Expert Reviews in Molecular Medicine

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Chimeric antigen receptor engineered T cells and their application in the immunotherapy of solid tumours

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Review

Cite this article: Mao R, Hussein MS, He Y (2022). Chimeric antigen receptor engineered T cells and their application in the immunotherapy of solid tumours. *Expert Reviews in Molecular Medicine* **24**, e7, 1–15. https://doi.org/10.1017/erm.2021.32

Received: 1 September 2021
Revised: 19 November 2021
Accepted: 20 December 2021

Keywords:
Adoptive cell transfer; chimeric antigen receptor; T cell engineering; tumour immunotherapy

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Abstract

In this article, we reviewed the current literature studies and our understanding of the parameters that affect the chimeric antigen receptor T cells (CAR-T's) activation, effector function, *in vivo* persistence, and antitumour effects. These factors include T cell subsets and their differentiation stages, the components of chimeric antigen receptors (CAR) design, the expression promoters and delivery vectors, and the CAR-T production process. The CAR signalling and CAR-T activation were also studied in comparison to TCR. The last section of the review gave special consideration of CAR design for solid tumours, focusing on strategies to improve CAR-T tumour infiltration and survival in the hostile tumour microenvironment. With several hundred clinical trials undergoing worldwide, the pace of CAR-T immunotherapy moves from bench to bedside is unprecedented. We hope that the article will provide readers a clear and comprehensive view of this rapidly evolving field and will help scientists and physician to design effective CAR-Ts immunotherapy for solid tumours.

1. Introduction

Over the last decade, immunotherapies with checkpoint blockades and adoptive cell transfer (ACT) have made remarkable progress in cancer treatment. The success of immunotherapy requires tumour-specific T cells (Ref. 1), which do not exist in most tumours. Engineering patient's T cells with T cell receptors (TCR) (Refs 2, 3) or chimeric antigen receptors (CAR) (Ref. 4) can provide tumour-specific T cells. ACT of the 'engineered' T cells (TCR-T and CAR-T) is a rapidly evolving research field and represents a promising approach of cancer immunotherapy (Refs 5, 6). CARs target surface antigen and do not have MHC restriction. Additionally, the antibody usually has a 1–3 log higher affinity than TCR. While TCR-T is still in the stage of trials,(Ref 7) CAR-Ts are approved for haematological malignancies (Table 1). In this review, we only focused on CAR-Ts. Readers are referred to two excellent recent reviews on TCR-Ts (Refs 7, 8). The complete control of blood cancers by CAR-Ts marks a new era in cancer treatment (Refs 6, 9, 10). There are ~253 ongoing CAR-T trials for different cancers (Ref. 11). CAR molecule is composed of the antigen-binding domain (ABD), a flexible hinge (H), transmembrane (TM), and intracellular signalling domain (SD) that include 1–2 co-stimulatory domain (CD) and CD3ζ (Fig. 1). The ABD and SD are from different sources and form into one artificial receptor (chimaera) that recognise tumour surface antigen independent of MHC. In this article, we will review the factors that affect the function and antitumour efficacy of CAR-Ts. We will cover the T cell subset selection, CAR design, delivery, expression, and the process of CAR-T production. We hope this review will help us to understand the role of each component in the CARs and to design effective CAR-Ts to generate potent and durable antitumour effects, especially for solid tumours.

1.1 T Cells

Although allogenic CAR-Ts can be pre-made as 'off-the-shelf' with better standardisation, the graft-vs-host diseases is a big hurdle for their success (Refs 12, 13). Currently, autologous T cells are the main source for CAR-T generation. The CAR-Ts approved by USA, Spain and China are generated from autologous T cells. The subset of T cells and their differentiation can affect CAR-T's function and antitumour effects.

1.1.1 Subsets

CD4 and CD8 T cells are important in antitumour immunity (Refs 14, 15) and used to generate CAR-Ts, alone or in their unselected composition (Refs 16–19). The cytotoxicity of CD8 CAR-Ts can be higher (Ref. 19), equal (Ref. 16) or lower (Ref. 17) than CD4 counterparts. Although more IFNγ–producing CD8 CAR-Ts were observed upon antigen stimulation (Refs 17, 19), CD4 CAR-Ts produced a higher amount of IFNγ, TNFα and IL-2 (Refs 16, 19). The CD4 CAR-T outperformance was also observed in the glioblastoma-targeted IL13Ra2-specific (IL13-41BBζ) CAR-Ts (Ref. 17), possibly because CD4 cells are less prone to exhaustion than CD8 (Refs 17, 20). The composition of blood CD4 and CD8 cells vary

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Table 1. CAR-Ts approved for treating haematological cancers

Trade Name	KYMRIAH	YESCARTA	TECARTUS	BREYANZI	ABECMA	ARI-0001	Relma-cel
proper name	tisagenlecleucel	axicabtagene ciloleucel	brexucabtagene autoleucel	lisocabtagene maraleucel	idecabtagene vicleucel		Relmacabtagene Autoleucel
T cell sources	autologous T cells	autologous T cells	autologous T cells	autologous CD4+,CD8+	autologous T cell	autologous T cell	autologous CD4+ and CD8+
Year approved	30 August 2017	18 October 2017	23 July 2020	5 February 2021	26 March 2021	10 February 2021	6 September 2021
Approved by	FDA	FDA	FDA	FDA	FDA	AEMPS	NMPA
ScFv (Ab)	FMC63	FMC63	FMC63	FMC63	Murine	A3B1 murine	FMC63
Linker Virus	(GGGGS)3 Lentivirus	(GGGGS)3 Retrovirus	(GGGGS)3 Retrovirus	Lentivirus	Lentivirus	Lentivirus	Lentivirus
Promoter	EF1a	MSCV	MSCV	?	MND	EF1a	
Target	CD19	CD19	CD19	CD19	BCMA	CD19	CD19
Disease	(R/R) B-ALL, DLBCL, etc.	DLBCL, etc.	(R/R) MCL	(R/R) LBCL, DLBCL, etc.	(R/R) multiple myeloma	(R/R) ALL	(R/R) LBCL
H/T domain	CD8a	CD28	CD28	IgG4 (H), CD28 (TM)	CD8a	CD8a	
Signalling domain	4-1BB, CD3ζ	CD28, CD3ζ	CD28, CD3ζ	4-1BB, CD3ζ	4-1BB, CD3ζ	4-1BB, CD3ζ	4-1BB, CD3ζ
Other factor Generation	2nd	2^{nd}	T cell enrichment 2nd	EGFRt 2nd	2nd	2nd	EGFRt 2nd
Company	Novartis	Kite Pharma	Kite Pharma	Juno Therapeutics	BMS	Hospital clinic (spain)	JW Therapeutics

FDA, U.S. Food and Drug Administration; AEMPS, The Spanish Agency of Medicines and Medical Devices; NMPA, the National Medical Products Administration of China.

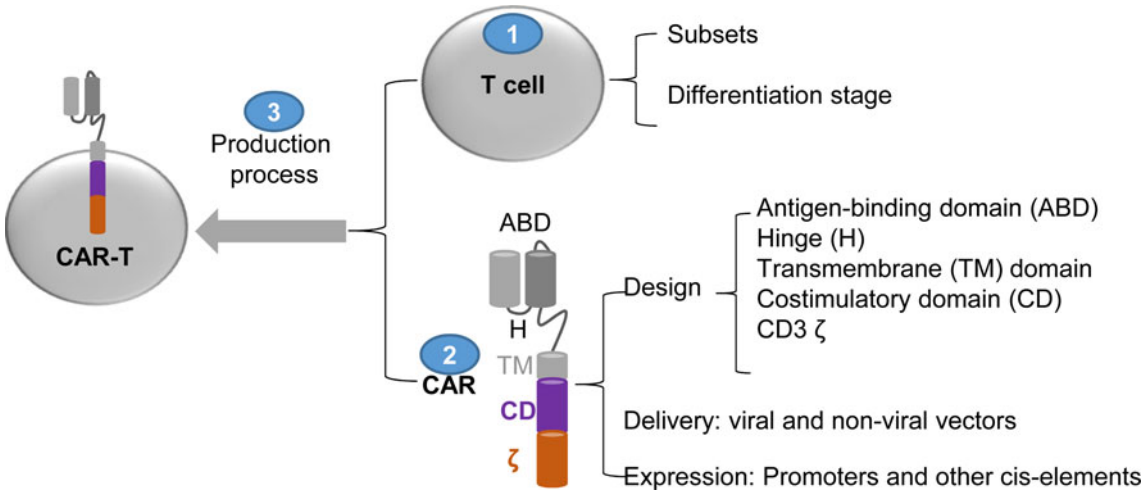


Figure 1. The parameters that affect the function and antitumour effects of CAR-T cells.

in their activation and differentiation state, especially for cancer patients receiving chemotherapy, which can affect the therapeutic effect and toxicity of CAR-Ts (Refs 18, 21). The antitumour effects of CD4 and CD8 CAR-Ts may vary due to their origin, CAR-T design and tumour types, but CD8 and CD4 CAR-Ts have synergistic antitumour activity (Refs 18, 19). The unselected T cells with both CD4 and CD8 are frequently used to generate CAR-Ts. In this approach, CD8 CAR-Ts can undergo rapid activation and provide a prompt response against the tumour, whereas CD4 CAR-Ts persist longer and generate a durable antitumour effect. Together, they work more effectively than their own. Though CAR-Ts are usually generated ex vivo, a recent study reported in vivo selective transduction CD8 and CD4 cells by CAR genes. Using lentivirus with either CD8α- (CD8-LV) or CD4-specific DARPin (designed ankyrin repeat protein) (CD4-LV) as entry receptor, Agarwal et al. selectively delivered CAR gene into CD8 or CD4 T cells in NSG mice (Ref. 20). CD4-LV exhibited superior antitumour effects compared to CD8-LV alone or even in their combination.

1.1.2 Differentiation stages

T cells undergo differentiation from naïve (T_N) to stem-like memory (T_{SCM}), central memory (T_{CM}), effector memory (T_{EM}), and effectors (T_E) (Ref. 22). In a mouse study, ACT of the activated tumour-specific CD8 cells derived from T_{CM} generated stronger antitumour effects compared to T_{EM} CD8 cells (Ref. 23). Then, they showed that the activated CD8 T cells derived from T_N generated stronger antitumour effects than that from T_{CM} (Ref. 24). In a non-human primate study, the CD8 T cells with T_{CM} phenotype persisted longer *in vivo* (Ref. 25). Similarly, the CAR-T's proliferation and persistency are affected by their differentiation states as well (Ref. 26). CAR-Ts derived from T_N, T_{SCM} and T_{CM} have a greater proliferative potential and longevity (Refs 27, 28). In one study (Ref. 19), investigators found that human CD4/CD8 CD19 CAR-Ts derived from T_N had the highest cytokine secretion, followed by T_{CM} and T_{EM}. The in vivo antitumour efficacy of the CD4 CAR-Ts derived from different differentiation stages follows the same order: $T_N > T_{CM} > T_{EM}$. In contrast, for CD8 CAR-Ts, those derived from T_{CM} generated longer antitumour effects ($T_{CM} > T_N = T_{EM}$). Importantly, a combination of the T_{CM}-derived CD8 CAR-Ts with the T_N-derived CD4 CAR-Ts resulted in complete tumour eradication in treating leukaemia xenografts. Thus, it is generally believed that CAR-Ts generated from defined T cells subsets (T_N and/or T_{CM}) may be more effective.

1.1.3 CD26hi T cells

CD26 is a marker for terminal T_E. However, in contrast to conventional reasoning, one study showed that the CAR-Ts generated from using $CD26^{hi}$ CD4 T cells persisted and regressed solid tumours (Ref. 29). Further study showed that the $CD26^{high}$ CD4 T cells are a distinct cell population and can be used to develop CAR-Ts with enhanced antitumour effects in treating solid tumours (Ref. 30).

2. CAR design

CAR, the other component of CAR-T, has four major domains: ABD, H, TM, and SD (Fig. 1) (Ref. 21). The selection and design of each domain affect CAR-T activation and antitumour effects.

2.1 ABD

The ABD recognises tumour antigen and confers specificity to CAR-Ts.

2.1.1 Targets

An ideal target should have high coverage to recognise all tumour cells, high specificity to prevent toxicity, and stable expression to maintain durable CAR-T recognition (Ref. 31). However, such an 'ideal' target is rare. Instead, shared antigens are frequently targeted. For example, CD19 is on both normal and malignant B cells. Although CD19 CAR-Ts lead to B cell aplasia, this 'on-target/off-tumour' toxicity is tolerable and can be managed by immunoglobulin replacement (Ref. 32) or by CAR-T removal (Ref. 33). Loss of antigen is a common mechanism of CAR-T therapy failure (Refs 34, 35). Even initial response was outstanding in multiple trials, long-term follow-up revealed that a notable proportion of patients received KYMRIAH (Ref. 36) or YESCARTA (Ref. 37) relapsed with CD19-negative leukaemia. Other B-cell restricted surface molecules such as CD20, CD22, and CD123 are detected in patients with CD19-negative relapses (Refs 38, 39). CD19 CAR-T failure due to loss of CD19 was overcome by using another single-targeted (Ref. 40), sequential infusion of 2 single-targeted CAR-Ts (Ref. 41), or dual/multi-targeted (Refs 39, 42) CAR-Ts. **Antigen density** on tumour cells also affects the cytotoxicity and cytokine production of CAR-Ts (Refs 43, 44). Using CD20 CD28ζ CAR-Ts and target cells with various levels of CD20, Watanabe *et al.* demonstrated that the antigen threshold was ~200 and a few thousand molecules per target cell for lytic activity and cytokine production, respectively (Ref. 44).

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Similarly, low expression of targets CD19 (Ref. 43), CD22 (Ref. 45), EGFR (Ref. 46), and anaplastic lymphoma kinase (ALK) (Ref. 47) might negatively affect CAR-T function and persistence. The **epitope location** (membrane-proximal or distal) determines the synapse cleft distance and kinetic segregation of phosphatases like CD45. This will affect the signalling strength and cytotoxic granule delivery (Refs 21, 48). The consensus view is that CAR-Ts targeting membrane-proximal epitopes generate stronger effects. The CAR-Ts targeting the membrane-proximal epitope of mesothelin (Ref. 49) and CD33 (Ref. 50) or membrane-distal epitope of CD22 (Ref. 51) could be modified by regulating the length of the hinge to enhance antitumour function (see section 2.2).

2.1.2 scFv (single-chain variable fragment)

Although other molecules, such as IL13 (Ref. 17), adnectin (Ref. 52), DARPin (Refs 20, 53), and nanobodies (Ref. 54), are used in CAR design, mAb scFv is the most common ABD due to its size, affinity, and specificity (Ref. 55). The scFv consists of variable heavy (VH) and light (VL) chains connected by a flexible linker of 10 to 25AA in the format of VH-linker-VL or VL-linker-VH (Refs 56, 57). The scFv derived from murine mAb (FMC63) is used in 5 approved CD19 CAR-Ts (Table 1). While one report showed that both VH-linker-VL and VL-linker-VH orientations had the similar antigen-binding capacity and CAR-T function (Ref. 58), other found that the antigen recognition could be maintained only in particular VH and VL orientation (Ref. 59). Murine mAbs are used in approved CAR-Ts, they are immunogenic and may induce anti-drug antibodies (ADA) that cause side effects, CAR-T loss, and therapy failure (Ref. 60). Humanised CD19 CAR-Ts (Ref. 61) and fully human anti-CD19 CAR-Ts (Ref. 62) maybe more effective.

2.1.3 Linkers

$(G4S)_3$ and $(G4S)_4$, the 3 and 4 repeats of pentapeptide (Gly–Gly–Gly–Gly–Ser), are the common linkers between VL and VH (Ref. 63). Glycine and serine contribute for flexibility and solubility, which are critical for scFv proper folding and antigen-binding. Recent studies reported that short scFv linker $(G4S)_1$ enhanced 'tonic signalling' in CD22-BBζ and CD33-BBζ CAR-Ts (but not CD19-BBζ CAR-Ts) without causing more exhaustion, but leading to better anti-leukaemic function (Ref. 64). In contrast to this beneficial effect, a previous study showed tonic signalling in anti-GD2 CD28ζCAR-Ts resulted in exhaustion and decreased antitumour activity (Ref. 65), which was ameliorated by replacing CD28 with 41BB. These data suggest that the effect of tonic signalling on CAR-T function depends on multi-factors including target, co-stimulatory domain, and even the length of the linker. Thus, tonic signalling is not always bad for CAR-T function, but needs to be taken into consideration in CAR design (Ref. 66).

2.1.4 Affinity and avidity

The affinity (single pair of molecular interaction) of scFv and the avidity (multiple pairs of molecular interaction) can affect CAR-T's function and therapeutic effect (Refs 67–69). Conventionally, scFvs of high affinity are preferred in CAR-Ts for increased antigen recognition, binding capacity, and tumouricidal effect (Ref. 21). However, CAR-T's avidity beyond a certain threshold would not increase efficacy (Ref. 70) and even exhibited inferior responses (Ref. 71). A recent report showed that a low-avidity CD19 CAR-T enhanced expansion and persistence in treating refractory acute lymphoblastic leukaemia (Ref. 72). Drent *et al.* studied 8 anti-CD38 scFvs covering an affinity of 10–1000 folds (Ref. 73). In the study, CD38 CAR-Ts from scFv with ~1000 folds lower affinity could effectively lyse $CD38^{high}$ multiple myeloma cells but not $CD38^{low}$ healthy haematopoietic cells. Recently, how the biophysical properties of CAR (affinity, avidity, and antigen density) contributed to CAR-T function was further studied using CD28ζ CAR-Ts with varying avidity to the same antigen, HLA-A2/$Tyrosinase_{369–377}$ (Ref. 74). The CAR-T derived from the highest-affinity scFv (4 nM) showed a lower lytic response determined by CD107a, as compared to CAR-Ts from intermediate-affinity scFv (35 and 16 nM). The CAR expression and CAR-T avidity, measured by Tyr/HLA-A2 monomer and multimer binding respectively, correlated with each other in CAR-T function. Increasing avidity resulted in high sensitivity to low antigen concentrations. However, the polyfunctional CAR-Ts (IFNγ, TNFα and IL2 triple-positive) was more in the medium-avidity CAR-Ts. In summary, the intermediate avidity CAR-Ts can reduce the 'on target/off tumour' activity by recognising only antigen-high tumour cells (Ref. 73), but also are more polyfunctional, correlating to better clinical efficacy (Refs 62, 63) (Table 2).

The underlying mechanism how intermediate avidity CAR-Ts generated a strong and durable antitumour effect is unclear. Weber *et al.* reported that *in vivo* persistence and antitumour effects of CAR-Ts could be improved by temporarily suppressing CAR expression (Ref. 75), suggesting that transient disengagement between CAR-T and tumour may avoid exhaustion or to restore their functionality. Recently, we developed a novel 8F8 mAb that recognised a human glypican 3 epitope next to that of the widely used GC33 mAb, but had 17 times lower affinity (Ref. 76). Despite different avidity, 8F8 and GC33 CAR-Ts possess similar *in vitro* function. However, *in vivo* study showed more 8F8-BBζCAR-Ts in the tumour than GC33 CAR-Ts. Importantly, the tumour-infiltrating 8F8 CAR-Ts were less exhausted and better functional than GC33 CAR-Ts, generating durable antitumour effects in treating HCC xenografts. The low-avidity 8F8 CAR-Ts were in a 'lightly exhausted' state ($PD1^{lo}$), whereas high-avidity GC33 CAR-Ts exhibited 'deeply exhausted' ($PD1^{hi}$) (Ref. 77). Compared to high-avidity CAR-Ts, the unstable engagement (Slow on/Fast off) between the low-avidity CAR-Ts and tumour cells may provide a transient break for CAR-Ts and prevent them from exhaustion and apoptosis inside solid tumours.

2.2 Hinge (H)

Hinge is the spacer between scFv to TM. It contributes to the flexibility of scFv and the synaptic distances during CAR-antigen engagement (Refs 21, 78, 79). The optimal hinge length depends on the location of the antigen epitope and steric hindrance on cancer cells. The length of the hinge should provide adequate synaptic cleft distance and enhance epitope binding by scFv (Refs 48, 69). In general, a short hinge is more suitable for CAR targeting membrane-distal epitopes (Refs 69, 78), whereas a long hinge is flexible and more effective for CAR targeting membrane-proximal epitopes that are often sterically hindered (Ref. 80). Two types of the hinge are in current CARs.

2.2.1 IgG-based hinges

Are from human IgG (IgG1 and IgG4) CH2 and CH3 domains (Ref. 57). The CH2 domain of IgG1 and IgG4 can interact with Fcγ receptors, resulting in 'off-target' toxicities and activation-induced cell death. Modification or deletion of the CH2 domain could minimise this side effect (Refs 21, 57). The IgG4 hinge is in one FDA-approved CAR-Ts (BREYANZI).

2.2.2 CD8α and CD28 hinges

Are naturally without FcγR binding site and used in most FDA-approved CAR-Ts (Refs 21, 57). Alabanza *et al.* demonstrated that CD19 CARs with H/TM from human CD28 or

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Table 2. Summary of 14 completed clinical trials of solid tumour CAR-T therapy: scFv affinity vs clinical efficacy

Target	Target binding Domain	ICD	*In vitro* or Preclinical	Clinical trials
FRα (folate binding protein)	scFv based on MOv18 K_D: ~**0.2 nM** (Ref. 210)	FcεRIγ	Dual specific T cells (allo-TCR and FRα CAR) inhibited tumour growth (Ref. 211)	NCT00019136 No reduction in any of 12 patients (Ref. 212)
GD2	scFv from mAb Hu3F8 (Ref. 213) K_D: **11 nM**	CD28-BB-CD3ζ		NCT02765243 4/10 SD for 1 yr DOI:CSBOLDSTART CSBOLDEND10.21203/rs.3.rs-803629/v1
	scFv from 14.G2a (Ref. 213) K_D: **77 nM**	CD3ζ	EBV-CTL expressing 14.G2a-CD3ζ CAR were expanded and maintained long-term in the presence of EBV-infected B cells (Ref. 214). However, CAR-ATC (activated general T cells) were not expanded by GD2.	NCT00085930: Initial report (Ref. 148) found CAR-CTL (EBV-CTL transduced with CAR) generated better expansion than CAR-ATC, but a later report (Ref. 215) showed that CAR-ATC persists 4 years. Clinical outcome: 3 CR (2 sustained for > 4 years), 1PR, 1SD out of 11 patients.
		CD28-OX40- ζ	14g2a-CD28-OX40-Z Construct Signalling Induces Sustained Clonal Expansion (Ref. 149)	NCT01822652 (Ref. 216). 5/11 SD, among the SD, 2 became CR after salvage treatment. Higher dose plus chemo and PD1 extended survival.
	KM8138 (Ref. 217) K_D: **149 nM**	CD28- ζ		NCT02761915 (Ref. 218): No response in 12 patients, but some response in bone marrow disease for 1 patient.
CD171 (L1-CAM)	CE7 scFv K_D: 0.01 nM **(patent US7354762)**	CD3ζ		NCT00006480 (Ref. 219): 1 PR/ 6 patients but only for 56 days. CAR-Ts disappears in a week in high tumour burden and 42 days in limited tumour burden.
CEA	scFv based on hMN14 K_D: **3.4 nM** (Ref. 220)	CD28-ζ		NCT01373047: Hepatic artery injection of CAR-Ts with (3) or without (3) IL2 support. 1 SD/6 (Ref. 221)
	scFv based on MFE23 K_D: 2.3 nM (Ref. 222)	CD3ζ		NCT01212887 (Ref. 223). No clinical responses, Low persistence of CAR-Ts
Claudin 18.2	hu8E5 scFv (Ref. 224) EC50: 45 nM	CD28-ζ		NCT03159819 (Ref. 225). 1CR, 3PR, 5SD/12
C-Met	scFvof Onartuzumab K_D: **1.2 nM** (Ref. 226)	4-1BB- CD3ζ		NCT01837602 (Ref. 227). Intratumoural injection of mRNA transduced CAR-Ts, no clinical effects
EGFR	scFv based on E10. K_D: 20 nM (in nanobody) (Ref. 228)	4-1BB- CD3ζ		NCT01869166 (Refs 229, 230). 1 CR, 2 PR out of 28 patients
EGFRvIII	scFv based on 3C10-2173 K_D of 2173: 101 nM (Ref. 231)	4-1BB- CD3ζ	Weak antitumour effects in NSG mice (Ref. 231)	NCT02209376 (Ref. 232). **No significant clinical effect**
GPC3	scFv of GC33 K_D (by us): 1.38 nM	CD28- ζ		2 PR out of 13 patients (Ref. 233). One patient survive more than 2 years.
PMSA	scFv based on 3D8 K_D: 22.5 nM (Ref. 234)	CD3ζ		NCT01929239. 2 PR effect but only last 1–2 months (Ref. 235)
ROR1	scFv based on UC-961 K_D: 2 nM (Ref. 236)	4-1BB- CD3ζ		NCT02706392 (Ref. 237). Limited mix responses in 2 /5

CD8α had similar anti-tumours in mice (Ref. 81). However, CARs with CD8α-H/TM may have a clinical advantage due to lower cytokine production. On the other hand, Majzner *et al*. reported that CD28-H/TM had a more stable and efficient immunological synapse that could enhance CD19 CAR-Ts to recognise low antigen (Ref. 43). In addition, CAR-T with CD28-H showed stronger lytic activity although the CAR level of CD8α-H and CD28-H are similar. CAR-Ts with H/TM from CD8α or CD28 had higher antigen-specific cytotoxicity compared to the basic CD3ζ CAR-T, indicating that TM is another crucial factor to affect CAR-T function. However, it was still difficult to identify which domain (H or TM) contributed to the different CAR-T function as the H and TM were changed simultaneously. In addition, the H length affects CAR-T function. In a recent study, six anti-VEGFR2 CARs were generated in which the H/TM from CD4, CD8α, and CD28 was exchanged individually in the basic

https://doi.org/10.1017/erm.2021.32 Published online by Cambridge University Press

CD3ζ CAR (Ref. 82). The data showed that longer H length (CD8α:65AA and CD28:36AA) had a higher expression that CAR with shorter H (CD4:23AA and CD3ζ:9AA). In another study (Ref. 83), a series of anti-CD19 CARs were generated based on CD19 CD8α-H/T BBζ prototype (71aa) (Kymriah) by altering H and intracellular domain, but not TM. The CD19-BBζ CAR (86aa) variant contains 86aa from CD8α with a longer extracellular fragment (55aa *versus* 45aa) and a longer intracellular sequence (7aa *versus* 3aa). These CD19-BBζ (86aa) CAR-Ts had a lower level of expansion and cytokine production than Kymriah, but achieved complete or partial remission with no neurotoxicity or CRS in 15/25 patients. This data further indicates that hinge plays a key role in CAR-T function and safety.

2.3 TM

TM was frequently selected together with upstream H and/or downstream SD. It anchors scFv to the cell membrane and contributes to CAR expression, stability, and CAR-T function (Ref. 82). CD19 CAR with CD28-TM domain was more stable on T cell surface than CD19 CAR with a CD3-ζ TM domain (Ref. 84). However, CD28-TM but not CD8 TM domain in the CAR could form a heterodimer with the endogenous CD28, which may affect CAR-T's function (Ref. 85). The TM can also regulate CAR signalling and CAR-T function by CAR expression level (Ref. 82). H/TM domains from CD8α and CD28 are used in the FDA-approved CAR-Ts KYMRIAH and YESCARTA, respectively. In addition, the inducible co-stimulator (ICOS), including its TM, improved the anti-tumour response and increased persistence of the 3rd generation ICOS-41BBζ CAR-Ts (Ref. 86).

2.4 SD

The chimeric molecule of antibody and TCR was first created in the late 1980s, in which the VL and VH of mAb were linked to TCRα and TCRβ separately (Refs 87, 88). In the following study, scFv was linked to CD3ζ to develop the 1st generation CARs (Ref. 89). The CD3ζ remains an essential component of SD in all following generations of CARs. However, CD3ζ alone did not show significant antitumour effects in vivo (Ref. 90). The addition of 1–2 CD improved CAR-T proliferation, function, persistence, and antitumour effects. The number of CD classify the CARs into 1st (CD3ζ only), 2nd (one CD + CD3ζ), or 3rd (more than one CD + CD3ζ) generation. In general, a CD is placed proximal to the cell membrane, followed by CD3ζ (Ref. 91). Different CD may generate different effects on CAR-T activation, expansion, function, and survival (Refs 92–94).

2.4.1 CD28 and 4-1BB

Are the most frequently used CD in CAR design. All the FDA-approved CAR-Ts use CD28 or 4-1BB CD. In general, CD28 CARs confer greater functionality (Ref. 95) and higher sensitivity (Ref. 43). Using cell lines with different CD19 level, Majzner et al. demonstrated that CD28 CAR outperformed 4-1BB CAR against antigen-low tumours, whereas both CD28 and 4-1BB CARs had a similar effect against tumours with high antigen (Ref. 43). In addition, for low-avidity CAR-Ts, the CD28 domain may enhance their cytotoxicity and cytokine secretion (Ref. 94). However, the higher functionality of CD28 CAR-Ts comes at a cost. They are less persistent in vivo (Ref. 96). In contrast, 4-1BB CAR-Ts have longer persistence with more Tcm (Ref. 97). Metabolically, CD28 enhances glycolysis and 4-1BB increases mitochondrial biogenesis in T cells (Ref. 97). Interestingly, 4-1BBζ CARs can be re-engineered to enhance functional activity against antigen-low tumour and maintain persistence by incorporating 2 copies of CD3ζ or by replacing the CD8α-H/T domain with CD28 H/T domain (Ref. 43). Furthermore, the use of both CD28 and 4-1BB could improve the antitumour effect of CAR-Ts with very-low affinity scFvs (Ref. 94).

2.4.2 Other CDs

From other CD28 and TNFR families were also used in CARs. **(1) ICOS** belongs to the CD28 family. Incorporation of ICOS resulted in Th17 differentiation and enhanced persistence of both CD4 and CD8 CAR-Ts (Ref. 86), probably due to ICOS-induced PI3 K/Akt signalling (Refs 86, 98, 99). The CD4 CAR-Ts with ICOS have a' helper effect' that increase the persistence of CD8 cells expressing either CD28- or 4-1BB–based CARs. Furthermore, the 3rd-generation CAR-Ts using both ICOS and 4-1BB displayed superior antitumour effects and increased persistence in vivo compared to the 2nd-generation with ICOS or 41BB alone (Ref. 86). **(2) OX40** is a member of the TNFR family. Different from CD28 required for T cell activation and IL2 production, OX40 is expressed after activation and needed for ongoing T cell proliferation and cytokine production (Ref. 100). Recently, the antigen-independent effect of various co-stimulatory receptors on T cell proliferation was evaluated by transducing T cells with CD20-BBζCAR with an additional P2A-linked full length of 12 different co-stimulatory receptors (Ref. 101). Interestingly, compared to original CD20-BBζCAR-Ts, expression of OX40 showed the greatest increase in proliferation, followed by CD27-expressing, and GITR-expressing CAR-Ts. In addition, the OX40-expressing CAR-Ts possess enhanced cytotoxicity and reduced apoptosis and exhaustion. Moreover, a co-stimulatory combination of CD28 and OX40 benefited the survival and cytotoxicity of the 3rd-generation CAR-Ts (Refs 102, 103). **(3) CD27** enhanced effector cytokine production and cytotoxicity, and reduced apoptosis with upregulation of Bcl-X_L protein (Ref. 104). In vivo, CD27 signalling enhanced CAR-T's survival compared to CD28, and generated strong antitumour activity similar to CD28ζ and 4-1BBζ CAR-Ts. In a recent study, the T2–27ζ CAR-Ts targeting trophoblast cell surface antigen 2 (T2) was found to exhibit more durable antitumour activity compared to T2–28ζ CAR-Ts via promoting IL7Rα expression to enhance survival and to reduce exhaustion (Ref. 105). On the other hand, Wang et al. proposed that CD27 co-stimulation might display dual-function: the transient CD27–CD70 interaction between $CD27^+$ CAR-Ts and tumour cells enhanced antitumour potency, but constitutively CD27 expression induced CAR-T exhaustion and apoptosis (Ref. 106). **(4) CD3ϵ** improves CAR-T function by mediating CD3ζ signal (Refs 107, 108). CD3ϵ is the only CD3 chain that downregulates Lck activity and associates with CD3ζ phosphorylation and signal transduction by recruiting inhibitory tyrosine kinase CSK. The incorporation of CD3ϵ cytoplasmic domain into CD19-28ζ CAR at membrane-proximal position results in improved antitumour effect, by negatively regulating excessive cytokine production.

3. CAR delivery vectors and expression promoters

3.1 CAR gene delivery vectors

The majority of the CAR-Ts in development and all seven FDA-approved CAR-Ts were generated by delivering CAR genes with lentivirus or retrovirus (Refs 109, 110). A small fraction of CAR-Ts was developed by transposon such as sleeping beauty (SB) and PiggyBac (PB) (Ref. 111). Compared to viral vectors, gene delivery via transposon is less immunogenic, generating stable genome integration and higher gene expression, which may benefit their clinical application (Ref. 111). However, the transduction efficacy of non-viral vector needs improvement.

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3.2 Promoters and CAR expression level

The CAR level can affect CAR avidity and CAR-T function. One study found that targeted insertion of CD19–28z CAR into native TCR loci reduced CAR level by two-fold, generating a better antitumour effect by preventing tonic signalling and CAR-T exhaustion (Ref. 112). In contrast, high-level CARs damages CAR-T's antitumour effects (Ref. 113). The LTR (long terminal repeat) promoter in γ retroviral vector expresses a higher level CAR and causes more CAR-T apoptosis than EF-1α promoter (Ref. 114). On the other hand, in the lentivirus-mediated CAR-T transduction, among the four promoters (CMV, EF-1α, hPGK and RPBSA), EF-1α promoter yielded the strongest cytotoxicity, especially for longer genes (Ref. 115). In another study, Ho, et al. reported that the replacement of EF1α with MND promoter in the CD19-BBζ could reduce CAR density and increase the transduction rate (Ref. 116). The MND-driven CAR-Ts had prolonged activity with low IFNγ and toxic effect in the preclinical study. ABECMA, an MND-driven CAR-T targeting B Cell Maturation Antigen, was recently approved by FDA for refractory/relapsed multiple myeloma.

4. CAR-T production

The CAR-T production process includes T cell isolation from patient peripheral blood, activation by anti-CD3/CD28 coated beads, CAR transduction, and CAR-T expansion. The optimal manufacturing process is an important step to generate high-quality CAR-Ts (Ref. 117). (**1**) The ratio of anti-CD3/CD28 coated beads versus T cells needs to be optimised so that the T cells are sufficiently activated, but not over-activated to be driven into exhaustion (Ref. 118). (**2**) In the process of CAR-T production, IL2 is present in media. The use of other common γ chain cytokines (γc-cytokines) such as IL7, IL15 and IL21, alone or in combinations IL7/IL15 (Refs 119–122), IL4/IL7, and IL4/IL7/IL21 (Ref. 123) have been reported to produce superior CAR-Ts with a higher percentage of T_{CM}.

5. CAR signalling and CAR-T function

Proper CAR signalling is critical for CAR-T's activation, effector function and antitumour effects (Fig. 2). As CAR contains CD3ζ and CD, CAR signalling may follow TCR signalling pathways. Two recent reviews compared the signalling of TCR and CAR (Ref. 124) and among different CARs (Ref. 125). Here, we highlight the main points.

5.1 TCR signalling

TCR signalling is well delineated (Refs 126, 127). Briefly, co-receptor CD4 or CD8 helps TCR-MHC/peptide engagement and bring leukocyte-specific tyrosine kinase (Lck) to the TCR complex. The Lck phosphorylates the immuno-tyrosine activation motif (ITAMs) in CD3ζ, CD3δ, CD3ϵ, and CD3γ, which recruit and phosphorylate Zap70. Zap70 phosphorylates a membrane-associated scaffolding protein, linker for activation of T cells (LAT), which become a docking site for other signalling proteins, including Grb2, Sos, Slp-76, and PLC-γ1. The Sos/Grb2 activates RAS guanyl nucleotide-releasing protein (RasGRP)-MAPK-Erk and transcription factor AP-1. The activated PLC-γ1 cleave phosphatidylinositol triphosphate (PIP2) to generate diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG, a membrane-associated lipid, activates protein kinase C θ (PKC-θ), participating in the pathway leading to the nuclear entry of NF-κB. DAG also activate RasGRP-MAPK pathways, leading to activation of AP-1. IP3, on the other hand, stimulates the efflux of Ca2 + from ER to the cytoplasm. Elevated Ca2 + activates calcineurin, which dephosphorylates NFAT. Dephosphorylated NFAT enters the nucleus to join AP-1 and NF-κB to induce specific gene transcription. Productive T cell activation also needs co-stimulation from CD28, CD2, CD5, CD30, 4-1BB, OX40, LFA-1 and ICOS.

5.2 CAR signalling

CAR combines CD3ζsignal 1) and co-stimulatory (signal 2) and may use similar signalling machinery as natural TCR (Refs 124, 125, 128) (Fig. 2A). However, there are several major difference between TCR and CAR signalling. (**1**) The signal triggering of TCR and CAR may be different (Ref. 124). While TCR does not need clustering (Ref. 129), CAR requires oligomerisation (Ref. 130), to initiate signalling. Thus, soluble antigen does not normally activate CAR-Ts. (**2**) CAR forms a nonclassical immunological synapse (Ref. 131). (**3**) The sensitivity of TCR and CAR is different. TCR can detect as few as one MHC/peptide complex, while CAR requires at least hundreds of antigen even though it has a higher affinity (Ref. 44). (**4**) Though CD28 and 4-1BB CD are associated with PI3 K-Akt and TNFR-associated factors (TRAFs), respectively (Ref. 125), they activated similar signalling proteins based on phosphor-proteomic analysis (Ref. 128). In addition, compared to 4-1BBζ, CD28ζ CARs activated faster and higher-magnitude changes in protein phosphorylation, correlating with effector T cell phenotype and function. (**5**) The 3rd gen CD28-BBζ CAR-Ts showed enhanced activation by phosphorylation level of the signalling proteins than 2nd gen CAR-Ts (Ref. 132), and may benefit antitumour effect of CAR-Ts with very-low-affinity scFvs (Ref. 93). However, a contradictory report does exist, which showed 2nd gen CAR promoted stronger TCR signalling than 3rd gen CARs (Ref. 133).

5.3 CAR-T function

CAR-Ts may use a similar mechanism as natural T cells to exert their cytotoxicity and to undergo exhaustion after strong and persistent engagement with tumour cells (Ref. 134) (Fig. 2B, 2C). (**1**) **Fast-acting lytic activity**. This fast-acting cytotoxicity depends on the perforin/granzyme B (Ref. 135). Granzyme B induces caspase-dependent and independent apoptosis whereas perforin enables granzyme B enters target cells by forming pore (Ref. 136). (**2**) **Slow-acting lytic activity.** Fas/FasL mediates caspase-dependent apoptosis (Ref. 136). The Fas/FasL pathway plays a key role in the on-target killing of antigen-positive as well as off-target 'bystander' killing of antigen-negative tumour cells (Ref. 137), preventing tumour escape. But, Fas/FasL also cause the death of stimulated T cells (Ref. 138). A recent report showed that CAR-Ts were prone to Fas-mediated cell death (Ref. 139), whereas the use of Fas-DNR (dominant-negative receptors) engineered CAR-Ts resulted in intrinsic disruption of Fas signalling, and leading to longevity and superior antitumour efficacy (Ref. 140). (**3**) **IFNγ and TNFα mediated cell death**. Activated CAR-Ts secrete IFNγ and TNFα into the target cell by exocytosis and induce tumour cell death (Ref. 141). Similar to Fas/FasL, TNFα and IFNγ can induce bystander eradication of antigen-lost tumour variants. However, the role of TNFα and IFNγ on tumour progression and immunotherapy is paradoxical. TNFα can promote tumour progression by inducing TNFα-dependent tumour cell dedifferentiation (Ref. 142), triggering TNF/TNFR1 signalling and T cell death (Ref. 143), and promoting Treg (Ref. 144). Similar to TNFα, IFNγ exposure reduced antigen expression (Ref. 145). IFNγ is also a strong inducer of PD-L1 expression on tumour cells, which increase CAR-T exhaustion. Furthermore, the increase of these cytokines may lead to cytokine release syndrome and activation-induced cell death (Refs 146, 147).

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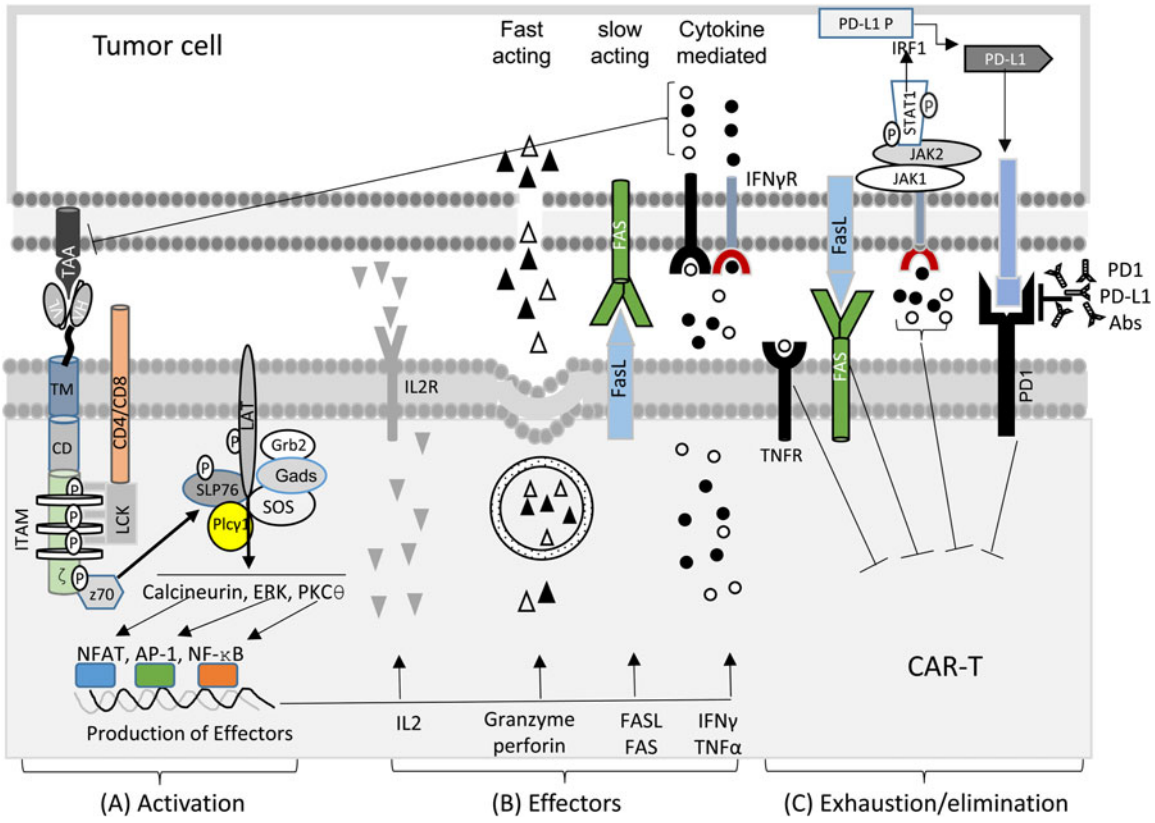


Figure 2. The schematic diagram of CAR signalling and CAR-T activation (A), the effector function of CAR-Ts to kill tumour cells (B), and the exhaustion/death of CAR-Ts by persistent tumour antigen stimulation (C).

6. CAR-Ts for solid tumours

Most human tumours are solid tumours. Scientists and physicians had been studying CAR-T therapy on solid tumours for nearly two decades (Refs 148–150). However, compared to the ~80% complete remission in blood cancers (Ref. 151), CAR-T therapy in solid tumours generated limited effects even to this day. One meta-analysis of 22 clinical trials and 262 patients showed only a 9% of response rate (Ref. 152). In another review of CAR-T solid tumour therapy (Ref. 153), the authors summarised 42 clinical trials of 375 patients. There were 13 complete responses, 35 partial responses, four mixed responses, and 121 stable diseases. The low efficacy of solid tumours may attribute to various reasons, which include antigen heterogeneity, low tumour infiltration, suppressive TME, short CAR-T survival, and life-threatening toxicities. In this section, we are going to review potential strategies to improve the efficacy of CAR-Ts in treating solid tumours.

6.1 Optimise CAR design for better tumour recognition

As discussed in section 2, recognition of tumour cells by CAR-Ts can be optimised by adjusting CAR avidity, epitope location, the hinge, TM, and the signalling domain (Ref. 43). In addition, optimal CAR design includes targeting multiple epitopes on the same antigen (Refs 61, 154), a 2nd different target antigen (Ref. 40), 2 or more antigens sequentially (Tandem CARs) (Ref. 41) or simultaneously (Ref. 155).

6.2 Co-express protein payloads

The 4th generation CAR-Ts, also named as Truck (T cells redirected for antigen-unrestricted cytokine-initiated killing), are CAR-Ts with a constitutive or inducible transgenic 'payload' including cytokines (IL7, IL12, IL15, etc.) (Ref. 156) or chemokines (CCL19) (Refs 157–159), which improve TME (Ref. 160), prevent CAR-T exhaustion (Ref. 120), reactivate endogenous innate lymphocytes to exert their anti-tumour responses (Ref. 159), or increase CAR-T trafficking and infiltration (Ref. 161). Furthermore, the 5th generation CAR-Ts were being developed by incorporating the JAK-STAT activation domain between CD28 and CD3ζ, leading to increased proliferation and persistence but decreased differentiation of CAR-Ts (Ref. 162).

6.3 Co-express immunostimulatory RNA

In the latest study (Ref. 163), June and colleagues reported that co-expression of RN7SL1 RNA enhanced CAR-T expansion and persistence, and reduced exhaustion. Importantly, the RN7SL1 RNA could activate myeloid-derived dendritic cells via RIG1/MDA5 pathway and activation of the endogenous T cell responses, generating broader antitumour immune responses to target tumour variants with antigen-loss.

6.4 Develop variants of mAb/TCR chimaera

The native TCR complex contains 10 ITAMs on CD3ζ, γ, ϵ, and δ. In CAR, there are only three ITAMs in the CD3ζ. The use of chimeric VL (VH) with different TCR chains will likely incorporate a native TCR complex to improve CAR-T sensitivity and reduce toxicity. Several studies reported the combination of Fab or scFv with native TCR or co-receptors (Fig. 3).

6.4.1 TRuC (TCR fusion construct)

In TRuC, scFv is fused to one of the intact TCR chains (except ζ chain) (Ref. 164). The data show that some TRuC (ϵ-TRuC, scFv

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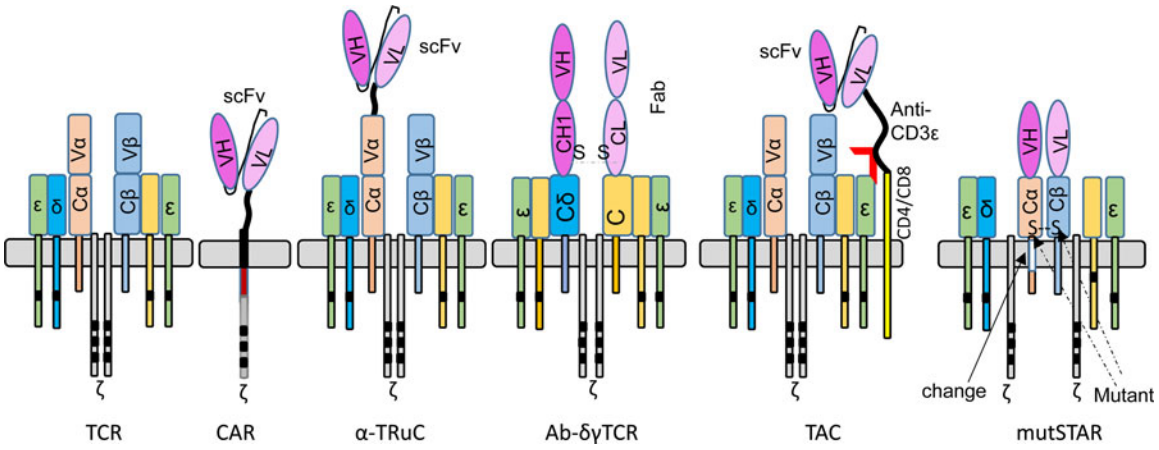


Figure 3. Different variants of chimeric antibody-TCRs.

fused to intact CD3ϵ) generated better antitumour effects with less cytokine-mediated toxicity.

6.4.2 Ab-γδTCR

Another format of mAb/TCR chimaera is the Ab-γδTCR, which has the Fab domain of H and L chain links to CD3δ or CD3γ chain (Ref. 165). The CD19 Ab-γδTCR engineered T cells are less exhausted and lower cytokine production and, but generated compared antitumour effects.

6.4.3 TAC (T cell antigen coupler)

TAC has the scFv for antigen binding fused anti-CD3ϵ domain and then linked to CD4 or CD8 molecules (Ref. 166). While the scFv binds tumour cells, its anti-CD3ϵ domain engages with native TCR. In solid tumour models, HER2 TAC-T cells outperformed CD28ζ CAR-Ts with increased tumour infiltration and anti-tumour efficacy and reduced toxicity.

6.4.4 muSTAR

Different from the original CAR design (Ref. 87), in the muSTAR (mutated Synthetic TCR with Antigen Receptor) (Ref. 167), the VL and VH were connected to mutant mouse TCRα or β constant region. The mutant TCR constant region will pair with each other and reduce mispairing with endogenous TCRα and β chains. The muSTAR engineered T cells had overall advantages compared to CAR-Ts. They (**1**) recognise both antigen high or low tumour cells to avoid 'tumour escape'; (**2**) have less auto-activation and tonic signalling; (**3**) generate stronger tumour killing capacity; (**4**) have earlier and higher tumour infiltration; (**5**) are less exhausted. Further improvement in persistence, activity/proliferation of CAR-Ts could be achieved by developing dual targeting muSTAR and by incorporating IL2Rβ/ costimulatory domain. Thus, the muSTAR has superior potential for solid tumour treatment by combining the advantage from CAR for antigen-specific recognition and advantage from TCR for proper recruitment of other molecules for signal transduction. The muSTAR leads to increased T cell activity and less toxic effect, probably by becoming an integral part of the TCR complex (Ref. 164).

6.5 Improve CAR-T traffic and infiltration into tumour

For solid tumours, insufficient CAR-T migration, infiltration, and intratumour expansion and accumulation are the likely causes of failure in therapy (Refs 56, 168–171). Solid tumour mass is characterised by abnormal angiogenesis and stromal formation, altered chemokine expression, inhibitory checkpoint receptors and hypoxia environment, which are in favour of recruiting immunosuppressive cells such as Tregs but preventing effector immune cells (Ref. 172). Several approaches can overcome these obstacles to enhance CAR-T tumour infiltration.

6.5.1 Chemo and irradiation

They are an integral part of ACT as they are often used to precondition patients. They induce lymphodepletion to free space for incoming CAR-Ts and modify TME to attract T cells and create a favourable milieu for them to exert their function (Ref. 173). A short course of radiotherapy or chemotherapy before CAR-T treatment benefited CAR-T migration/infiltration by depleting the immunosuppressive cells such as Treg in TME (Refs 168, 174). For example, cyclophosphamide treatment generated pro-inflammatory myeloid and T cell signatures in tumours, and enhanced the recruitment of antigen-presenting cells, as well as endogenous and adoptively transferred T cells, resulting in long-term anti-tumour immunity (Ref. 175). In another study, Lin *et al.* reported that ionising irradiation enhanced IL8 secretion by brain tumour cells and that the CD70-specific CAR-Ts co-expressing IL8 receptors CXCR1 and CXCR2 markedly enhanced CAR-T tumour infiltration and antitumour effects (Ref. 176).

6.5.2 Targeting stroma cells

Facilitates CAR-Ts across the extracellular matrix (ECM) barrier into tumour lesion. Recent studies showed that CAR-Ts co-expressing heparanase (often lost during CAR-T preparation), an enzyme that degrades heparan sulphate proteoglycans, the main components of ECM, significantly increased tumour infiltration (Ref. 177). Another study showed that inhibition of the NADPH oxidase 4 (NOX4), an enzyme upregulated by cancer-associated fibroblast in the stroma, increased CAR-T tumour infiltration (Ref. 178).

6.5.3 Chemokine/chemokine receptors

Play important role in CAR-T migration and infiltration into tumours. Tumour cells can produce certain chemokines in their abnormal growth whereas CAR-Ts often do not express the chemokine receptors. Improved CAR-T infiltration have been reported via the expression of IL7/CCL9 (Ref. 179) that is essential for the recruitment of T cells and DCs from the periphery. In addition, expression of CCR2 (Ref. 180), CXCR2 (Ref. 181) or CCR4 (Ref. 161) on CAR-Ts improved infiltration by binding to the CCL2 or IL8 or CCL17/CCL22 elevated in TME. Other pairs of chemokine/chemokine receptors such as CCL5 and CXCL9 may also improve CD8 CAR-T infiltration (Ref. 182). On the other hand, CXCL12 produced by stromal fibroblasts may prevent T cell infiltration and should be avoided (Ref. 183).

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6.6 Improve CAR-T survival

CAR-T survival inside the tumour is a critical factor for generating antitumour efficacy. Several strategies are being studied to enhance their survival. (**1**) **The CAR-Ts derived from** T_N/T_{CM} have an enhanced cytokine production with longevity (Refs 18–20, 184). (**2**) **The affinity of ABD** may be important for CAR-T survival, especially in solid tumour mass. We analysed 14 complete trials of CAR-T therapy in solid tumours (Table 2). We found that CAR-Ts constructed from intermediate affinity scFv (K_D from 20–100 nM) generated some clinical responses in all 4 trials. In contrast, 8 other clinical trials with high-affinity CAR-Ts (K_D from 0.2–11 nM) showed no clinical responses except one. All the responders have a longer persistence of CAR-Ts. Intermediate avidity CAR-Ts can prevent them from exhaustion and apoptosis due to transient break from continuous stimulation inside solid tumours (Refs 75, 77). (**3**) **4-1BB** enhanced mitochondrial biogenesis that benefit for CAR-T persistence and function in hypoxia environment (Ref. 97). (**4**) The **3rd generation CAR-Ts with ICOS-4-1BB** (Ref. 86) or **CD28-OX40** have longer survival than the 2nd generation CAR-Ts (Refs 102, 103). 4) **Co-expression of stimulatory cytokines and chemokines**: IL4/IL7 (Ref. 185) and IL-4/IL21 (Ref. 186) receptors increase STAT5/STAT3 phosphorylation and improve CAR-T persistence.

6.7 Improve and reprogram TME

Hostile and suppressive TME severely hinder CAR-Ts to exert their function inside tumour lesion. Improving or reprograming TME (Ref. 187) could enhance CAR-Ts efficacy in solid tumours. (**1**) **Counteract immune inhibitory molecules:** Co-expression of TGF_β receptor (Ref. 188) (or TGF_β dominant-negative receptor (DNR) (Ref. 189) by CAR-Ts can competitively bind TGF_β to block its immunosuppressive effect. Secondly, co-expression of PD1 DNR (Ref. 190) or Fas DNR (Ref. 140) on CAR-Ts can block the PD1-PD-L1 inhibitory interaction. Similarly, a combination of PD1-PD-L1 checkpoint inhibitor (Ref. 191) and tyrosine kinase inhibitor (dasatinib) (Ref. 75) also enhanced TME and improved CAR-T survival and activity. In addition, CAR-Ts with 'payload' cytokines of IL12 (Ref. 192), IL18 (Ref. 193) or IL23 (Ref. 194) inhibited Treg in TME. (**2**) **Deplete or reprogram suppressive cells**: Treg could be depleted to increase CD8 T effector function and antitumour immunity (Ref. 195). Interestingly, down-regulation of Helios could convert Treg into T effectors (Ref. 196). Another study showed knockout of Nrp 1 made Treg lost its inhibitory function and promote antitumour immunity (Ref. 197). Secondly, tumour associated macrophages could also be reprogrammed to become tumouricidal (Ref. 198). Thus, reprograming tumour macrophages and other myeloid cells is another strategy to improve TME (Ref. 199). (**3**) **Remodel tumour vasculature**: The neovasculature in the tumour lesion can be targeted to change TME. A study showed that angiogenesis inhibitor Bevacizumab could remodel tumour vasculature to increase tumour infiltration of anti-GD2 CARTs and enhance antitumour effects (Ref. 200). (**4**) **Other approaches** such as targeting metabolism (Ref. 201) and tumour stroma (Ref. 202) may also improve TME and create a favourable environment for CAR-Ts to exert their antitumour function.

6.8 Reduce CAR-T toxicity

CAR-T toxicities include on-target/off-tumour toxicity, cytokine release syndrome (CRS), and neurotoxicity. Optimising parameters in the 'CAR design' (section 2) can reduce CAR-T toxicity. Other strategies are also studied to reduce CAR-T's toxicity that are well-reviewed (Ref. 203). Here, we focus on two novel strategies in CAR design to reduce toxicity.

6.8.1 The SynNotch switch

Notch is a transmembrane receptor. The transmembrane domain (TM) and its connected part of the extracellular domain form the 'notch core'. Upon engaging with the ligand, the intracellular domain will be released downstream of the notch core by protease. Lim's group found that both the extracellular and intracellular domains could be replaced as long as the notch core is retained to create SynNotch (Refs 204, 205). This modular synNotch design provides extraordinary flexibility in engineering T cells with scFv or other ligands targeting specific tumour antigen. Upon engagement, it releases transcriptional factor to drive CAR expression to target another tumour antigen to initiate killing activity. Such design makes sure the CAR-Ts needs to target 2 tumour antigens to initiate killing to avoid toxicity. It will also restrict the cytotoxicity inside the tumour mass (Refs 206, 207).

6.8.2 Co-LOCKR protein switch

Recently, Lajoie *et al.* designed a co-localisation dependent protein switch, latching orthogonal cage–key protein (LOCKR) (Ref. 208). This Co-LOCKR switch enables AND, OR and NOT Boolean logic operations. (**1**) They implemented AND gates to redirect T cell target tumour cells expressing two surface antigens while avoiding off-target recognition of single-antigen cells. (**2**) They also tested the three-input switches that add NOT or OR logic to avoid or include cells expressing the third antigen. Such Co-LOCKR design will increase specificity against tumour cells and thus reduce toxicity.

7. Expert and topical summary

In this review, we gave a comprehensive analysis of different factors that may affect CAR-T's antitumour efficacy. While a clear strategy for designing effective CAR-Ts for solid tumours is still lacking, we think the following aspects should be considered in CAR design for solid tumours. (**1**) **CAR-Ts capable of strong in situ expansion and persistence inside tumour mass**: The CAR-Ts should undergo antigen-driven expansion, persist and exert their tumour killing activity inside tumour lesion, and generate memory immune responses after tumour regression. Based on current analysis, a combination of T_N/T_{CM} subsets and intermediate avidity CAR may generate expandable and persistent CAR-Ts. As demonstrated in Table 2, 7 of 8 high avidity CAR-Ts trials ended up with no clinical responses, future clinical trials should test more on the intermediate affinity CAR-Ts. In addition, innovative design of CAR-Ts that allow CAR expression only when they encounter target tumour cells may help avoid CAR-T exhaustion and deletion, achieving potent antitumour effects in solid tumours. (**2**) **Invoking endogenous antitumour immunity**: Due to the antigen heterogeneity of solid tumours, it is unlikely that CAR-Ts targeting one antigen will generate persistent antitumour immunity to achieve complete responses. Designing CARs targeting multiple tumour antigens in tandem or simultaneously may not only prevent immune escape and relapse, but also help avoid off-tumour toxicity. In addition, we think one important outcome of CAR-T immunotherapy should include the activation of endogenous immune cells to invoke broad antitumour immunity. In this case, CAR-Ts kill tumour cells and release tumour antigens (shared or neoantigens) that can be taken up by proper dendritic cells to cross-prime a patient's own immune system. Thus, CAR-Ts will serve as an immune initiator in addition to the direct killing of tumour cells. The incorporation of an innate activator in the CAR-T to activate dendritic cells will facilitate the activation of the patient's

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own antitumour immunity. A recent report demonstrated that CAR gene engineered macrophage (CAR-M) could effectively activate endogenous antitumour immune response (Ref. 209), suggesting that CAR-Ts and CAR-M may work together to eradicate tumour mass and invoke endogenous broad spectrum of antitumour immunity. **(3) Targeting TME**: Co-expression of immune therapeutics may create a favourable TME for CAR-Ts to exert effector function to generate antitumour effects inside tumour lesions. Delivery of immune-stimulatory cytokines and chemokines, immune-stimulatory RNA, or immune checkpoint inhibitors may not only improve TME, but also can achieve direct antitumour effects and invoke endogenous immune system to achieve complete and persistent antitumour immunity.

Acknowledgement. The authors like to thank current and previous members of Dr He's laboratory. In addition, over the years, research in He's lab has been funded by NIH/NCI grants (CA168912 and CA235159) and Augusta University startup package.

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