; PSCA, prostate stem cell antigen; PSMA, prostate-specific membrane antigen; ROR1, receptor tyrosine kinase like orphan receptor 1; ROR2, receptor tyrosine kinase like orphan receptor 2; TM4SF1, Transmembrane 4 L Six Family Member 1; TnMUC1, Tn glycoform of mucin-1

Table 2. Clinical outcomes of T-cell therapy trials with on-target, off-tumour toxicity

Antigen target	CAR/ TCR	Tumour type(s)	Cross-reactivity	Description of toxicities	Clinical trial identifier
MART-1[7]	TCR	Metastatic melanoma	Melanocytes in the skin, eye, and ear	Epidermal melanocyte toxicity, uveitis, synechiae, hearing loss	NCT00509288
gp100[7]	TCR	Metastatic melanoma	Melanocytes in the skin, eye, and ear	Epidermal melanocyte toxicity, uveitis, synechiae, hearing loss	NCT00509496
CEA[8]	TCR	Metastatic colorectal cancer	Gastrointestinal epithelium	Severe transient inflammatory colitis	NCT00923806
CAIX[9]	CAR	Metastatic renal carcinoma	Bile duct epithelium	Liver toxicity	DDHK97-29/P00.0040C
MAGE-A3[10]	TCR	Metastatic cancer, metastatic renal cancer, and metastatic melanoma	Brain	Lethal neurotoxicity	NCT01273181
MAGE-A3[11]	TCR	Metastatic melanoma	Myocardium	Lethal cardiac toxicity	NCT01350401
MAGE-A3[11]	TCR	Advanced myeloma	Myocardium	Lethal cardiac toxicity	NCT01352286
HER2[13]	CAR	Metastatic cancer	Lung epithelium	Lethal pulmonary toxicity	NCT00924287

Abbreviations: CAR, chimeric antigen receptor; TCR, T-cell receptor; MART-1, melanoma antigen recognized by T cells 1; gp100, glycoprotein 100; CEA, carcinoembryonic antigen; CAIX, carboxy-anhydrase-IX; HER2, human epidermal growth factor receptor 2

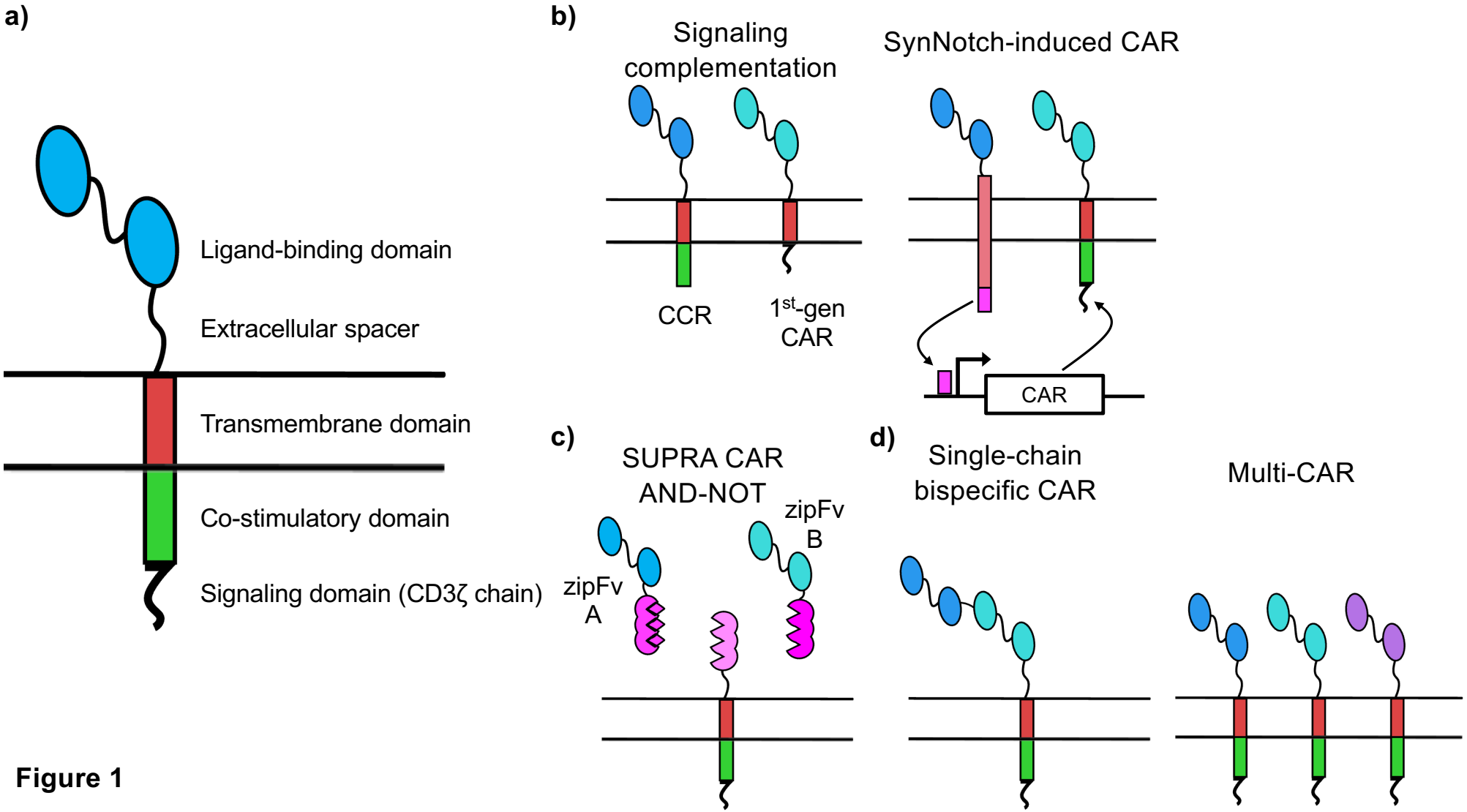
a)
Ligand-binding domain
Extracellular spacer
Transmembrane domain
Co-stimulatory domain
Signaling domain (CD3ζ chain)
b)
Signaling complementation
CCR
1st-gen CAR
SynNotch-induced CAR
CAR
c)
SUPRA CAR
AND-NOT
zipFv A
zipFv B
d)
Single-chain bispecific CAR
Multi-CAR

Figure 1

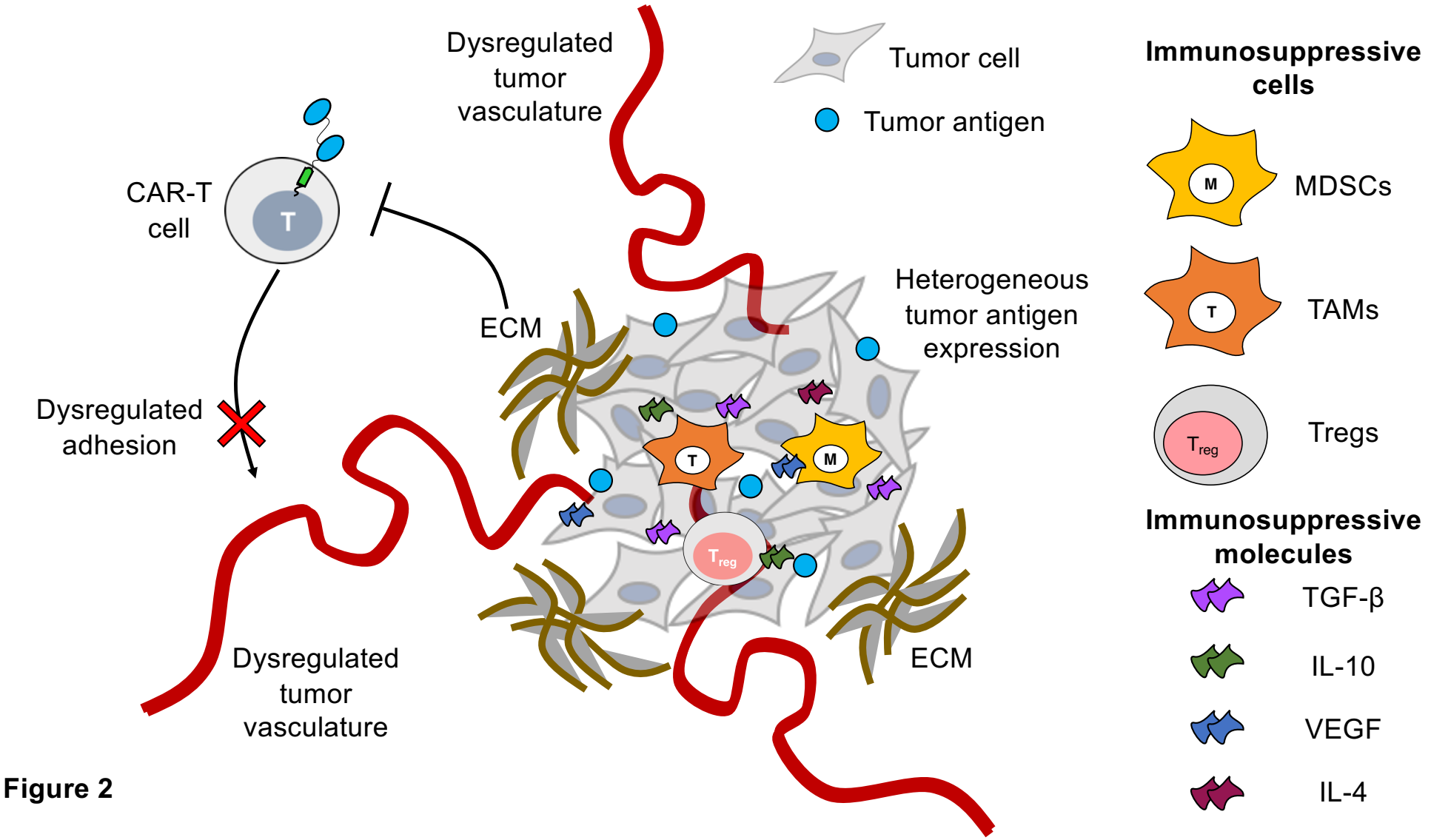
Dysregulated tumor vasculature
Tumor cell
Tumor antigen
Immunosuppressive cells
CAR-T cell
T
ECM
Heterogeneous tumor antigen expression
MDSCs
M
TAMs
T
Dysregulated adhesion
Tregs
Treg
T
M
Treg
Immunosuppressive molecules
TGF-β
IL-10
VEGF
IL-4
Dysregulated tumor vasculature
ECM

Figure 2

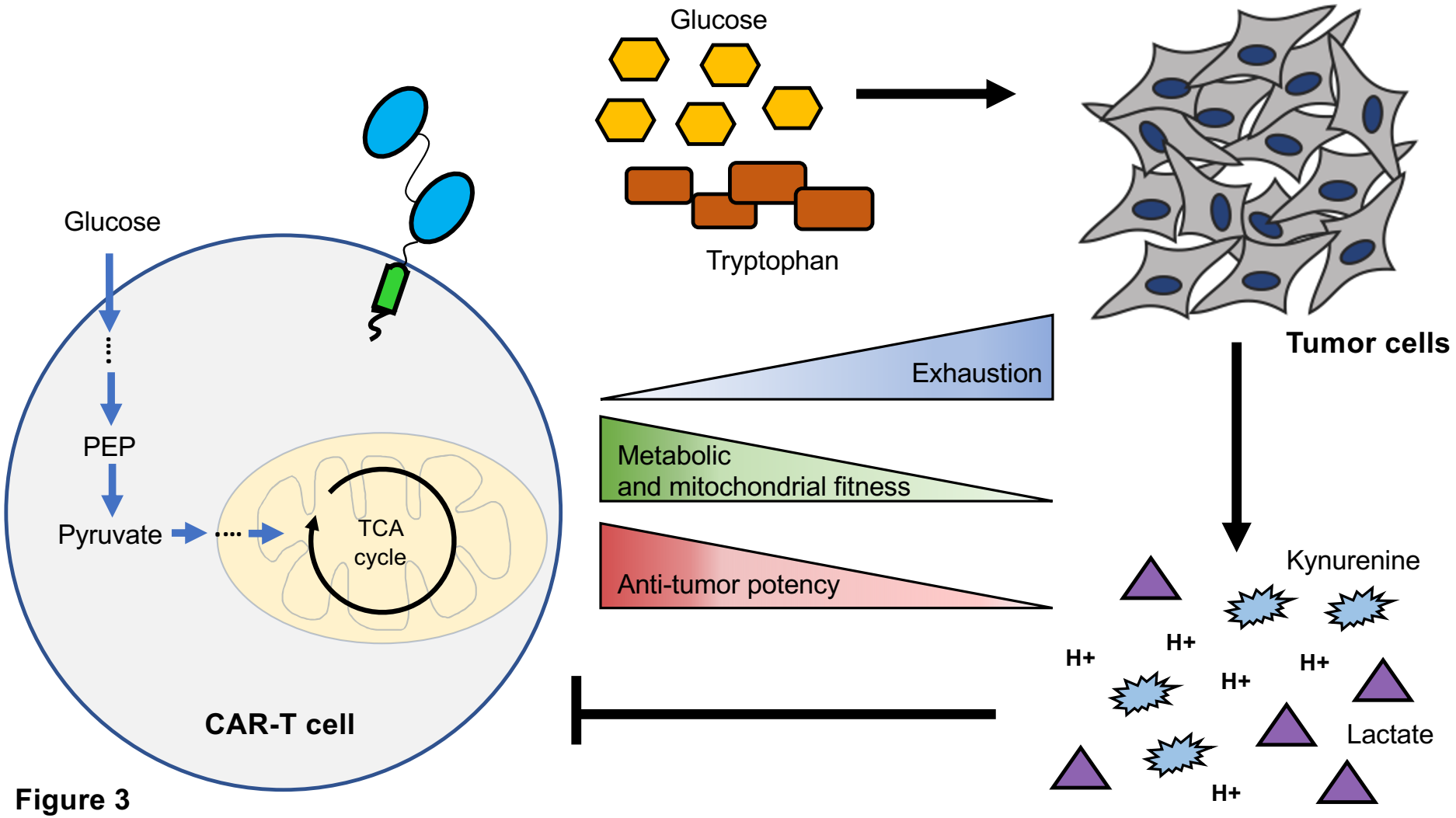
Glucose
Tryptophan
Tumor cells
Glucose
PEP
Pyruvate
TCA
cycle
CAR-T cell
Exhaustion
Metabolic
and mitochondrial fitness
Anti-tumor potency
Kynurenine
H+
H+
H+
H+
H+
Lactate

Figure 3

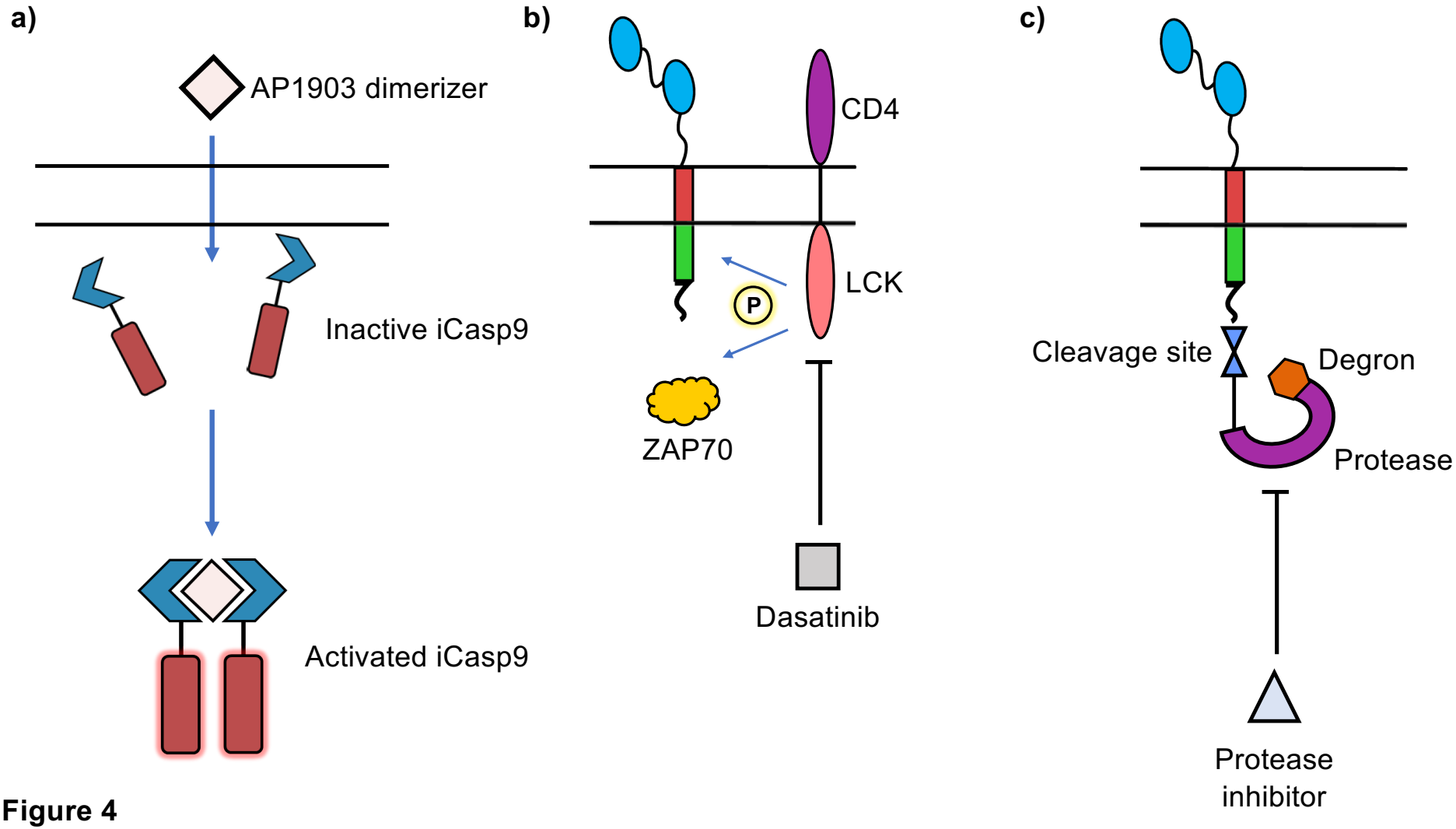
a)
AP1903 dimerizer
Inactive iCasp9
Activated iCasp9
b)
CD4
LCK
P
ZAP70
Dasatinib
c)
Cleavage site
Degron
Protease
Protease
inhibitor

Figure 4

FIGURE LEGENDS

Figure 1. CARs are synthetic, modular receptors with programmable antigen recognition

a. CARs — from the N-terminus to C-terminus — include a ligand-binding domain (typically an scFv), extracellular spacer, transmembrane domain, and intracellular signaling domains consisting of one or two co-stimulatory domains (typically CD28 or 4-1BB) for second- and third-generation CARs, respectively, and an activation domain (typically CD3ζ). Target specificity can be programmed by incorporating different ligand-binding domains.

b. AND-gated antigen recognition can be programmed by signal complementation, or alternatively by ligand-induced CAR expression, to confer greater specificity. In signal complementation, each of two receptors must engage its own cognate antigen for a full T-cell signaling response to occur. Given that T cells require both CD3ζ signaling in addition to co-stimulation in order to be fully activated, signal complementation can be achieved by pairing a CCR — which lacks ζ-chain signaling — with a first-generation CAR — which lacks co-stimulatory signaling. In synNotch-induced CAR expression, a constitutively expressed synNotch receptor triggers CAR expression upon cognate antigen binding. The CAR's target antigen must also be present to activate the engineered T cells. For example, EpCAM and B7-H3–targeting synNotch receptors have both been engineered to trigger downstream expression of ROR1-targeting CARs. The CAR's target antigen must also be present to activate the engineered T cells.

c. Enhanced specificity through AND-NOT–gated antigen recognition can be achieved through the SUPRA CAR platform, which consists of zipFvs (scFv sequences fused to leucine zippers) and T cells expressing zipCARs (CARs whose extracellular domains consist of a leucine zipper). AND-NOT–gated computation is achieved with a zipCAR and two zipFv sequences, where a HER2-targeting zipFv can pair either with the zipCAR to trigger downstream T-cell activation, or with an AXL-targeting zipFv, which acts as a competitive inhibitor for zipCAR binding. When target cells express only HER2 and not AXL, the HER2 zipFv can bind the zipCAR and trigger downstream T-cell signaling. However, when target cells express both HER2 and AXL, the AXL zipFv outcompetes the zipCAR for binding to the HER2 zipFv, and T cells remain unstimulated.

d. To combat antigen heterogeneity, CAR-T cells can be programmed to target multiple tumour antigens. This can be achieved by either engineering a single-chain bispecific CAR which encodes two ligand-binding domains in a single receptor, or by co-expressing multiple receptor chains in a single T cell.

Abbreviations: synNotch, synthetic Notch; SUPRA, split, universal, and programmable.

Figure 2. T-cell–extrinsic factors limiting treatment efficacy against solid tumours

Adoptively transferred T cells are limited in their ability to infiltrate, target, and kill tumour cells. Barriers to infiltration include tumour vasculature with downregulated expression of adhesion molecules necessary for T-cell extravasation from the endothelium into tumours, as well as a dense, fibrotic network of ECM proteins that hinders T-cell motility. Heterogeneous antigen expression poses a further challenge for CAR T cells, where cytolysis is dependent on target antigen recognition. Finally, the presence of immunosuppressive cell types and immunosuppressive cytokines — which are produced by and can polarize or attract suppressor cells — can dampen the anti-tumour function of infiltrating CAR-T cells. For instance, suppressor cells such as Tregs can produce TGF-β, which can inhibit T-cell cytotoxicity through various mechanisms such as downregulation of granzymes as well as increased ECM deposition. TGF-β also promotes further polarization of $CD4^+$ T cells into Tregs and can induce a more suppressive phenotype in myeloid cells. Suppressive myeloid cells in the TME include TAMs and MDSCs, which can inhibit T-cell function through upregulated expression of the inhibitory ligand PD-L1, as well as secretion of inhibitory cytokines such as TGF-β and IL-10. **Abbreviations:** ECM, extracellular matrix.

Figure 3. CAR-T cells face intrinsic and extrinsic metabolic challenges in the TME
The potency of CAR T cells can be limited by both intrinsic and extrinsic metabolic factors in the TME. Exhausted T cells in the TME, which have poor anti-tumour function, are found to be impaired in glycolytic and mitochondrial metabolism and mitochondrial function necessary to mount effective anti-tumour CAR T-cell responses Tumour cells can out-compete CAR T cells for nutrients essential for effective CAR T-cell activation, such as glucose and tryptophan, in the TME. Furthermore, metabolites secreted by tumour cells, such as lactic acid and kynurenine, can directly suppress CAR-T cell function by inhibiting lactic acid export by effector T cells and exerting anti-proliferative and cytotoxic effects against effector T cells, respectively. **Abbreviations:** PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

Figure 4. CAR-T cell safety and control can be managed through genetic or pharmacological strategies

a. Small molecule AP1903 dimerizes inactive iCasp9 in its monomeric form to re-constitute activated caspase 9 signaling leading to apoptosis.
b. The CAR signaling cascade is initiated by LCK-mediated phosphorylation of CD3ζ ITAMs. ZAP70 binds to phosphorylated CD3ζ, which then triggers LCK-mediated phosphorylation of ZAP70, thereby activating CAR signaling. Tyrosine kinase inhibitor dasatinib inhibits LCK phosphorylation of CD3ζ and ZAP70, which in turn inhibits CAR signaling.
c. SWIFF-CARs include — from the N-terminus to C-terminus — a CAR, a protease cleavage site, a protease, and a degron. In the absence of protease inhibitor, the protease-degron fusion is removed from the CAR, relieving the CAR from degron-mediated degradation. In the presence of protease inhibitor, the protease-degron fusion remains connected with the CAR, resulting in CAR degradation.

Abbreviations: iCasp9, inducible Caspase 9; LCK, lymphocyte-specific protein tyrosine kinase; ZAP70, ζ-chain T cell receptor-associated protein kinase 70 kDa, ZAP70; SWIFF-CAR, switch-off CAR.

BOXES

Box 1. Comparing CARs, TCRs, and hybrid receptors

Both CAR and TCR engineering can direct T-cell cytotoxicity towards tumour cells. Conventional CARs derive their antigen-targeting moiety from antibodies and, unlike TCRs which are MHC-restricted, recognize peptides in an MHC-independent manner [224,225]. While conventional CARs derive their signaling component from the CD3 complex, they have distinct signaling properties from TCRs. TCRs form complexes consisting of a CD3εδ heterodimer, a CD3εγ heterodimer, and a CD3ζ homodimer, whereas conventional CARs incorporate a single CD3ζ domain[224,225]. Despite the fact that the ligand-binding domain of CARs frequently has substantially higher binding affinity for its target ligand compared to TCRs, TCRs can respond to a single pMHC complex whereas CARs can require substantially higher antigen densities to induce a robust T-cell response[224,226,227]. These unique characteristics of binding and signaling preclude head-to-head clinical comparisons of T cells engineered with CARs versus TCRs, and the ideal choice of receptor may depend on properties of the target antigen, including antigen density and ease of surface presentation.

Emerging strategies aim to combine the benefits of MHC-independent recognition of antigens by CARs and robust signaling from TCRs. In one strategy, an antibody–T-cell receptor (AbTCR) was designed by fusing a Fab with TCR γ and δ domains[228]. In another strategy, TCR fusion constructs (TRuCs) were engineered by fusing an antibody-based binding domain to various TCR subunits which reconstituted complete TCR complexes[229]. A third strategy introduces T-cell antigen couplers (TACs), which combine extracellular antigen-targeting and TCR-recruitment domains with either a CD4- or CD8α-based co-receptor domain[230]. Importantly, all three CAR/TCR hybrid receptors conferred greater anti-tumour efficacy than their conventional CAR counterparts in leukaemia, lymphoma, and ovarian carcinoma xenograft models, with lower cytokine release levels[228–230]. The pre-clinical data show promise that CAR/TCR hybrid strategies can obviate MHC-restriction, enhance anti-tumour potency, and increase the safety profile of adoptive T-cell therapy.

Box 2. The emerging roles of TOX and TCF-1 on T-cell exhaustion for CAR-T cell therapy

The complete story on T-cell exhaustion has yet to be fully understood, but recent work has cemented TOX and TCF-1 as key players in the regulation of exhausted T cells. TCF-1 plays a dominant part in regulating the bifurcation of chronically stimulated T cells into either terminal effectors or progenitors of exhausted T cells[187–189]. Subsets of TCF-1$^+$ T cells have been shown to possess stem-like qualities despite expression of multiple co-inhibitory receptors. TCF-1$^+$ TILs exhibit central-memory–like transcriptional signatures and have the ability to self-renew or differentiate into TCF-1$^-$/PD-1$^+$ terminally exhausted T cells[187,188]. The heterogeneous TCF-1$^+$ progenitor population can be further stratified into four subsets as defined by the presence or absence of CD69 and Ly108, a surface-bound proxy for TCF-1, with each subset showing varying degrees of stem-like or exhausted phenotypes[189].

In mouse models of chronic viral infection, TOX expression is high and sustained in exhausted CD8$^+$ T cells, but low in functional T-cell populations, a shared finding reported by several research teams[180–183]. TOX can interact with diverse chromatin remodeling proteins[180] and fix T cells into an epigenetic state of T-cell exhaustion and dysfunction[183]. These findings could be

highly relevant to adoptive T-cell therapy as TILs derived from patients with melanoma, non-small-cell lung cancer (NSCLC), and hepatocellular carcinoma show high levels of TOX expression[180,181]. Applying these findings to CAR-T cell engineering, double knockout of TOX and TOX2, another HMB-box transcription factor, enhanced the anti-tumour potency of CAR-T cells in a melanoma mouse model[231]. However, TOX-knockout T cells expressing a SV40 large T antigen-specific TCR were dysfunctional upon adoptive transfer despite low co-inhibitory receptor expression[181]. Further investigation in complementing CAR-T cell therapy with strategies that preserve TCF-1–associated stemness and/or counteract TOX-driven exhaustion may yield next-generation CAR-T cell therapies with further improved efficacy.

Box 3. Gene-editing strategies to guide CAR-T–cell engineering efforts

Recent advances in clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9)-mediated gene editing have enabled robust, targeted genomic modifications in primary human T cells. In fact, T cells with targeted knockouts of endogenous TCR and PD-1 combined with viral integration of the tumour-antigen–specific NY-ESO TCR are currently being evaluated in the clinic[232].

However, the utility of CRISPR/Cas9 editing lies not only in the ability to knock out genes with known deleterious functions, but also in its capacity to serve as a discovery tool for previously unknown regulators of T-cell function. Several recent studies have used CRISPR/Cas9 library screens in primary human T cells to identify novel gene-knockout targets that enhance T-cell fitness and promote robust anti-tumour responses, such as REGNASE-1[233] and p38 kinase[234]. Separately, knock-in library screens identified synthetic receptors that program optimal T-cell function both *in vitro* and *in vivo*[109]. The ability to precisely eliminate genetic drivers of T-cell dysfunction — and simultaneously introduce novel T-cell programs — should prove an invaluable tool for overcoming T-cell–intrinsic deficiencies encountered in the solid TME.

GLOSSARY TERMS

T-cell exhaustion: An evolving term that broadly describes a state of T-cell hypofunctionality, characterized by distinct epigenetic, metabolic, and phenotypic signatures, as a consequent of chronic stimulation.

B-cell aplasia: Depletion of healthy B cells in a patient receiving CAR-T cells targeting pan B-cell markers.

Mesothelial cells: Cells that line internal body cavities, including organs such as the heart and lungs.

Boolean AND-gate: A logical computation in which both condition “A" AND condition “B” must be met in order for the outcome to be true.

Bispecific T-cell engager (BiTE): A bispecific antibody consisting of a tumour-targeting antibody and a T-cell stimulating, anti-CD3 antibody.

Boolean AND-NOT-gate: A logical computation in which condition “A” must be true AND condition “B” must NOT be true in order for the outcome to be true.

Immunoreceptor tyrosine-based activation motifs: A conserved sequence found in the cytoplasmic region of CD3 T-cell receptor chains, and when phosphorylated, serve as docking sites for downstream signaling molecules.