Chimeric antigen receptor (CAR) T-cell therapy has been clinically proven to efficiently combat haematological malignancies. However, continuous efforts are required to increase the specificity of CAR T-cells against tumour versus normal tissues, and are essential to improve their antitumour activity in solid tumours. This review summarises the structure of major CAR designs, and strategies to overcome immunosuppressive tumour microenvironment, and reduce toxicities. Along with reviewing currently available techniques that allow the elimination of CAR T-cells after they fulfil their desired functions, using suicide genes, drug elimination strategies are also introduced. A better understanding of the strengths and pitfalls of CAR T-cell therapy will provide fundamental knowledge for the improvement of engineered T-cell therapy in the near future.

Versatile CAR T-cells for cancer immunotherapy

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Introduction

Chimeric antigen receptor (CAR) is one of the fast-developing techniques in immunotherapy, which directs CAR-expressing immune cells (usually T and natural killer cells) to recognise specific target antigens expressed by tumour cells [1–4]. The basic construct of CAR comprises of a signal peptide sequence, the binding moiety (i.e., single chain variable fragment, scFV), and a spacer region at the extracellular domain, a transmembrane domain, and an intracellular signalling domain (i.e., CD3ζ). The first-generation CAR is mainly the fusion of scFv with a CD3ζ cytoplasmic domain. The second- and third- generation CARs incorporate one or more costimulatory domains other than CD3ζ domain, such as CD28, 4-1BB or both, which contributes to prolonged T-cells activation and expansion [5–11]. The constructs of the first-, second- and third- generation CARs are listed in Fig. 1A. Engagement with antigen via scFV leads to the activation of CD3ζ-mediated activation signals and CD28, 4-1BB-mediated costimulatory cascades, which subsequently induce cytotoxic activity of the engineered immune cells.

Adoptive T-cell therapy was first employed in 1988 by Rosenberg *et al*. using *ex vivo* expanded tumour-infiltrating T-cells (TILs) in melanoma patients [12]. Other researchers also used T-cells expanded from peripheral blood mononuclear cells in tumour immunotherapy [13, 14]. Adoptive transfer of T-cells engineered with scFV specifically targeting CD19 (named as CD19-CAR) made great success in anti-B-cell malignancy [15, 16]. CD19-CAR T-cell therapy was recently approved by the Food and Drug Administration (FDA) for immunotherapy in relapsed and refractory (r/r) B-cell acute lymphoblastic leukaemia (ALL) for paediatric and young adult patients, and for adult patients with r/r diffuse large B-cell lymphoma (DLBCL), who have failed two or more prior therapies. The high response rates in patients treated by CD19-CAR T-cells led to great efforts by researchers and oncologists to design novel CAR constructs based on current available T-cell engineering strategies along with the recently generally used CRISPR/Cas9 method. This review summarises the current progression in CAR T-cell design and its associated T-cell engineering strategies, and discusses the potential of next generation CAR T-cells for haematological tumours to solid tumours.

First generation of CAR

Kuwana and Eshaar first demonstrated that synthetic CARs, fusion of scFv against hapten with a CD3ζ cytoplasmic domain, can overcome MHC restriction and TCR low affinity, leading to more efficient recognition of tumour targets [1, 6]. This is called the first-generation CARs, which included the zeta-chain of the CD3 complex (CD3ζ) that facilitates TCR signal and activates T-cells with modest toxicity. This kind of CARs lack co-stimulatory signals, which limits the proliferating capabilities of T-cell upon exposure to repeated antigens. T-cells with first-generation CARs are easily arrested at the G-G1 phase of the cell cycle, similar to T-cells activated with anti-CD3 alone *ex vivo* [17].
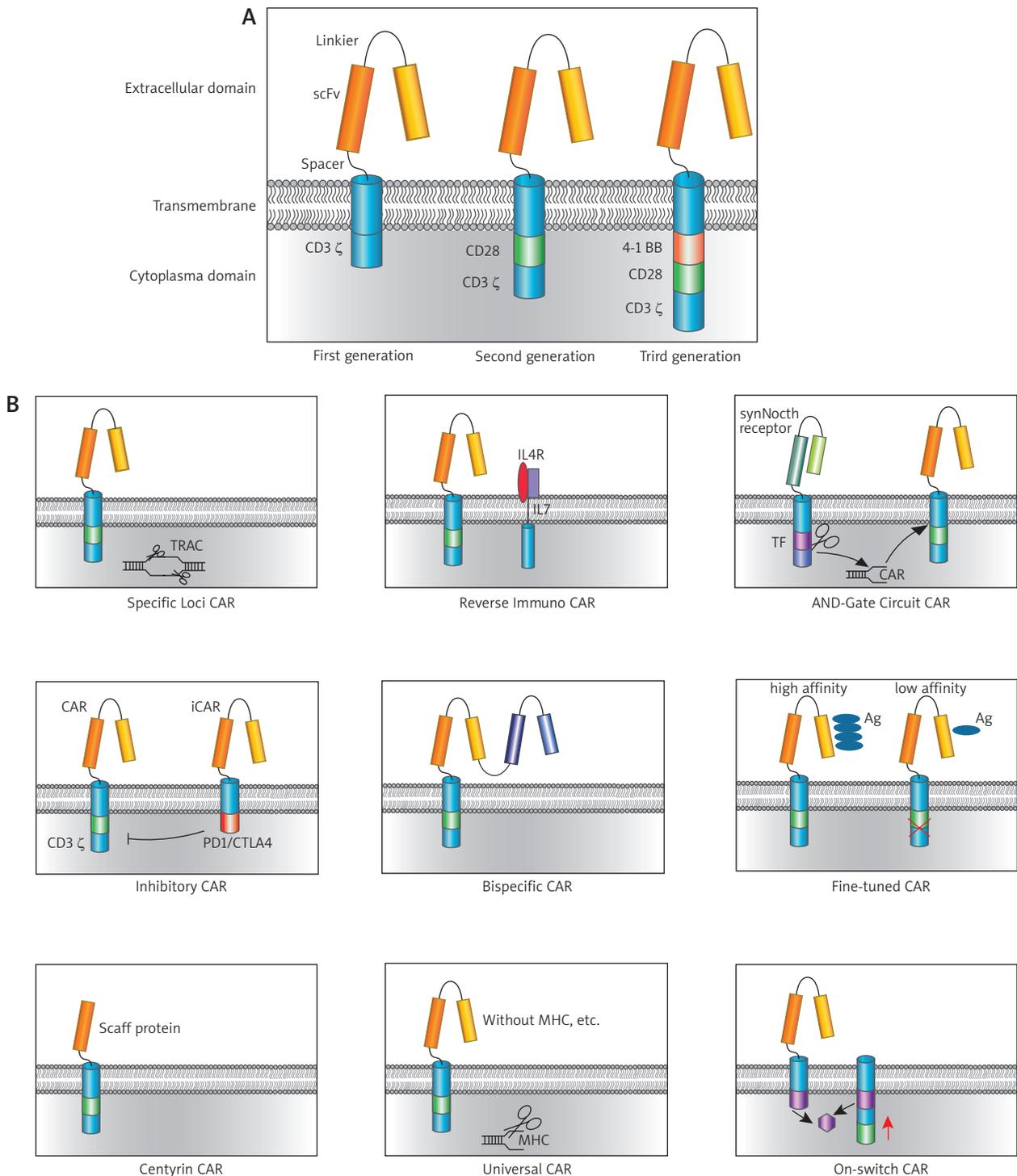
Fig. 1. Chimeric antigen receptor (CAR) structure and design. A) Three generations of CARs. CAR compromises 3 domains: extracellular domain; transmembrane domain; and cytoplasmic domain. First generation CAR only has CD3ζ domain; second-generation CAR incorporates CD28 or 4-1BB, while the third-generation CAR incorporates both or more. B) Primary CARs design and evolution

Second- and third-generation CARs

The introduction of co-stimulatory CD28 or 4-1BB into the signalling domain of CARs led to impressive clinical benefits, especially in B-cell malignancies [8–11]. It is well understood that coupling of CD28 and CD3ζ signals augments TCR signalling, increases cytokine production, promotes proliferation and anti-apoptosis, and affects the epi-genetic structure and metabolism of T-cells [8–10]. These functions are mediated by phosphoinositide 3-kinase (PI3K)-AKT pathway and activated following the phosphorylation of the cytoplasmic tails of CD3ζ and CD28. Thus, incorporation of CD28 signals into the second-generation CAR promotes the proliferation and persistence of engineered CAR T-cells *in vivo* [8–10, 18, 19].

Table 1. List of CAR targets in haematological and solid tumor

I. Haematological tumor CAR targets		
Target	**Gene**	**Description**
BCMA	TNFRSF17	TNF receptor superfamily member 17\B-cell maturation protein
CD123	IL3RA	interleukin 3 receptor subunit α\CD123 antigen
CD138	SDC1	syndecan 1
CD19	CD19	CD19 molecule
CD20	MS4A1	membrane spanning 4-domains A1
CD22	CD22	SIGLEC2
CD38	CD38	CD38 molecule
CD5	CD5	lymphocyte antigen T1/Leu-1
Ig κ chain	IgK	immunoglobulin κ locus
LeY	FUT3	fucosyltransferase 3 (Lewis Blood Group)
NKG2D ligand	NKG2D	killer cell lectin like receptor K1/CD314
ROR1	ROR1	receptor tyrosine kinase like orphan receptor 1
WT1	WT1	Wilms' tumour antigen 1
II. Solid tumour CAR targets		
Target	**Gene**	**Description**
C-Met	MET	MET proto-oncogene
CAIX	CA9	carbonic anhydrase 9
CD133	PROM1	prominin 1
CD171	L1CAM	L1 cell adhesion molecule
CD70	CD70	tumour necrosis factor ligand superfamily member 7
CEA	CEACAM5	carcinoembryonic antigen related cell adhesion molecule 5
EGFR	EGFR	epidermal growth factor receptor
EGFR vIII	EGFRVIII	epidermal growth factor receptor variant 3
Ep-CAM	EPCAM	epithelial cell adhesion molecule
EphA2	EPHA2	EPH receptor A2
FAP	FAP	fibroblast activation protein α
GD2		disialoganglioside
GPC3	GPC3	glypican 3
HER2	ERBB2	Erb-B2 receptor tyrosine kinase 2
HPV16-E6	HPV E6	human papillomavirus E6 protein
IL13Ra2	IL13RA2	interleukin 13 receptor subunit α2
LeY	FUT3	fucosyltransferase 3 (Lewis Blood Group)
MAGEA3	MAGEA3	MAGE family member A3
MAGEA4	MAGEA4	MAGE family member A4
MART1	MLANA	melan-A
Mesothlin	MSLN	mesothelin
MUC1	MUC1	mucin 1, cell surface associated
MUC16	MUC16	mucin 16, cell surface associated
NY-ESO-1	CTAG1B	cancer/testis antigen 1B
PD-L1	CD274	CD274 molecule
PSCA	PSCA	prostate stem cell antigen
PSMA	FOLH1	folate hydrolase 1
ROR1	ROR1	receptor tyrosine kinase like orphan receptor 1
VEGFR2	KDR	kinase insert domain receptor/vascular endothelial growth factor receptor 2

Another strategy is the addition of 4-1BB into the second-generation CAR. 4-1BB can be induced transiently by TCR and CD28 signalling via ERK and JNK signalling pathways. Researchers have found that 4-1BB supports T-cell survival, with effects more evident and durable in CD8+ T-cells than CD4+ T-cells [20–22]. The most important function of 4-1BB signalling is to facilitate memory T-cell formation and robust expansion upon antigen re-stimulation [23, 24]. When comparing CD28 and 4-1BB CARs, Sadelain *et al.* found that both two second-generation CARs demonstrated similar anti-tumour effect, while 4-1BB CARs persisted longer *in vivo* even after the tumour had been eradicated [7, 19, 25].

In addition to CD28 and 4-1BB, other co-stimulatory molecules were also introduced into CARs signalling domains, including CD27, OX-40, ICOS, CD40L, CD137, LAP10, etc. [19, 26–28]. When more than two co-stimulatory signalling are incorporated in addition to CD3ζ in CARs, they are named the third-generation CARs. The effects of different constructs are still under investigation due to limited data. The paragraphs below will introduce different designs CARs currently under investigation (Fig. 1B).

Target CARs to specific *loci*

Current CARs vectors are delivered into T-cells via mammalian plasmid transfection, mRNA transfection, viral transduction, or transposon/transposase [29–36]. The random integration of CAR into the host genome may cause severe harmful results, such as clonal expansion, oncogenic transformation, unpredicted transgene expression, and gene silencing. To avoid the above-mentioned uncontrolled events, Sadelain *et al.* used the CRISPR/Cas9 method to guide the integration of CD19-CAR into a specific TCRα constant (TRAC) *locus* [37]. In a mouse model of acute lymphoblastic leukaemia, they showed that the TRAC-CAR T-cells induced greater responses and more prolonged survival than randomly transduced CARs. In addition, T-cells with engineered TRAC-CAR presented more memory characteristics and less exhausted phenotype. The locus-targeted CAR demonstrated optimal expression of CARs on the T-cell surface. The TRAC-CAR dynamic expression seems to be regulated by the TCR enhancer/promotor in response to repeated stimulation by antigen, mimicking a natural procedure of TCR regulation [37]. This study is an example of a successful T-cell genome editing by CRISPR/Cas9. However, similar strategies have not been extrapolated in clinical settings and the associated side effects are unclear.

Reverse immunosuppressive CAR

Tumours can employ an immune suppressive microenvironment to evade host immune cells cytotoxicity. Tumours and their surrounding matrix produce inhibitory cytokines, including interleukin-4 (IL-4), IL-10, TGF-β, and leukaemia inhibiting factor (LIF), which promote tumour growth and protect the tumour from immune destruction [38, 39]. One of the obstructs that limit CARs function in solid tumour is the inhibitory tumour microenvironment. In order to reverse the suppressive situation, Mohammed, *et al.* re-engineered their prostate stem cell antigen (PSCA-

) CAR by expression of IL-4 receptor on the T-cell surface with cytoplasmic domain replaced by IL-7 (4/7 ICR T-cells). In the presence of IL-4 and OKT3 (both of which mimic tumour inhibitory factors and tumour antigen), they found that CAR T-cells not only proliferated but also expanded in the absence of IL-2. In an *in vivo* study, the 4/7 ICR PSCA-CAR T-cells showed more effective memory features, superior antitumour activity, and increased expansion, compared to CAR without 4/7 ICR. Thus, the inhibitory effects of tumour derived IL-4 inverted into T-cell proliferation and enhanced the PSCA-CARs antitumour activity [40]. Another advantage of this strategy is to limit the off-target effects of CAR T-cells to tumour antigens expressed at low level in normal tissues that are not enriched with inhibitory factors (IL-4). These results suggest that a combination of costimulatory molecules with anti-inhibitory signals in the CAR-signalling domain may further improve the clinical benefits of CAR T-cell therapy. However, this strategy does not work in second-generation CARs with CD3ζ and CD28 due to unidentified mechanisms.

Pressor *et al.* replaced PD-1 signalling domain with CD28 counterpart on CTLs; when PD-L1 on tumour cells engaged with the altered PD-1 on CTLs, enhanced CTL cytotoxicity was confirmed [41]. In a similar vein, the PD1/CD28 signalling domain swap has successfully transferred to CAR engineering, and PD1/CD28 PSCA-CARs outperformed the conventional PSCA-CAR alone or PD1 antibodies [42]. Several groups also reported enhanced cytotoxicity by the depletion or knockdown of PD1 in CAR T-cells in solid tumour models [43, 44]. Therefore, transgenic expression of two factors may increase CARs specificity, and invert more inhibitory factors simultaneously in solid tumours.

Combinational antigen recognition: dual CAR AND-Gate

Determination of tumour-specific surface antigens is the most critical step for CAR specificity. Very rare tumour surface antigens express exclusively in tumour tissues; and even tiny amounts of some "so-called" tumour-specific antigens in normal tissues or organs will cause severe side effects or even lethal results upon infusion of large quantities of CARs T-cells. Distinct strategies have been used to develop CARs, referred to as dual CAR AND-gate, that simultaneously recognises two or more tumour-specific antigens [45, 46]. This type of CAR T-cells mediates more specific killing of target cells that bear both antigens, with low efficacy in tissues that express either antigen alone, thus reducing the undesirable side effects. A more recent strategy in the dual CAR AND-gate used a synthetic Notch (synNotch) receptor carrying the binding moiety for one antigen, whose activation can subsequently induce the expression of a second CAR. Upon engagement with the first antigen, the synNotch receptor automatically clips its cytoplasmic domain and gains transcription factor function. This process in turn induces the second CAR expression in 4 hours [47]. The method showed high efficiency to clear the tumour cells expressing both antigens, and left the normal tissues unattacked. Such methods open the possibility that tumours could be targeted based on multiple antigens. Furthermore,

synNotch receptor could also be used to induce effective molecules and downstream genes to tumours sites [47–50].

Inhibitory CARs (iCAR): brake of CARs

Inhibitory CAR (iCAR) is normally delivered together with conventional CAR into T-cells. iCAR recognises a distinct antigen from conventional CAR, and its scFV is fused to PD-1 or CTLA-4 cytoplasmic domain. When the targeT-cells express CAR-specific antigen, CARs are activated and execute cytotoxicity; when targeT-cells express both antigens, iCARs are activated and transmit negative signals that dampens the function of the CARs [51]. iCAR is used to reduce the bystander killing by targeting proteins that are expressed on healthy tissues, but reduced in tumours.

Bispecific CARs

Tumour antigens often evolve during tumourigenesis due to somatic mutations and epigenetic modifications. For example, patients treated with CD19-CARs relapse due to CD19 loss. Therefore, another tumour antigen is required to enhance CARs efficacy. A number of researchers designed a bispecific CARs that fused two scFv, which can recognise dual antigens. CD19-CD20-CAR T-cells were able to control both wild-type B-cell lymphoma and CD19-mutants with equal efficiency *in vivo* [52]. Another group constructed a CD19-HER2-CAR, which simultaneously targets the CD19 and the human epidermal growth factor receptor 2 (HER2). The efficacy of CD19-HER2-CAR was determined in a mouse model of B-cell lymphoma, which induces distinct T-cell reactivity against each antigen, and synergistic enhancement of effector functions when both antigens were presented [53]. The bispecific CARs help to overcome the inefficiency of adoptive cell therapy due to the loss of tumour antigens.

Affinity-tuned CARs

Based on antibody-antigen affinity, one can screen antibodies that discriminate antigens expressed at different density in normal tissue versus tumour. The CAR with low-affinity scFv selectively targets cells overexpressing EGFR, but shows no effects on cells with lower levels of EGFR [54]. Through such a strategy, the application of affinity-tuned scFvs in CARs offers a wider range of choices against antigens that are physiologically present in normal tissues but are remarkably upregulated in tumours.

Synthetic binding proteins CARs

Over the last two decades, great progress has been achieved in technologies involving synthetic binding proteins. Several platforms have been reported in generating proteins to diverse targets with high affinity and specificity, which are superior to antibodies in terms of smaller size and freedom from disulphide bond formation. Centyrin is one type of scaffold molecules being engineered to bind to target proteins with an interface of similar size to those used by antibodies. Janssen Biotech has developed a number of Centyrin libraries that are used for in vitro selection of Centyrin molecules that bind to targets with high affinity and specificity [55]. Recently, Janssen Biotech

has authorised Poseida to develop Centyrin-based CARs, a B-cell maturation antigen (BCMA)-specific Centyrin has been developed to replace scFv part in the CAR construct. So far, little information about Centyrin CARs has been disclosed publicly, but our laboratory cooperating with Poseida has found that BCMA-specific Centyrin-based CAR treat has demonstrated similar effects to conventional CAR in treating multiple myeloma. In addition to Centyrin, there are also other well-established synthetic binding protein platforms, such as Monobodies, Anticalin, Affibody, and DARPin [56]. They all have the potential to be applied in biomedical fields that traditionally use antibodies.

Universal CARs

Conventional CARs are engineered and generated from autologous T-cells, and they are relatively safe in comparison to allogeneic cell transplantation, which commonly induce graft-versus-host-disease (GVHD). On the other hand, adoptively transferred T-cells also express major histocompatibility complex (MHC) class I, which can be attacked by allogeneic host T-cells to mount host-versus-graft disease (HVGD) [57]. However, the generation of autologous CAR T-cells is time-consuming and expensive, and depends heavily on the availability of patient-derived T-cells. To circumvent this problem, researchers explored ways to eliminate TCRs and MHC-related molecules on CAR T-cells, which can help to minimise GVHD and HVGH when infused into allogeneic recipients. Based on this idea, Cooper *et al*. knocked out TCR α/β chains by Sleeping Beauty transposon/transposase system. As expected, the allogeneic TCR-CD19-CAR T-cells are deficient in TCR signalling, but retained the capability to respond to CD19-expressing tumour [58]. Later, they completely disrupted HLA-I molecules in CAR T-cells and human embryonic stem cells by zinc finger nucleases assay, which allows the application of allogeneic CAR T-cells from a single donor into multiple recipients [29]. The CRISPR/Cas9 system greatly facilitated T-cell engineering as a simple and highly efficient way for multi genomic *loci* editing [59, 60]. Recent studies reported that CRISPR/Cas9 can be used to eliminate TCR, β-2 m, and PD-1 triple *loci* simultaneously in CAR T-cells [61], which demonstrated significantly reduced allogeneic reactivity and enhanced anti-tumour function [62].

Side effects of CAR T-cell therapy

Due to the limited data for other CARs, we will focus our discussion on CD19-CARs in this section. The major side effects of CD10-CARs in the clinic include cytokine release syndrome (CRS), neurological toxicity, and anaphylaxis. Toxicity control has become a critical step in CAR T-cell therapy.

Cytokine release syndrome (CRS): Corticosteroids could rapidly reverse CRS without compromising the desired antitumour effects. However, prolonged exposure to high-dose corticosteroids will result in severe side effects. IL-6 receptor-neutralising antibody, tocilizumab, has also been approved by FDA to reduce CRS [15, 63]. In addition, one dose of methylprednisolone is used to directly block

T-cell activation and stop CAR T-cell therapy, when tocili-zumab fails to control CRS [63].

Neurologic symptoms: The causative pathophysiology of neurologic side effects remains unknown. It is hypothesised that elevated cytokines leads to neurological toxicity. No direct CAR T-cell-mediated toxicity has been observed in the central nervous system. In most cases, the neurological side effects are often self-limiting without treatment [64].

B-cell aplasia: CD19-CAR T-cells kill not only tumour cells expressing CD19, but also normal $CD19^+$ B cells. Therefore, patients who receive CD19 CAR T-cells therapy will develop B-cell aplasia, leading to hypogammaglobulinaemia. To prevent secondary infections caused by B-cell aplasia, IV immunoglobulin infusion is administered monthly to patients as long as aplasia persists [65].

Immunogenicity-related toxicity is another issue that requires attention. A case report showed that human anti-mouse IgG antibody (target mouse original scFV) responses were elicited following infusion of CAR T-cells [30]. It was the same group that found that CAR T-cell infusion can also induce anaphylaxis and cardiac arrest, which is probably due to IgE antibodies specific to the CAR [32].

Control systems in CARs

Despite of the above-mentioned strategies to control CAR-associated over activation, selective depletion of engineered CARs is an alternative strategy under massive investigation.

Suicide genes: To quickly remove infused CAR T-cells to avoid T-cells over activation, suicide genes are introduced in the construct of CARs. Fas and human inducible caspase 9 (iCasp9) are two suicide genes that have been investigated, which can be triggered by small molecules to cause T-cell apoptosis. Previous studies demonstrated an iCasp9 dimerising agent (AP1903) could eliminate 90% of iCasp9-engineered T-cells within 30 minutes following administration of a single dose [66-68].

Elimination genes: Another strategy to eliminate T-cells is to engineer a targetable moiety in genetically modified T-cells. For example, CAR T-cells can be enforced to overexpress CD20 and which can be targeted by rituximab. The CD20-expressing CAR T-cells will be eliminated through antibody-dependent T-cellular cytotoxicity (ADCC), upon infusion of rituximab. Such a strategy is also used by EGFR, which can be targeted by cetuximab, an FDA-approved mAb. Marin *et al.* reported that iCasp9 and CD20 are the most efficient controlling molecules that can induce rapid cell-death with sound safety and superior efficacy [69, 70].

ON-switch CAR

ON-switch CAR comprises two physically separate constructs, one containing the conventional CAR but with the CD3ζ activation domain is replaced by an inducible binding domain, and the other one contains the CD3ζ domain but lacks the antigen recognition scFC portion. The activity of ON-switch CAR is triggered by orally-administered small molecules on a dose-dependent manner. When a small mol-ecule is administered, the split constructs form heterodimer, which subsequently activates T-cells [71]. ON-switch CAR yields antigen-specific and titratable killing of target T-cells depending on the dose of small molecules. Such a strategy can allow physicians to control CAR responses feasibly.

Challenges in solid tumour

CAR T-cells therapy has demonstrated remarkable success in haematological malignancies, but has limited effects in solid tumours. First, identification of antigens uniquely expressed in solid tumours remains as the critical challenge. Ideal tumour antigens should be expressed exclusively in tumours but not in normal tissues, or at least much more robust in tumour than in normal tissues. So far, nearly 30 antigens for solid tumours are currently under investigation as targets for CAR T-cell therapy [19, 28, 48, 72]. Second, tumour microenvironment in solids presents more complex suppressive factors than haematological malignancies, which impairs the trafficking and infiltration of CAR T-cells through mechanisms involving oxidative stress, nutrient starvation, low pH, and hypoxia [38, 73]. Third, inhibitory soluble proteins, including adenosine, Prostaglandin E2 (PEG2), TGF- , and IL-10, secreted by tumour and tumour stromal cells suppress the function of CAR T-cells [38, 73]. Fourth, suppressive immune cells within solid tumour microenvironment, including Tregs, myeloid-derived suppressor cells, and tumour-associated macrophages/neutrophils are known to present as a barrier against CAR T-cell function. Thus, CAR T-cell design should combine strategies to overcome these negative factors, or should use strategies in combination with immune checkpoint blockade or other approaches [19, 26, 38, 48, 73, 74].

Perspectives

The CD19 CAR T-cell therapy is a revolutionary treatment in haematological malignancies [16, 75–77], as shown by the unprecedented response rate. The success of CAR T-cells in clinics has inspired tremendous interests of physicians, translational researchers, as well as industries to identify new targets for tumour immunotherapy. A range of versatile CAR constructs targeting a variety of novel tumour antigens have been reported and some of them are tested in phase I/II clinical trials. However, more optimised designs of CARs are required to reduced toxicity, multiply targeting, and increase cost-benefit efficiency, etc. With advanced technologies, such as CRISPR/Cas9, universal CAR T-cells with improved efficacy could be developed and applied in the near future.

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The authors declare no conflict of interest.

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