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Recent advances and discoveries on the mechanisms and functions of CAR T cells

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Abstract

This Review discusses the major advances and changes made over the last three years to our understanding of chimeric antigen receptor (CAR) T cell efficacy and safety. Recently, the field has gained insight into how various molecular modules of the CAR influence signaling and

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M.V.M. and R.C.L. researched the data for the article and selected the content and otherwise contributed equally to writing the article and to review and/or editing of the manuscript before submission.

Competing interests
M.V.M. and R.C.L. have intellectual property on certain CAR T cells and antibodies (not licensed yet). M.V.M. receives consulting income from several industry sponsors that market CAR T cell therapies; M.V.M. serves on several scientific advisory boards and has equity in TCR2 and Century therapeutics.

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function. We report on mechanisms of toxicity and resistance as well as novel engineering and pharmaceutical interventions to overcome these challenges. Looking forward, we discuss new targets and indications for CAR T cell therapy expected to reach the clinic in the next one to two years. We also consider some new studies which have implications for the future of CAR T cell therapies, including changes to manufacturing, allogeneic products, and drug-regulatable CAR T cells.

Table of contents summary

This Review outlines the major advances that have been made to the efficacy and safety of chimeric antigen receptor (CAR) T cell therapies over the last three years and looks to new findings which will have consequences for the future of this immunotherapy.

Introduction

After two decades of fine-tuning T cell engineering, the tremendous clinical success of chimeric antigen receptor (CAR) T cells in patients with leukemia and lymphoma has led to an exponential growth in research within the field. The US Food and Drug Administration (FDA) approval of CAR T cells in 2017 catapulted the field into an era of fast-paced and innovative research. Here we discuss what the field has learnt since this milestone and how it will affect the future of CAR T cell therapy. We start with a brief review of the basic CAR design and discuss what has been discovered in the last few years about each component's effect on the signaling and function of the engineered cell. Interestingly, it now seems that each component of the CAR matters for determining its function, with even single amino acid changes resulting in alterations in signaling threshold for antigen binding, exhaustion, and persistence. We also describe the toxicities that appear to be a class-effect of CD19 CAR T cells: cytokine release syndrome and neurotoxicity, with updated findings from advanced clinical trials. Until very recently preclinical CAR T cell research was limited by a lack of adequate animal models, but recent advances in more humanized approaches have enabled systematic testing of potential interventions and also elucidated mechanisms underlying toxicities. We next review our current understanding of resistance to CAR T cells, and how it can be overcome with innovative CAR T cell design. Finally, we discuss a selection of promising new targets and indications as well as manufacturing innovations that will likely have a major effect on the future of CAR T cell therapy.

CAR engineering

The first generation of CARs consisted of an extracellular antigen-recognizing single chain variable fragment (scFv) developed from an antibody sequence fused to a transmembrane region and the intracellular signaling domain derived from the CD3ζ molecule of the endogenous T cell receptor (TCR)[1–4]. However, these CAR T cells had little efficacy in clinical trials owing to failed expansion and persistence[5]. Second generation CARs include a costimulatory domain, derived from either CD28 or 4–1BB and located between the transmembrane and CD3 signaling domains[6,7]. The first patients with B-cell leukemia treated with second generation CD19-targeted CAR T cells had profound and durable responses[8–10]. As a result, CAR T cell therapy revolutionized the treatment of hematological malignancies and was US FDA approved in 2017 owing to its efficacy against $CD19^+$ tumours[11,12] (see Table 1 for results from major published clinical trials).

CAR parts and assembly

The extracellular portion of a CAR is typically derived from the variable light (V_L) and variable heavy (V_H) regions of an antibody (scFv) against the tumour target of interest (Figure 1). The linker between these two domains is commonly derived from repeated glycine and serine residues, but other linker molecules have also been used (i.e. the Whitlow linker[13]). To overcome the structural and aggregation issues associated with scFvs, some CARs instead have been designed to use the single-domain V_{HH} of camelid antibodies, natural ligands, or artificial protein-binding constructs[14–16]. The hinge (also called the spacer region) links the antigen-recognizing extracellular domain to the transmembrane region. Some flexibility by this hinge is needed in order to allow the scFv domain to bind its cognate antigen. A variety of hinge regions have been used in CAR designs, including domains developed from CD28 and CD8[17]. The transmembrane portion of the CAR spans the cell membrane lipid bilayer and often is also derived from CD28 or CD8. It is thought that this domain can influence molecular interactions between CARs, forming homodimers or trimers based on the endogenous transmembrane association of the original protein[18,19]. All current US FDA-approved CAR T cell products are second generation design with either a CD28 or 4–1BB costimulatory domain[11,12]. In addition to CD28[20] and 4–1BB[21], other common costimulatory domains include OX40[22], CD27[23], and inducible T cell co-stimulator (ICOS)[24]. If two costimulation domains are used in one construct, this is considered a third generation CAR[22]. The CD3ζ cytoplasmic domain is the most distal intracellular part of the CAR. This molecule contains three immunoreceptor tyrosine-based activation motifs (ITAMs) which signal upon phosphorylation[25]. Some investigators have termed CARs with enhanced T cell function ‘fourth-generation’ CARs, particularly when they produce an additional protein molecule, such as cytokines or possess additional receptors like costimulatory ligands. These are also referred to as TRUCKs (T cells Redirected for Universal Cytokine Killing) or armored CARs[26,27].

CAR T cells are activated upon scFv recognition of antigen, which causes clustering and immobilization of the CAR molecules. Phosphorylation of the ITAM domains on the CD3ζ chain initiates signaling through the tyrosine kinase ζ-associated protein of 70 kDa (ZAP70), similar to TCR signaling[28]. This unleashes a T cell effector response including proliferation, release of cytokines, metabolic alterations, and cytotoxicity. CAR T cells are thought to mainly exert their cytotoxic function through secretion of granzyme and perforin, but there is some data to suggest that death receptors are also utilized, based on activation of downstream molecules such as BH3-interacting domain death agonist (BID) and FAS-associated death domain protein (FADD)[29–31]. Signaling from the costimulatory domain is dependent on the specific function of the domain chosen and can be modulated by specific mutations in canonical sequences[32,33].

CAR manufacturing

US FDA-approved CAR T cells are derived from the patient’s own cells (autologous), which circumvents issues of allogeneic rejection or graft-versus-host disease (GvHD) but requires individual bespoke manufacturing. Of note, CARs do contain foreign sequences and can be rejected, although this effect is partly mitigated by the use of the antimetabolite fludarabine in the preparative chemotherapy regimen, to facilitate lymphodepletion[34]. Autologous CAR T cell manufacturing typically begins with leukapheresis of the patient, although some investigators have used non-separated whole peripheral blood[35]. The T cells are then activated *ex vivo* with stimulation and costimulation, respectively through their TCR (CD3) and a chosen ligand (often CD28) in the presence of a cytokine cocktail. Stimulating antibodies can be added in soluble form, chemically conjugated to beads, or expressed on artificial antigen presenting cells (aAPCs)[36,37]. The T cells are typically grown in the presence of interleukin-2 (IL-2), but other cytokines including IL-7 and IL-15 are also used to manipulate the overall T cell phenotype with varying degrees of success[38–40]. After activation, the CAR construct is introduced into the T cells, typically by viral or non-viral vectors. Both retroviral and lentiviral vectors have been described as safe and effective, and both integrate randomly into the host T cell genome[41,42]. Electroporation of cells with non-viral vectors is another method used which has much lower costs, but safety and efficacy are still being assessed[43]. Some CAR T cell manufacturing now includes gene editing by CRISPR–Cas9 or transcription activator-like effector nuclease (TALENs)[44,45], which can be used in combination with an adeno-associated viral vector to target integration of the CAR into a particular locus. Finally, CAR T cells are grown on the scale of days in bioreactors and then delivered back to patients for infusion. Typically, CAR T cells are given as a single dose, or sometimes split over two or three days; regardless, this is still substantially different from most cancer drugs, which are given either daily or every 3–4 weeks until disease progression.

CAR signaling and exhaustion

What makes the ‘best’ CAR T cell remains a controversial question. A convoluting factor in clinical trials is that every clinically approved CAR T cell product is different, because it is made from the T cells of a specific patient. Clinical data have identified that responses correlate with expansion of CAR T cells, persistence, and a memory phenotype[46], so avoiding or delaying T cell exhaustion is considered to be a key goal.

Proliferative Capacity

In clinical studies with the CD19-targeted CAR, detectable CAR T cell expansion in patient blood is correlated with response[46]. Further evidence that sustained CAR T cell proliferation is beneficial was revealed by a very particular case study. A patient with chronic lymphocytic leukemia (CLL) treated with two infusions of CAR T cell therapy initially showed tumour progression in their bone marrow. However, two months after the second infusion, CAR T cell expansion peaked in the blood with corresponding tumour regression eventually leading to a complete response. Upon further investigation of the CAR T cell expansion, it was determined that a single CAR T cell clone had expanded, leading to the much delayed anti-tumour response. The cell had biallelic disruption of the tet methylcytosine dioxygenase 2 (*TET2*) gene owing to lentiviral integration in one allele and a hypomorphic mutation in the other. The *TET2* double knockdown caused a change in the epigenetic landscape leading to increased proliferation and a more central memory-like phenotype of the cells resulting in a robust anti-tumour response[47]. A genome wide CRISPR screen in human primary T cells identified additional genes which may have a similar effect if deleted: suppressor of cytokine signaling 1 (*SOCS1*), transcription elongation factor B polypeptide 2 (*TCEB2*; also known as *ELOB*), RAS GTPase-activating protein 2 (*RASA2*), and *CBLB* (encoding an E3 ubiquitin ligase). Disruption of these genes showed enhancement both in proliferation and *in vitro* cytotoxicity towards tumour cells[48]. Further investigation into viral integration sites in patient CAR T cells showed that responders have enriched insertional mutagenesis in genes encoding proteins in cell-signaling and chromatin modification pathways which promote proliferation. Based on these data, investigators were able to create a model where clinical outcome could be predicted based on vector integration site[49]. These studies suggest that CAR T cells with modifications in genes encoding proteins which influence proliferation may result in a more efficacious cell product, but this will need to be balanced against concerns over potential oncogenic transformation.

CAR T cells with increased proliferation and survival capacity have proven beneficial in a variety of disease contexts. $CD8^+$ T cells in patients with CLL have impaired T cell activation characterized by reduced glucose uptake after stimulation[50]. These impaired T cell responses are thought to be responsible for the lower response rate to CAR T cells observed in patients with CLL compared with other B cell malignancies; interestingly, patients with CLL who did have complete responses to CAR T cells were noted to have enhanced mitochondrial biogenesis in their $CD8^+$ T cells, which correlated with expansion and persistence of CAR T cells[50]. In multiple myeloma, it has been observed that T cells from patients with early onset disease have better fitness for CAR T cell manufacturing and potentially better efficacy compared to those from patients with relapsed or refractory disease[51].

Differences in Costimulation

CAR T cells with different costimulatory domains in their CARs have been noted to have different dynamics, with the presence of a 4–1BB costimulation domain conferring slower expansion and longer persistence compared to the presence of a CD28 costimulation domain, which leads to rapid expansion but less durability[52,53]. The molecular basis for this difference is not well understood, but previous studies have shown that CD28-based CAR T cells exhibit a more effector-like memory phenotype and have an enhanced glycolytic metabolism, whereas 4–1BB-based CAR T cells have a more central memory phenotype and rely on fatty acid metabolism[54–56]. Furthermore, it has been shown that cytotoxic $CD8^+$ lymphocytes receiving 4–1BB costimulation have superior proliferation, ex vivo expansion into memory $CD8^+$ T cells, and enhanced cytolytic activity compared to $CD8^+$ T cells with CD28 costimulation[57]. More recent investigation into the difference between these two costimulation pathways shows that 4–1BB CARs but not CD28 CARs activate noncanonical nuclear factor-κB (NF-kB) signaling after ligand engagement. Interfering with this signaling through overexpression of a dominant negative mutant (the C terminus of NF-κB-inducing kinase (NIK; also known as MAP3K14), which is a widely used strategy to block noncanonical NF-kB signaling) reduces the proliferation and survival of 4–1BB-based CAR T cells owing to an increase in expression of the apoptotic signaling molecule BIM (also known as BCL2L11)[58]. In addition to NF-κB, 4–1BB signaling is also reliant on tumour necrosis factor (TNF) receptor associated factors (TRAFs), which affect CAR T cell viability, expansion, and cytotoxicity in part due to regulating NF-κB. Overexpression of TRAFs in 4–1BB-based CAR T cells enhances their function[59]. Another comparison made between 4–1BB versus CD28 used a phosphorylated protein approach to show that the effector-like phenotype of CD28-based CAR T cells correlated with fast activation and larger changes in the magnitude of protein phosphorylation. The intensity of this signal is partly owing to constitutive association of the tyrosine kinase LCK with the CD28 intracellular CAR domain. In contrast, RNA sequencing shows that the lower degree of phosphorylation in activated 4–1BB-based CAR T cells is associated with higher expression of memory-associated genes relative to CD28-based CAR T cells[33,60]. Additionally, Li et al.[61] have shown that the persistence and memory phenotype of 4–1BB-based CAR T cells can be further enhanced by limiting the ubiquitination of the CAR (by mutating ubiquitin-targeted lysine residues in the CAR molecule). This increased recycling of the CAR molecule as opposed to its degradation enhances oxidative phosphorylation due to elevated endosomal 4–1BB signaling[61].

Conversely, when trying to design CARs for regulatory T (T_{reg}) cells, which function to protect tissue, the costimulation that elicits a desirable phenotype is reversed. CAR T_{reg} cells benefit from a CD28 domain which retains their suppressive activity *in vivo* against a desired target. This differs from a 4–1BB-based CAR T_{reg} cell, which can become increasingly cytotoxic due to the effects of the costimulation[62].

Various costimulatory domains beyond CD28 and 4–1BB have been used in combination with CD3ζ signaling for CAR T cell therapy. Recent work shows that ICOS increases persistence of CAR T cells[63]. Additionally, ICOS in combination with 4–1BB costimulation as a third generation CAR design shows superior efficacy in solid tumour mouse models over 4–1BB-based second generation CARs. Interestingly, the membrane proximal intracellular costimulatory domain has the dominant effect in third generation CAR T cells[63].

Tuning CAR signaling

In addition to augmenting the proliferative capacity of CAR T cells, limiting their exhaustion is also beneficial to the anti-tumour response. T cell research has shown that strong T cell activation drives exhaustion[64,65] and so several efforts have been made to tune CAR signaling to alleviate exhaustion. Antigen and CAR molecule density both have a large impact on efficacy – low expression of either contributes to a limited anti-tumour response[66]. Tuning CAR signaling with different linkers[67,68], hinge and transmembrane domains[69–71], costimulation[69], ITAMs[25], and promoters (to moderate CAR molecule expression)[72] can all influence CAR functionality depending on the amount of tumour antigen present (Table 2).

Primarily, alterations in the spacing regions between the V_L and V_H regions and the transmembrane domain affect the flexibility of the CAR construct, therefore determining its ability to access and bind its target antigen epitope[73,74]. More recently, Singh et al.[67] showed that a CAR based on the same scFv targeting CD22 but with different linker lengths (20 amino acids versus 5) had different clinical responses. Despite similar binding affinities, the CAR with a longer linker formed monomers in solution whereas the shortened version formed homodimers which was associated with increased tonic signaling. The shorter linker CAR demonstrated superior anti-tumour function both *in vitro* and *in vivo*, leading to a new clinical trial using a shortened-linker CD22 CAR (NCT03620058[75] and NCT02650414[76])[67]. Qin et al.[68] also explored the effects of linkers in the design of bispecific CAR T cells targeting CD19 and CD22 and showed that linker length had a profound effect on CAR construct potency.

In addition to linker length, it is known that hinge length needs to be optimized. How distal (shorter hinges) or proximal (longer hinges) the target antigen epitope is to the target cell membrane determines the formation of an immunological synapse[73,74,77,78]. More recently, hinge and transmembrane domains have also been shown to have a much more important role than initially expected, and much work is now going on to optimize CAR constructs based on the proper combination of these clearly more-than structural components[70]. For example, in a direct comparison of CARs bearing a CD8α hinge and transmembrane domain or a CD28 hinge and transmembrane domain, CD8α-based CARs had lower levels of both cytokine production and activation-induced cell death (AICD)[71]. Furthermore, Majzner et al.[69] have also recently showed that differences in costimulation molecules, once believed to be the main drivers of differences in CAR T cell phenotypes, can be manipulated to behave more similarly in the context of low levels of the tumour antigen by changing the hinge–transmembrane region of the CAR T cell construct.

It has also been demonstrated that manipulating the ITAM domains of the CAR construct alters signaling. The activation of CD28-based CAR T cells can be calibrated using ITAM mutants to change the binding affinity of ZAP70. Through these mutations, CAR T cells can be directed to different fates, enabling a CD28-based CAR T cell with mutations in ITAM1 and ITAM2 domains to persist with long-lived memory but retain effective anti-tumour function[25]. Engineering additional ITAM domains into 4–1BB-based CAR T cells enables them to recognize low antigen density-expressing target cells (typically a threshold only reached by CD28-based CAR T cells)[69].

Another approach is to mimic the signaling of the endogenous TCR, which has evolved to fine tune endogenous T cell responses. Using CRISPR–Cas9, CAR integration can be targeted to the endogenous TCRα chain (TRAC) locus, resulting in expression controlled by the endogenous T cell promoter. This results in uniform CAR expression, but more importantly enhances T cell potency by averting tonic CAR signaling. CARs are internalized and re-expressed upon exposure to antigen, which delays T cell exhaustion and terminal differentiation to an effector T cell fate[72]. In a novel design, the scFv of an antibody has been combined with the γ and δ chains of the TCR to serve as the effector domains. These so-called antibody-TCR (AbTCR) cells retain the cytotoxic activity of CD28- or 4–1BB-based CAR T cells but with less cytokine release resulting in less exhaustion[79]. Additionally, two groups recently published that insertion of the CD3ε molecule into the CAR enhances anti-tumour function by tuning CAR signaling with less cytokine production and longer persistence[80,81].

Emerging mechanisms of toxicity

The two hallmark clinical toxicities associated with CAR T cell therapy are cytokine release syndrome (CRS) and neurotoxicity, and neither was predicted by animal modeling before clinical translation. CRS typically manifests in patients within the first week of CAR T cell therapy as fever, hypotension, and respiratory insufficiency with high serum cytokine levels[82,83] (Figure 2). Neurotoxicity is exhibited as temporary working memory loss, delirium, seizures and rarely acute cerebral edema[84]. Both CRS and neurotoxicity are attributed to rapid activation and expansion of the T cells which secrete cytokines. The current understanding is that cytokines secreted by the CAR T cells and/or ligand–receptor interactions activate additional immune cells of the myeloid compartment which in turn release more cytokines to create a loop of activating inflammation called a cytokine storm. Naturally occurring adrenaline in patients has also been shown to contribute to this self-amplifying production loop[85,86]. Although neurotoxicity has been clinically associated with CRS, the mechanisms involved appear to be different[87].

In some cases, neurotoxicity or CRS-associated multi-organ failure can be lethal. High tumour burden and higher CAR T cell expansion have been correlated with higher grades of CRS[88]. Overall, CARs with a 4–1BB costimulation domain seem to cause less neurotoxicity and CRS than those with a CD28 domain[84]. In a study of 53 adult patients who received CD28-based CD19-targeted CAR T cells for the treatment of acute lymphoblastic leukemia (ALL), 62% of patients developed neurotoxicity, of which 67% were considered severe. Of the patients with severe neurotoxicity, 72% developed seizures, though these effects were transient[89]. In a similar study with 133 patients treated with 4–1BB-based CD19-targeted CAR T cells for ALL, 40% had a neurologic toxicity, with 8% of those patients experiencing seizures. In this case, most patients with high grade neurotoxicity were involved in dose escalation later determined to be above the maximum tolerated dose[87]. Severe neurotoxicity in patients has been correlated with high levels of proinflammatory cytokines (IL-6, IL-8, CC-chemokine ligand 2 (CCL2), and CXC-chemokine ligand 10 (CXCL10)) in the cerebrospinal fluid (CSF)[89]. Evidence of blood–CSF barrier disruption correlates with neurotoxicity grade but white blood cell count or CAR T cell count in the CSF does not[89]. Despite these observations, the mechanisms of neurotoxicity are currently unknown. However, we speculate that the increase in pro-inflammatory cytokines may activate endothelial cells in the brain vasculature, which leads to areas of increased permeability and capillary leak (known as vasogenic edema), which manifest as focal edema and neurotoxicity[87]. Another possible explanation suggested by a recent study is that that brain mural cells express CD19 and that on-target CAR T cell activity against these cells results in neurotoxicity[90]. Clinically, the main treatment to overcome CRS is to break the feedback loop of cytokines through treatment with an IL-6 receptor monoclonal antibody, tocilizumab. Corticosteroids are often also used in combination with tocilizumab to reduce inflammation and vasogenic edema in the brain. Yet, neither of these interventions appear to block CAR T cell expansion in patients[88].

One of the challenges in designing new strategies to prevent or mitigate CRS is the set of limitations inherent in mouse modeling. The NOD–SCID–IL-2 receptor gamma null (NSG) mice typically used as preclinical models for CAR T cell therapy carry mutations which severely affect their immune compartment, including myeloid cells. However, the advantage of NSG mice is that human tumour cells and human T cells can be engrafted more efficiently than with other strains. In an attempt to better model human CRS, researchers have turned to other mouse strains, animal species or humanized mouse models. Giavridis et al.[91] developed a mouse model of CRS using SCID-beige mice which, similarly to human CRS, develops 2–3 days after CAR T cell injection. These mice have a less impaired IL-1 response to interferon-γ (IFNγ) priming compared to NSG mice. In their model, 75% of these mice die due to CRS within 48 hours. Interestingly, the CAR T cell cytokines themselves were not driving the CRS, although this could be in part due to the reliance of the model on cross-reactivity between human T-cell produced cytokines and mouse myeloid cell receptors. Despite limited cross-reactivity, mouse CRS was driven by production of mouse IL-6, IL-1, and nitric oxide produced by macrophages activated by the human CAR T cells[91]. In another example of an advanced model, Taraseviciute et al.[92] developed a rhesus macaque model of neurotoxicity in which CD20 CAR T cells caused a cascade of immune activation leading to atypically high levels of proinflammatory cytokines and chemokines in the CSF and infiltration of CAR^+ and CAR^- T cells in the brain parenchyma leading to encephalitis. The animals also exhibited symptoms characteristic of CRS including fever and elevated C-reactive protein (CRP) levels[92]. Another group utilized the human CD19 transgene in mice to show increased levels of cytokines compared with the lack of CRS observed using mouse CD19. Furthermore, antibody-mediated neutralization of IL-6 or IFNγ in this model alleviated T cell induced weight loss[93]. Another potential animal model utilizes a triple transgenic NSG (SGM3) mouse, which expresses human stem cell factor, granulocyte macrophage-colony stimulating factor (GM-CSF), and IL-3 to mimic the innate immune component of CRS involvement. However, this model still relies on some mouse cytokines as part of the cytokine response, most importantly IL-6[86]. In an effort to improve this CRS model to better mimic the human clinical phenomena, sublethally-irradiated newborn SGM3 mice can be injected intrahepatically with human hematopoietic stem and progenitor cells (HSPCs). This more rapidly reconstitutes hematopoiesis and results in the production of human B cells, monocytes, and T cells compared with non-transgenic NSG mice. As proof of principle of the effectiveness of this more advanced model, characterized by high fever and elevated human IL-6 levels, it was shown that tocilizumab can prevent CRS but does not protect against meningeal inflammation leading to lethal neurotoxicity[94].

Additional interventions to manage toxicity beyond IL-6 receptor blockade are under investigation, both in pre-clinical models and in clinical trials. Inhibiting GM-CSF signaling, either through monoclonal antibody neutralization (lenzilumab) or by genetically altering the CAR T cells to knockout GM-CSF, can limit CRS and neuroinflammation[95]. Giavridis et al.[91] and Norelli et al.[94] have demonstrated that disruption of the IL-1 pathway with anakinra, an IL-1 receptor antagonist, mitigates both CRS and lethal neurotoxicity in mice[91,94]. Anakinra is now being tested clinically to prevent neurotoxicity in CAR T cell treated patients[96]. In addition to cytokine intervention, others have shown that pharmacological blockade of catecholamine with metyrosine can protect mice from the lethal complications of CRS[86]. Other groups have sought to control CAR T cell toxicity not through addressing the toxicity itself, but by controlling the CAR T cell load and potency with the use of dasatinib, a US FDA-approved tyrosine kinase inhibitor. Dasatinib limits

CAR T cell signaling by preventing LCK phosphorylation and nuclear factor of activated T-cells (NFAT) induction after CAR T cell engagement[97]. Unlike suicide switches which would irreversibly affect CAR T cell efficacy[98], dasatinib suppresses CAR T cell activation and function without killing the cells. This could be implemented in a clinical setting when early symptoms of CRS arise, whereby CAR T cells could be transiently turned off using dasatinib to prevent further cytokine secretion[97,99].

Mechanisms of resistance

One of the common mechanisms associated with relapse after CAR T cell therapy is loss of antigen (Figure 3). Flow cytometry analysis of B cell-acute lymphoblastic leukemia (B-ALL) cells from patients with relapsed or refractory B-ALL that had relapsed after 4–1BB-based CD19-targeted CAR T cells therapy showed that 12 of the 17 patients had progressed with CD19 negative disease. Sequencing of the genomes of the B-ALL cells revealed all 12 patients had mutations in CD19 making the molecule unrecognizable to CAR T cells[100]. Selective pressure to mutate CD19 is the most common mechanism of antigen loss, and many relapsing patients are now screened to determine if CD19 is still present. Although the most common, mutagenesis of CD19 is not the only documented mechanism of antigen loss. Ruella et al.[101] reported a patient with B-ALL who relapsed 9 months after receiving CD19 CAR T cell therapy. By quantitative polymerase chain reaction (qPCR) it was determined that the patient still had CAR sequences in blood cells, but this did not correlate with an expansion of CAR T cells by flow cytometry which instead showed an expansion of CD19 negative leukemic cells. Further investigation revealed that the tumour cells had originated from one leukemic B cell that had been transduced with CAR during manufacturing. The CAR molecule ‘masked’ CD19 on the surface of the cell, preventing it from being recognized by CAR T cells[101]. This is considered to be a rare event but has serious implications for the safety of manufacturing. In addition to complete loss of CD19, a third mechanism of antigen loss has been reported by Hamieh et al.[102] which explains how antigen-low tumour escape can occur. Through a process called trogocytosis, CARs remove target antigen from tumour cells and internalize it, leading to decreased antigen density on tumour cells. This affects both CD28- and 4–1BB-based CAR T cells, but with differential consequences because CD28-based CARs are less sensitive to decreases in antigen density[102].

Antigen loss explains some relapses, but not all patients who relapse have antigen negative disease[103], suggesting there may be additional factors leading to resistance, such as exhaustion of CAR T cells. A study of responders and non-responders (with primary resistance) to CD19-targeted CAR T cells in CLL revealed that T cells in infusion products had a more effector-like signature, including differentiation, glycolysis, exhaustion, and apoptosis, in non-responding patients. In contrast, T cells in infusion products from responding patients had upregulation of IL-6 and signal transducer and activator of transcription 3 (STAT3) signatures. An elevated frequency of memory-like T cells ($CD27^+CD45RO^-CD8^+$) prior to CAR T cell generation is also associated with sustained remission[46]. Whole genome-wide loss-of-function screens in ALL cell lines have identified impaired death receptor pathways as another mechanism of resistance to CD19-targeted CAR therapy. Loss of FADD, BID and tumour necrosis factor-related apoptosis-inducing ligand 2 (TRAIL2) in tumour cells renders them more resistant to CD19-targeted CAR T cell efficacy, both *in vitro* and *in vivo*, owing to lower cytotoxicity and proliferation of the CARs[29,30]. Furthermore, prolonged CAR stimulation by ALL cells lacking one of these death receptor molecules leads to T cell exhaustion[29,46].

Most of the experience of the field with CAR T cell therapy is in the setting of hematological malignancies, where the tumour microenvironment (TME) is thought to play much less of a role in response. However, as the field looks beyond CD19-targeted CAR T cells to solid tumours, a suppressive TME may also generate resistance to CAR T cell treatment. This is especially a concern with difficult to penetrate, highly immunosuppressive solid malignancies like glioblastoma or pancreatic cancer. The lack of chemokines to attract the CAR T cells, an abnormal vasculature, and ECM-producing cancer-associated fibroblasts (CAFs) can all make it difficult for CAR T cells to even traffic to the tumour. Once there, immunosuppressive cells and cytokines, including T_{reg} cells, myeloid-derived suppressor cells (MDSCs), transforming growth factor β (TGFβ), IL-6, IL-10 and prostaglandin E_2 (PGE_2), inhibit CAR T proliferation and effector functions[104].

Overcoming resistance

Overcoming antigen loss

The most straightforward approach to preventing antigen escape is with the development of CARs targeting other antigens, which could be used in series or in tandem. CAR T cells targeting CD20 and CD22 are in clinical development both in stand-alone and tandem scFv formats[105,106]. Another group has leveraged natural CD40–CD40 ligand (CD40L) cytotoxicity: through constitutive expression of CD40L, CAR T cells are able to engage CD40 on both $CD40^+$ tumour cells (directly engaging cytotoxicity) and also on tumour-adjacent antigen presenting cells (APCs). Activated APCs then increase expression of costimulatory molecules and major histocompatibility complex class II (MHC-II) to recruit additional effector cells like endogenous T cells which can assist in tumour killing through their endogenous TCRs, even in the absence of tumour cell expression of the CAR antigen[107]. Another approach is to increase antigen expression on the target cells. Small-molecule γ-secretase inhibitors have been shown to increase B-cell maturation protein (BCMA; also known as TNFRSF17) expression on myeloma cells by impairing cleavage of surface-expressed BCMA. This combination treatment with BCMA-targeted CAR T cells is now in a clinical trial (NCT03502577[108]). Similarly, Bryostatin 1 has been shown to upregulate CD22 and increases the efficacy of CD22-targeted CAR T cell therapy in preclinical models, though the mechanism of this CD22 upregulation remains to be elucidated[109]. In addition, scFv affinity can be tuned to the expression pattern of the tumour. Liu et al.[110] have shown when comparing similarly designed CARs against the same antigen but with various affinities, higher affinity scFvs are able to recognize antigen at low expression levels and even at levels which are undetectable by flow cytometry.

Overcoming exhaustion

The field of cancer immunotherapy was revolutionized with the development of immune checkpoint blockade. Programmed cell death protein 1 (PD1) is upregulated on T cells upon activation and binds its natural ligand PD1 ligand 1 (PDL1), to dampen the T cell response, both in terms of proliferation and effector function[111]. Studies showed that blockade of the PD1–PDL1 axis could be leveraged to overcome T cell inhibition owing to PDL1 expression in tumours[112]. This translated into US FDA-approval of monoclonal antibodies against PD1 and PDL1, which have since had great clinical success in oncology[113]. What has been learnt with immune checkpoint blockade in overcoming T cell exhaustion is now being applied to CAR T cells, including combinatorial treatment, autocrine secretion of anti-immune checkpoint molecules from CAR T cells, and genetic perturbation of immune checkpoint genes in CAR T cells themselves. For example, Cherkassky et al.[114] have shown that CAR T cell combinatorial treatment with a PD1 antibody can restore CD28-based CAR T cell effector function in mouse models where they typically become exhausted. There are now many ongoing clinical trials evaluating the effects of combinatorial immune checkpoint blockade (targeting either PD1 or PDL1) with CD19-targeted CAR T cells (NCT02650999[115], NCT02706405[116], NCT02926833[117], NCT03310619[118]). Early results suggest combinatorial treatment is safe and has a low toxicity profile. One case report has even demonstrated the benefit of combinatorial treatment: a patient with diffuse large B cell lymphoma (DLBCL) who progressed post-CD19-targeted CAR T cell therapy received anti-PD1 therapy at day 26 post-CAR T cell transfusion. By day 45, significant tumour regression was observed and by three weeks post-PD1 blockade, the patient was able to return to work[119]. To avoid the need for two separate therapeutics, researchers have also tested CAR T cell-intrinsic secretion of antibodies against immune checkpoint molecules including anti-PD1 and anti-PDL1 in preclinical models[120,121]. Similar benefits to those achieved with combinatorial treatments were observed including prolonging T cell function and limiting exhaustion. The additional benefit of local secretion of antibodies against immune checkpoint molecules is the potential to limit systemic toxicities [122]. Another approach is to engineer the CAR T cells to be resistant to PDL1. One study showed exhaustion can be limited by transducing CAR T cells with a PD1 dominant negative receptor to act as a sink to minimize PD1 signaling through the endogenous receptor[114]. An alternative method is to knockout the gene encoding PD1 in CAR T cells using CRISPR–Cas9. This improves anti-tumour immunity *in vitro* and within *in vivo* models in the presence of $PDL1^+$ tumours[123]. This method of genetic knockout has also been shown to be tolerated clinically with PD1 knockout T cells persisting in patients for up to 9 months[45].

Additional approaches not directly targeting immune checkpoints have also been utilized to overcome exhaustion. Expression of a constitutively signaling IL-7 receptor along with a CAR has the benefit of augmenting the CAR T cells after antigen exposure but avoids stimulating bystander cells (unlike cytokine-secreting CAR T cells). Specifically, the co-expression of this receptor with a CAR increases T cell proliferation, persistence, and anti-tumour response without T cell dysfunction[124]. An alternative approach to limit exhaustion has been investigated in a mouse model of CAR tonic signaling. Exhaustion is associated with increased accessibility of activator protein 1 (AP1) transcription factor motifs, which leads to terminal differentiation of T cells. Overexpression of the canonical AP1 factor JUN combats the exhaustion resulting in increased functional capacity and improved anti-tumour potency of the CAR T cells[125].

Overcoming the immunosuppressive tumour microenvironment

Innovative approaches to increase trafficking and limit suppression by anti-inflammatory cytokines and cells in the TME are also in development. One strategy is to increase the CAR T cell response through secretion of proinflammatory soluble factors. Overexpression of IL-7 and CCL19 in CAR T cells, which are required for formation of the T cell zone in lymphoid organs, increases infiltration of pro-inflammatory dendritic cells and T cells into solid tumour tissues and enhances tumour regression in mouse models[126]. Armored CAR constructs overexpressing proinflammatory cytokines like IL-12 and IL-18, have also been shown to activate more pro-inflammatory endogenous immune cells and enhance the anti-tumour response[127,128]. Alternatively, CARs engineered to express the IL-12b p40 subunit produce IL-23 upon T cell activation which activates STAT3 signaling to promote proliferation. These CARs have superior efficacy in xenograft and syngeneic solid tumour mouse models. In addition, they have fewer side effects compared with IL-18 producing CARs, which may be due to reduced activation of bystander cells since IL-23 works in a predominantly autocrine manner[129].

Another strategy is to redirect or circumvent the suppressive TME. In prostate cancer, transduction of a dominant-negative TGFβ receptor (TGFβR) as a second transgene in the same vector as a prostate-specific membrane antigen (PSMA)-targeted CAR acts as a sink for the high localized levels of TGFβ in the tumour to limit its immunosuppressive effects[130]. This particular CAR construct is now in a Phase I clinical trial (NCT03089203)[131]. Similarly, CRIPSR–Cas9 mediated knockout in CAR T cells of the endogenous TGFβRII led to CAR T cells that exhibited increased efficacy, less conversion to T_{reg} cells, and less exhaustion in xenograft solid tumour models[132]. Another approach to overcoming T_{reg} cell-mediated immunosuppression is to alter the activity of the T_{reg} cells themselves. This idea stemmed from a CAR T cell clinical trial in glioblastoma targeting the epidermal growth factor receptor class III variant (EGFRvIII) which showed high intratumoural T_{reg} cell infiltration post-CAR T cell infusion [133]. In a follow-up study, researchers developed an EGFR-bispecific T cell engager (BiTE) that is secreted by EGFRvIII-targeted CAR T cells and redirects conventional T cells and T_{reg} cells to exert cytotoxic function against the tumour[134]. This is a novel use of CAR T cells to co-opt immunosuppressive cells in the TME to promote an anti-tumour response. In a similar approach, an oncolytic adenovirus is used to deliver genetically encoded BiTEs to tumour cells in combination with CAR T cell therapy. Two solid tumour models of different cancer types, pancreatic and colon, show significant improvement to CAR T cell efficacy when treated in combination with the oncolytic virus BiTE[135]. Another group has generated an enhanced anti-tumour response by utilizing vaccination of amphiphilic CAR T ligands in combination with CAR T cell therapy. The strategy relies on the injected amphiphilic ligands trafficking to lymph nodes and presenting to APCs to prime CAR T cells leading to massive expansion[136].

Promising new targets and indications

Multiple Myeloma

The leading target for multiple myeloma is BCMA (NCT02658929[137], NCT02546167[138]). However, as is seen with CD19 negative relapse following treatment with CD19-targeted CAR T cells, BCMA negative relapse can occur[139,140]. Two groups have suggested alternative, ligand-based CAR T cells which target both BCMA and another molecule expressed on multiple myeloma cells, transmembrane activator and CAML interactor (TACI; also known as TNFRSF13B). These CARs are based on a truncated form of the molecule a proliferation-inducing ligand (APRIL), which binds surface BCMA and TACI and is typically secreted by myeloid cells in the bone marrow. Lee et al.[14] used a single truncated (to prevent cleavable secretion and amino-terminal interactions with proteoglycans) APRIL molecule in a third generation design with CD28 and OX40 costimulatory domains[14]. This design was in the clinical trial AUTO2 (NCT03287804[141]) but was subsequently terminated due to a lack of preliminary efficacy. Schmidts et al.[19] designed a second generation CAR with 4–1BB costimulation based on three repeated truncated APRIL molecules to mimic the trimeric soluble secreted form of the protein, which binds surface BCMA and TACI to promote myeloma growth[19]. In multiple myeloma xenograft preclinical models, the trimeric form shows increased efficacy over the single APRIL CAR design[14,19].

Smith et al.[142] have identified an additional target for multiple myeloma, G protein-coupled receptor, class C group 5 member D (GPRC5D), which has an expression pattern on $CD138^+$ multiple myeloma cells independent of BCMA expression. Using a phage display system to generate scFvs, the authors screened 42 constructs using a Nur77 reporter system (Nur77 is an early indicator of TCR signaling) to identify an optimal second-generation CAR based on spacer length and low tonic signaling. In normal tissues, the GPRC5D protein is only expressed in the hair follicle[142]. To address concerns of on-target toxicity, Smith et al. used both mouse and non-human primate cynomolgus monkey models to show that there was a lack of alopecia when GPRC5D was targeted. GPRC5D-targeted CAR T cells were able to clear mice with a mixture of BCMA positive and negative multiple myeloma cells, whereas mice treated with BCMA-targeted CAR T cells succumbed to the disease[142]. Several other targets are also in development for multiple myeloma, including CD38, CD138, and SLAM family member 7 (SLAMF7)[143].

Solid Tumours: Pan Cancer

A major concern in the field of CAR T cell therapy is its applicability beyond hematological malignancies. Many factors contribute to the hurdle of making an efficacious CAR T cell for solid tumours, including but not limited to a lack of tumour-specific antigens and an immunosuppressive TME. Many leaders in the field believe there are less than a dozen feasible CAR T cell targets beyond CD19, and identifying targets that have high, homogenous expression in the tumour but not in healthy tissue (to limit on-target, off-tumour toxicity) has proven to be difficult.

The B7-H3 (also known as CD276) immune checkpoint molecule has been a popular immunotherapy target over the past few years, with a few promising clinical trials targeting the molecule with monoclonal antibodies (NCT02982941[144,145], NCT02381314[146,147], NCT03406949[148,149], NCT02628535[150,151], NCT03729596[152,153]). However, one of the main concerns with bringing forward a CAR T cell product targeting B7-H3 is on-target toxicity owing to low expression on normal tissue[154]. Using an antibody that has been found to only recognize high levels of B7-H3, Majzner et al.[154] designed a CAR T cell based on this specific scFv in an attempt to limit on-target toxicity of healthy tissues with low level B7-H3 expression. They showed that in the context of high antigen density, which is true of several pediatric cancers including medulloblastoma, osteosarcoma, and Ewing sarcoma, B7-H3-targeted CAR T cells were efficacious *in vitro* and *in vivo*. However, when antigen density is low, the B7-H3-targeted CAR T cells have much lower activity, suggesting that there may be a therapeutic window for an efficacious, non-toxic B7-H3-targeted CAR T cell therapy in the clinic[154].

He et al.[155] have designed a so-called 237CART which can recognize multiple cancer-specific antigens. The 237CART cells are based on an antibody initially developed to recognize Tn-glycosylated podoplanin (Tn-PDPN). The basis of the tumour-specific recognition by 237CART cells is the result of loss of function mutations in *COSMC*, which encodes a molecular chaperone thought to be required for expression of active T-synthase, the only enzyme that galactosylates the Tn antigen during mucin type O-glycan biosynthesis. This differential pattern in Tn galactosylation, which results from COSMC mutation, has been shown to occur in ovarian cancer, leukemia, breast cancer, sarcoma, and neuroblastoma. The 237CAR T cell is of particular interest due to this pan-cancer recognition. In addition, He et al. have shown that 237CART cells can recognize many Tn-glycopeptides, not just Tn-PDPN. This is promising for 237CART cell therapy effectiveness as antigen escape may be avoided[155].

CAR T cells targeting mesothelin have been pursued in recent years for a variety of indications, including pancreatic, lung and ovarian cancer. Preclinical models were promising, and several clinical trials are currently ongoing[156]. Initial results suggest mesothelin-targeted CAR T cells are safe and have evidence of anti-tumour activity, which is promising for the future of CAR T cells for solid tumours[157,158]. Additional clinical studies are evaluating whether the mesothelin-targeted CAR T cell therapy can be enhanced with combination immune checkpoint blockade to augment the T cell response[157]. Recently, early phase I results (NCT03907852) were announced using a mesothelin-targeted T cell receptor fusion construct (TRuC) based CAR[159]. The scFv of this CAR is fused to TCR subunits and lacks the traditional costimulatory domain. These CARs retain their cytotoxicity but have lower cytokine release and higher efficacy than traditional CAR T cells[160].

Solid Tumours: Brain

Brain tumours have remained relatively unresponsive to the wave of immunotherapies that have had incredible success and generated responses in other cancer types. However, there is potential for CAR T cell therapy to be used to treat brain tumours owing to the unique niche of the brain – unlike large molecules which frequently have difficulty gaining access to brain tumours because of the blood-brain barrier, T cells and T cell therapy can infiltrate the brain after intravenous transfusion[133]. The initial clinical trials of CAR T cell therapy against glioblastoma have targeted EGFRvIII, IL-13 receptor α2 (IL-13Rα2), and human epidermal receptor 2 (HER2), unfortunately with little efficacy likely owing to loss of target antigen[133,161,162].

In an effort to overcome the heterogeneity of target antigen expression often found with glioblastoma, Wang et al.[163] have designed a CAR T cell based on a chlorotoxin (CLTX) peptide which has potent anti-tumour activity even in the absence of other glioblastoma-associated antigens. Flow cytometry staining of patient derived glioblastoma cells for CLTX peptide revealed high and homogenous expression of an unknown surface ligand compared with the heterogeneous expression of IL-13Rα2 and HER2. Their study shows that the efficacy of CLTX peptide-targeted CAR T cells is dependent on surface expression of metalloproteinase-2 (suggesting the unknown antigen epitope is reliant on this proteinase to be recognized by the CAR T cell) and is non-toxic to normal tissues including peripheral blood mononuclear cells (PBMCs), induced pluripotent stem cells (iPSCs), and an immortalized fetal brain-derived neural stem cell line. This research suggests targeting the chlorotoxin peptide with CAR T cell therapy could be promising since it may not have the same issues with antigen heterogeneity that has been observed with previous targets[163].

Disialoganglioside GD2-targeted CAR T cells targeting neuroblastoma have been previously well tolerated in clinical trials[164]. Mount et al.[165] have additionally suggested GD2-targeted CAR T cells may be efficacious in diffuse midline gliomas with mutated histone H3 K27M (H3-K27M) which are a universally fatal pediatric cancer. While the group demonstrated efficacy in patient-derived orthotopic xenograft models, they also observed peritumoural neuroinflammation, which led to lethal hydrocephalus in some animals. This study suggests that with a cautious approach and aggressive neurointensive care management, GD2 CAR T cell therapy may be beneficial for H3-K27M^{+} diffuse midline gliomas[165]. Recently, a new clinical trial (NCT03294954[166]) has been initiated using Vα24-invariant natural killer T (NKT) cells transduced with an IL-15 expressing CAR targeting GD2. Interim analysis showed the GD2-targeted CAR-NKT cells were well tolerated in three patients. One patient achieved an objective response with regressing bone metastatic lesions[167].

Bosse et al.[168] used an RNA-sequencing-based pipeline to identify a novel target, a cell surface heparan sulfate proteoglycan GPC2, for neuroblastoma. GPC2 is highly expressed on neuroblastoma tissue, but not normal pediatric tissues. Using an antibody–drug conjugate based on the cytotoxic capacity of pyrrolobenzodiazepine (PBD), Bosse et al. showed reduced tumor growth by targeting GPC2 in a patient-derived xenograft model of neuroblastoma[168]. It is possible that GPC2 may prove to be an efficacious CAR T cell target as well.

Manufacturing Innovations

Gene transfer

A limitation of genetically reprogrammed T cell therapeutics is the use of viral vectors which have expensive and long production times for clinical use [169]. In addition, viral vectors have limited size constraints for the length of DNA that can be encoded (approximately 4kb for adeno-associated viruses, 8.5 kb for adenoviruses, and 10 kb for lentiviral vectors). Transposons have been explored over the past decade as a non-viral means of generating CAR T cells, as this method of gene transfer is more economical than viral-based methods. The Sleeping Beauty transposon-based system has been used to create CD19-targeted CAR T cells to treat lymphoma and leukemia patients who have relapsed following allogeneic hematopoietic stem cell transplants (NCT00968760[170], NCT01497184[171], NCT03389035[172])[43,173]. Similarly, the piggyBac transposon system has been used over the last several years as another method to generate CAR T cells. One biotechnology company currently has two clinical trials ongoing using piggyBac-based CAR T cells[174,175]. Roth et al.[176] have developed a new non-viral method for delivering DNA sequences (> 1 kb) to specific sites in the genome of primary human T cells using electroporation of CRISPR–Cas9 and double stranded DNA. Using this method a transgenic, cancer-specific TCR was introduced into the TCRα locus resulting in TCR-engineered T cells able to produce an anti-tumour response both *in vitro* and *in vivo*[176]. Further improvement on their design involves simultaneous orthotopic replacement of the endogenous TCRα and β locus, which avoids the mispairing of the endogenous and transgenic TCR chains (which can compete in typical transgenic TCR manufacture). As a result, they are able to recreate near-physiological T cell function [177]. However, it should be noted that currently this technique is still limited for large genetic loads (such as a large CAR construct), particularly for clinical use. In addition to changes in how genetic material is delivered, improved manufacturing of CAR T cells also involves how T cells are cultured *ex vivo*[40]. Groups have worked to determine the proper cocktail of cytokines for optimal growth conditions, and now others have joined the effort and introduced mimetic cytokines which may further enhance the production process[178].

Improving the effector cell type

Current manufacturing efforts use heterogeneous T cells from a patient (with the exception of $CD4^+$ T cell and $CD8^+$ T cell separation in some cases). However, there is evidence to suggest that some subsets of T cells may be more efficacious than others. Clinical trials have been conducted using central memory T cells for manufacturing CD28-based CD19-targeted CAR T cells (NCT01318317[179], NCT01815749[180]). Initial results show that these cells are efficacious but do not necessarily have more persistence compared with bulk manufactured CD19-targeted CAR T cells. However, it has been suggested that the costimulatory domain has a stronger effect on persistence than the T cell phenotype[181]. Although there has been interest in producing CARs from other more rare subsets (such as the T memory stem cells (T_{SCM})), commercialization becomes a difficulty as there is a need for large scale manufacture with good manufacturing practices (GMP)-quality reagents for cell sorting[182]. $CD26^{high}$ CAR T cells have also been investigated because they have superior function in solid tumour models as a result of increased cytokine production (IL-17A, IFNγ, IL-2, tumour necrosis factor (TNF), and IL-22), memory, stem-like properties (increased β-catenin and lymphoid enhancer-binding factor 1 (LEF1) expression) and trafficking (elevated CCR2 and CCR5 expression)[183]. γδ T cells are another potentially advantageous subset. The vast majority (95–99%) of typical CAR T cell infusion products are αβ T cells[184]. Both subsets show equivalent levels of cytotoxicity; however, γδ CAR T cells are less prone to exhaustion with lower levels of T-cell immunoglobulin mucin receptor 3 (TIM3) and PD1 expression post-activation. In addition, γδ CAR T cells express costimulation and antigen presentation molecules (CD86 and human leukocyte antigen-DR (HLA-DR)) and have been shown to cross present tumour antigens to other T cells in the TME[185]. Perhaps most convincingly, infiltration of γδ T cells in cancer is correlated with a favorable prognosis[186].

There are several approaches to making CAR T cell products more like a 'one-size fits all' drug (Figure 4). One approach, which still requires an autologous cell product but leverages one vector for multiple antigen specificities, is the split, universal and programmable (SUPRA) CAR with a 'zip' system to tune the T cell response. The CAR T cells express one portion of a 'zipper' protein extracellularly, with intracellular signaling domains. Then various protein therapeutics attached to the corresponding partner zipper can be used to change the target and signal strength of the CAR. There is also a combinatorial approach where two different zip systems are used, one with a CD3ζ signaling domain and the other with costimulation[187]. Other potential universal CARs include a biotin-binding immunoreceptor-based dimerizing system[188] and switch modules with neo-epitope tagging[189,190]. These CARs target a specific tag, such as fluorescein isothiocyanate (FITC) or biotin. The system then relies on a second antibody therapeutic against the target tumour antigen; this antibody is conjugated to the tag so that anti-tag CARs recognize it and are activated against the labeled tumour.

Allogeneic CARs

Third-party 'off-the-shelf' allogeneic CAR T cell therapy is highly sought after mainly to improve standardization of the product, patient wait times and logistics around coordinating manufacturing with patient urgency, and cost[191]. The two main barriers to use of allogeneic T cell therapies are the risk of causing GvHD, which is an important safety issue, and the risk of rejection of the product, an efficacy issue. The first clinical results obtained with allogeneic gene-edited T cells were published in 2017 where two infants with B-ALL were treated with TALEN-edited TCRα negative, CD52 negative 4–1BB-based CD19-targeted CAR T cells. Both patients achieved a complete response with minimal GvHD and went on to undergo an allogeneic stem cell transplant, which is standard of care for this disease when it is in remission[192]. Phase I/II clinical trials using this CAR in pediatric and adult ALL are ongoing (NCT02746952[193], NCT02808442[194]). Other allogeneic CAR T cell products involve multiplexed-genome editing via CRISPR–Cas9 technology. The methodology uses lentiviral delivery of a CD19-targeted CAR and electroporation of Cas9 and single guide RNAs targeting the endogenous TCR, β2 microglobulin and PD1 loci with the goal of reducing the risk of GvHD, rejection, and exhaustion, respectively[195]. More recently, Liu et al.[196] have also leveraged cord-blood derived natural killer (NK) cells and transduced these with a CD28-based CD19-targeted CAR retroviral vector (Box 1). Cord blood has the advantage of containing a larger proportion of NK cells (30% of lymphocytes compared with 10% in the peripheral blood) which lowers the risk of contaminating T cells causing GvHD. To improve NK cell persistence, the investigators also included a transgene for IL-15 in the construct; results from their first clinical trial demonstrated responses and sustained remissions in patients with lymphoma, and these products are now advancing in clinical development[197]. Daher et al.[198] have further improved the IL-15 design with cord blood derived CAR NK cells that have a *CISH* deletion to target the cytokine-inducible SH2-containing protein, which negatively regulates IL-15 signaling. As a result, these CARs have increased aerobic glycolysis and enhanced efficacy in eliminating lymphoma xenografts in vivo.

Concluding remarks

CAR T cell research has advanced rapidly into the clinic and now back to the bench, with trial results informing new mechanisms of efficacy, toxicity, and resistance, and catalyzing the search for novel targets, elucidation of signaling mechanisms, and application of novel technologies. Innovations in CAR design, transduction methodologies, and selection of the best cell types are bound to lead to improved responses and transform the treatment of patients with many different cancer types.

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Glossary terms:

Camelid antibodies

antibodies generated from Camelidae mammals, which have two identical heavy chains, and compared with typical antibodies are much smaller (15 kDa compared to 150 kDa) and lack a light chain

Graft-versus-host disease (GvHD)

a condition that can occur after allogeneic transplant owing to donor cells recognizing the host as foreign resulting in donor cell attack of the host body **Leukapheresis:** procedure in which white blood cells are separated from the blood and the remaining cells are returned to circulation

Artificial antigen presenting cells (aAPCs)

synthetic versions of APCs which activate immune cells; in the context of CAR T cells, aAPCs are engineered with T cell receptor stimulation and costimulatory molecules to expand T cells ex vivo

Transcription activator-like effector nucleases (TALENs)

DNA-binding domains fused to non-specific DNA-cleaving nucleases to target a specific sequence for gene alteration

Maximum tolerated dose

highest dose of treatment that does not cause intolerable side effects

Suicide switches

genetically encoded molecules included in a CAR vector that can be targeted to induce CAR T cell death

Hypomorphic mutation

altered gene resulting in lower expression and/or activity of the gene product

Tonic signaling

ligand-independent constitutive signaling of a CAR

Trogocytosis

a process where lymphocytes extract ligands from antigen presenting cells and express them on their own surface

Bispecific T cell engager (BiTE)

artificial bispecific antibodies made up of two single chain variable fragments (scFvs) – one that recognizes a specific antigen and the other that binds CD3 on T cells eliciting an activation response

Transposon

genetic segment which can be translocated in the genome from one location to another by a DNA transposase

Activation-induced cell death (AICD)

programmed cell death caused by repeated stimulation of T cells that serves as a negative regulator of activation

Amphiphilic

describes a molecule containing both hydrophobic and hydrophilic regions

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Box 1.

Beyond the conventional CAR T cell: CAR NK cell, CAR macrophage, and CAR T_{reg} cell

Natural killer (NK) cells have been pursued as the basis for development of allogeneic products owing to their intrinsic anti-tumour activity. NK cell-based therapy has the advantage of being cytotoxic when human leukocyte antigen (HLA) expression is downregulated, which is a relatively common tumour escape mechanism. In addition, allogeneic NK cells have been observed to have better efficacy than autologous NK cells[211,212]. A novel method involves deriving NK cells from cord blood and transducing them with an anti-CD19 chimeric antigen receptor (CAR) vector that has been engineered to ectopically produce of interleukin-15 (IL-15) to increase expansion and efficacy. These cord blood CAR-NKs are cytotoxic in vitro and have persistence and anti-tumour effects in vivo. With respect to toxicity, these CAR NK cells have a chemically inducible caspase 9 suicide gene and can be eliminated with treatment in vivo[196]. Attempts to make CAR cells from hematopoietic stem cells have shown that CAR expression during early lymphoid development suppresses *BCL11B*, thereby suppressing T cell associated genes. As a result, the CAR cells have NK-cell like properties, including NK cell receptor expression. These cells, termed CAR-induced killer (CARiK) cells, require a second generation CAR design with costimulation to have strong anti-leukemic effects. In a xenograft leukemia model, allogeneic CARiK cells showed increased survival with no observed graft versus host disease (GvHD) despite major histocompatibility complex (MHC) mismatch[213].

Klichinsky et al.[214] recently published a description of the first CAR macrophage (CAR-M), which demonstrated antigen-specific phagocytosis and pro-inflammatory M1 polarization in vitro. CAR-Ms were also able to cross-present antigen and activate T cells. Xenograft mouse models of lung metastases and intraperitoneal carcinomatosis treated with CAR-Ms had decreased tumour burden. In humanized mice, CAR-Ms enhanced T cell anti-tumour responses and single cell RNA sequencing showed induction of a pro-inflammatory tumour microenvironment[214].

CARs are also being used to redirect immunosuppressive regulatory T (T_{reg}) cells, as potential therapeutics in autoimmune disease and organ transplant. Several groups have shown prevention of allograft skin rejections using CAR T_{reg} cells in vivo[62,215,216]. Others have utilized CAR T_{reg} cells to ameliorate colitis[217]. However, the manufacture of CAR T_{reg} cells remains a concern for future clinical use due to difficulties purifying this cell subset from conventional T cells. Novel approaches including droplet-based sorting are currently being explored[218]. The first clinical trial for CAR T_{reg} cells was initiated in 2019 for the prevention of rejection following HLA-A2 mismatched kidney transplantation in patients with end stage renal disease.[219,220] CTLA4, cytotoxic T lymphocyte-associated antigen 4; IFNγ, interferon-γ; TGFβ, transforming growth factor β; TNF, tumour necrosis factor.

Figure 1: Schematic of a basic second generation CAR T cell.

The extracellular portion of the chimeric antigen receptor (CAR) molecule is typically generated from a monoclonal antibody against the target. The variable heavy (VH) and variable light (VL) chains, also known as the single chain variable fragment (scFv) from the antibody sequence are connected by a linker to form the antigen-specific region of the CAR molecule. The hinge or spacer region anchors the scFv to the transmembrane region which traverses the cell membrane. Intracellularly, the costimulatory domain and CD3ζ chain signal upon the scFv portion of the CAR recognizing and binding tumour antigen. Costimulatory signals are dependent on the costimulation domain used – CD28 is dependent on PI3K whereas 4–1BB requires tumour necrosis factor (TNF) receptor associated factors (TRAFs) and nuclear factor-κB (NF-κB). The CD3ζ chain contains three immunoreceptor tyrosine-based activation motif (ITAM) domains which upon phosphorylation, signal through ζ-associated protein of 70 kDa (ZAP70). Downstream signaling leads to T cell effector functions including release of perforin and granzyme leading to cell death of the target tumour cell.

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Figure 2: Current molecular understanding and therapeutic intervention of CAR T cell-induced cytokine release syndrome and neurotoxicity.

Upon recognition of tumour antigen, the anti-tumour response activated downstream in chimeric antigen receptor (CAR) T cells leads to activation of innate immune cells owing to secretion of inflammatory cytokines like granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-1 (IL-1). This leads to a self-amplifying inflammatory activation loop in macrophages causing release of IL-1 and IL-6. Therapeutic intervention at various stages of this response can mitigate neurotoxicity and cytokine releases syndrome (CRS). Antibody therapeutics targeting GM-CSF (lenzilumab), the IL-6 receptor (tocilizumab), and the IL-1 receptor (anakinra) have been used for this purpose clinically. Tyrosine kinase inhibitor dasatinib affects T cell signaling to reduce CRS and metyrosine inhibits macrophage inflammatory activation to achieve a similar effect. IFNγ, interferon-γ; TNF, tumour necrosis factor.

Figure 3: Antigen-dependent and antigen-independent resistance.

Resistance to chimeric antigen receptor (CAR) T cell therapy can be categorized according to the context of antigen loss or retention. Antigen loss is most commonly due to mutations in the gene encoding the antigen itself. However, there have been cases of antigen loss due to accidental transduction of a tumour cell leading to ‘CAR masking’ of the tumour antigen, resulting in relapse. In addition, reduced levels of tumour antigen can result in relapse and may be owing to trogocytosis in which CAR molecules on T cells remove tumour antigen from the surface, internalize it, and begin expressing the tumour antigen themselves. Antigen-independent resistance is due to exhausted T cells. This can result from a suboptimal infusion product with a terminal effector phenotype, loss of death receptor signaling on tumour cells making them resistant to CAR T cell killing, and an immunosuppressive tumour microenvironment due to regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSCs) and their respective soluble factors. IL-10, interleukin-10; PGE_2, prostaglandin E_2; TGFβ, transforming growth factor β.

Figure 4. CAR T cell subsets with increased efficacy and Universal CARs with interchangeable targets.

CD26 high chimeric antigen receptor (CAR) T cells have increased cytokine production, a more stem-like phenotype, and increased cell trafficking compared with conventional CAR T cells. CAR T cells with endogenous γδ T cell receptors (TCRs) have decreased exhaustion and increased costimulation compared with conventional CAR T cell products, of which 95% have αβ TCRs. Several versions of universal CARs have been published in recent years. The split, universal and programmable (SUPRA) CAR uses a ‘zipper’ system to interchangeably affect the CAR. The single chain variable fragments (scFvs) can be switched to target new antigens or multiple antigens at once and the zipper binding affinity can be changed to tune the strength of the CAR signal for an appropriate response. Gating strategies can also be used by having the CD3ζ signaling domain and the costimulatory domain on separate molecules so that both corresponding antigens must be present to have full CAR activation. Others have pursued universal CARs through tagging tumour cells with antibodies (which are transient and can be continuously changed) conjugated to tags like fluorescein isothiocyanate (FITC) or biotin and then developing CAR T cells against the tag to activate their cytotoxic function. CCR, CC-chemokine receptor; HLA-DR, human leukocyte antigen-DR; IFNγ, interferon-γ; IL, interleukin; LEF1, lymphoid enhancer-binding factor 1; PD1, programmed cell death protein 1; TIM3, T-cell immunoglobulin mucin receptor 3.Box 1.

Table 1.

Major Published Trials of CAR T cell therapy

Target Antigen	Disease (*indicates US FDA approval)	CAR	Clinical Trial Identifier	Sponsor	Number of patients	Median Age (yrs)	Response	Patients with CRS	Patients with neuro toxicity
CD19	B-ALL (pediatric)*	Tisagenlecleucel; 4–1BB costimulation; CTL019	NCT02435849 [53,199]	Novartis Pharmaceuticals	75	11	6 month relapse-free survival rate of 80%	77%	40%
CD19	Relapsed or refractory DLBCL*	Axicabtagene ciloleucel; CD28 costimulation; KTE-X19	NCT02348216 ZUMA-1[52,200,201]	Kite Pharma (a Gilead Sciences company)	101	58	83% objective response; 58% complete response	93%	67%
CD19	Refractory B cell lymphomas*	Tisagenlecleucel; 4–1BB costimulation; CTL019	NCT02030834 [202,203]	UPenn	28	58.5	64% overall response; 43% complete remission	57%	39%
CD19	Mantle cell lymphoma*	Axicabtagene ciloleucel; CD28 costimulation; KTE-X19	NCT02601313 ZUMA-2 [204,205]	Kite Pharma	68	65	93% objective response rate; 67% complete response	91%	63%
CD19	B-ALL	CD28 costimulation	NCT01044069 [206,207]	MSKCC	53	44	83% complete remission; median overall survival 12.9 months	85%	44%
CD22	Relapsed or refractory pre-B-ALL	4–1BB costimulation	NCT02315612 [106,208]	NCI	21	19	73% complete remission treated with higher dose	76%	unreported
BCMA	Relapsed or refractory multiple myeloma	Idecaptagene Cicleucel; 4–1BB costimulation; bb2121	NCT02658929 [137,209]	Celgene	33	60	85% objective response rate; 45% complete response rate	76%	42%
BCMA	Multiple myeloma	4–1BB costimulation	NCT02546167 [138,210]	UPenn	25	58	48% overall response rate	88%	32%

ALL, acute lymphoblastic leukemia; BCMA, B cell maturation antigen; CAR, chimeric antigen receptor; CRS, cytokine release syndrome; DLBCL, diffuse large B cell lymphoma; MSKCC, Memorial Sloan Kettering Cancer Center; NCI, National Cancer Institute; UPenn, University of Pennsylvania.

Table 2.

Tuning CAR signaling with each component

CAR component	Potential changes	Potential influences in function
Linker	Length67,68	Dimerization leading to differences in tonic signaling Potency
Hinge and transmembrane	Length[73,74,77,78], type (for example, CD8α, CD28)69–71	Immunological synapse formation Cytokine production and AICD Efficacy with low density target antigen expression
Costimulation	Type (for example, CD28, 4–1BB, ICOS)[33,57-]59,62,63	Initial expansion Persistence Durability of response Memory phenotype and cell fate
ITAMs	Number of functional domains[25,69]	Memory phenotype and cell fate Efficacy with low density target antigen expression
Promoter	Synthetic or endogenous[72]	CAR expression (number of molecules per cell) CAR internalization Differences in tonic signaling Memory phenotype and cell fate

AICD, activation-induced cell death; CAR, chimeric antigen receptor; ICOS, inducible T cell co-stimulator; ITAMs, immunoreceptor tyrosine-based activation motifs.