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Conjugated CAR T cell one step beyond conventional CAR T cell for a promising cancer immunotherapy

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ABSTRACT

Cancer immunotherapy aims to enhance the immune system reactivity against tumour cells. Chimeric Antigen Receptor (CAR), presented on the surface of the T cells, specifically redirects the cell and demonstrates significant promises in treating patients with different types of haematologic malignancies.

Although several cases of improvement have been reported, clinical experiences, such as excessive activity, poor control, toxicity and limited life span of conventional CAR T cells have emerged as treatment challenges associated with this therapeutic strategy. Recently, multiple switchable CAR T platforms have been made to enable better control in a dose-dependent manner, which is correlated to distinct characteristics of different switch molecules.

This review aimed at a brief represention of toxicities of the CAR T cells, the obstacles facing tumour treatments especially in solid tumours, and finally providing a framework for classification of the newly developed Conjugated/Split CAR-T cell technologies to overcome difficulties. Overall, Newly developed Conjugated CAR T cells using among with soluble switch molecules seems to be as responsive as the conventional CAR T cells, yet providing many new useful options to effectively overcome limitations and significantly improve patient safety.

1. Introduction

For several decades, cancer therapy was limited to surgery, chemotherapy, and radiotherapy. However, due to the high relapse rate and poor prognosis as the significant challenges of cancer treatment, these therapeutic methods were not usually useful. Nowadays, cell-based therapies have been more considered as new approaches in cancer treatment [1]. Recent advancements in synthetic biology and gene transfer has led to an efficient, robust and swift redirection of the innate immune system in humans, mainly polyclonal T lymphocytes [2]. In particular, the T cells engineered to express chimeric antigen receptor (CAR), besides their native T cell receptor (TCR), have been reported effective in eliminating cancer chemotherapy resistance [3]. These cells can proliferate in a large amount and make a robust response against malignant cells following the administration. Chimeric Antigen Receptor T (CART19) Cell, which is the CAR T cell engineered to target the B-cell antigen CD19, exhibited unexpected results in the treatment of relapsed or refractory leukaemia and lymphoma in clinical trials, approved by the Food and Drug Administration (FDA) in 2017 [4,5].

Accordingly, the conventional CAR T cell has improved medical equipment leading to a highly amplified response and persistence in treated patients resulting in the eradication of a considerable quantity of tumour cells through the weeks. Despite great advantages, numerous challenges, such as safety issues have slowed down clinical improvements in some patients treated with genetically modified *T*-cells [6].

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Due to the fixed antigen recognition capability of conventional CAR T cells directed only to one Tumour-Associated Antigen (TAA), and consequently their limited utilization in all cases, there is a great need to develop new advanced CAR T cells with varied specificities and specific for each patient. In the last few years, different switchable conjugated CAR T cells have been proposed, whose activation and proliferation are dependent on adaptor molecules that mediate the formation of immunological synapses between TAA and the target cell [7–11].

1.1. Conventional CAR T cells

Eshhar et al. designed the first CAR in 1989 [12], at the Weizmann Institute of Science, Israel and the first generation of CARs developed in 1993, when Eshhar engineered T cells using the first chimeric molecule, consisted of a portion of an antibody and a part of a TCR.

The CARs commonly is consisted of an extracellular antigen binding molecule bound to one or more intracellular signalling domains via a hinge region and a transmembrane domain. Single-chain variable fragments (scFvs) of the tumour antigen-specific antibodies are mostly used as an extracellular recogniser domain [13], which enables T cells to recognise targeted molecules of a selected cell, independently from major histocompatibility complex (MHC) restriction. The scFv is consisted of the variable fragments of the heavy and light chains of an immunoglobulin and it is served as the extracellular signalling peptide of the CAR as well as a flexible linker that fuses the heavy and light chains [14].

In the conventional CARs, a scFv with a simple ectodomain and other recognition components form the antigen-specific portion can recognise and strongly bind to the desired antigen and activate the T cell [15]. Spacer is a portion that connects the antigen binding domain to the transmembrane domain [16], which its simplest form is used in several scFv-based constructs at the hinge region of IgG1. The other portion of a CAR is the transmembrane domain which contains a hydrophobic alpha helix that spans the membrane and ensures the stability of the receptor [17]. Additionally, the ζ chain of complex TCR/CD3 (CD3ζ), which is considered as another transmembrane domain of the CAR, can bind the artificial TCR to the native TCR. It is able to reduce some of the complications of CAR T cell therapy, since it is naturally found in the native TCRs. CD28 transmembrane domain is by far the most stable receptor which is used to determine conventional CAR T cell systems [18].

All designed conventional CAR T cells have been divided into four generations which are varied in their *endo*-domain structure. The first generation of CARs comprises CD3ζ and they cannot produce enough interleukin-2 (IL-2), which is necessary to potentiate the immune system against tumour cells. It can be compensated through the administration the exogenous cytokines like IL-2 combined with 1st generation of CAR T cells with single-chain receptors [19]. However, no desirable outcomes have been reported in most of the studies using first generation of CAR T cells, which can be due to the inadequate proliferation, short life span *in vivo* and insufficient secretion of cytokines [20,21].

The second generation of CARs then appeared and benefitted from dual signal T cell activation: one is triggered by the antigen recognition (like 1st generation of CARs and through ectodomain and CD3ζ pathway), and other is produced by a co-stimulatory molecule, such as CD28/B7 or 4-1BB. Such co-stimulatory molecule was effective in enhancing the synthesis of IL-2, which promotes the activation of T cells and avoids apoptosis of CAR T cells *in vivo* [22]. The CD28ζ-CAR T cells can cause constitutive stimulation, proliferation, and growth. However, the 4-1BBζ-CAR T cells induce early exhaustion, which is able to limit the antitumour function of the cell compared with the other second generation of CAR T cell [23–25].

Along with the advancement of newer generations of CAR T cell, more co-stimulatory molecules were used to produce third generation of CAR T cells, which reported to have enhanced responses, using sequences of other co-stimulatory signals and CD3ζ domain, simultaneously. Some of the newer domains are OX40 (CD134), CD28, 4-1BB (CD137), CD27, DAP10, and some other domains. Several studies have shown that third generation CAR T cells display higher levels of efficacy and *in vivo* persistence compared to the second generation cells [26–28].

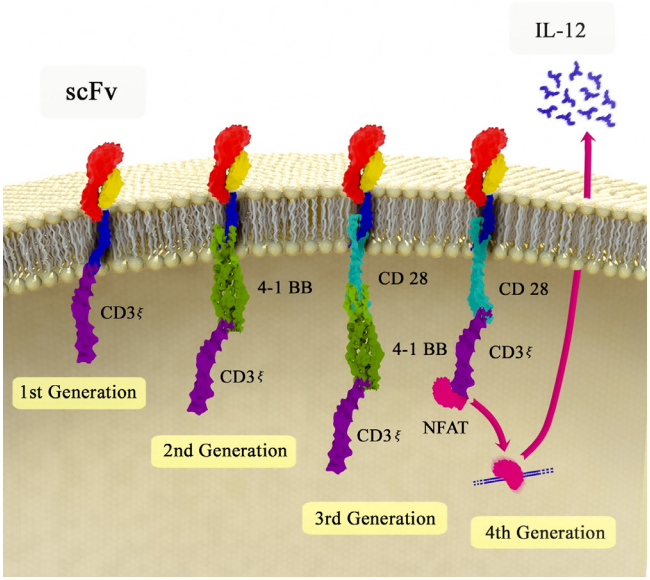

Fig. 1. A schematic demonstrating 4 generations of CAR T cells with their different signaling domains; 1st generation = CD3ζ + Transmembrane portion + scFv; 2nd generation = CD3ζ + 4-1BB + Transmembrane portion + scFv; 3rd generation = CD3ζ + 4-1BB + CD28 + Transmembrane portion + scFv; 4th generation = NFAT* + CD3ζ + CD28 + Transmembrane portion + scFv. *: NFAT induces production and secretion of IL-12 which is an important growth factors for T cells.

The fourth generation of CAR T cell were produced by adding IL-12 to the base structure of a second generation of CAR and they then were known as the "T cell Redirected for Universal Cytokine-mediated Killing" (TRUCKs) [29]. The most important benefits of using the TRUCKs include augmentation of T cell activation, activation and attraction of other innate immune cells, and the significant potentiation of the immune response to eliminate the cancer cells. Secretion of some immune modifiers and consequently modification of the inhibitory tumour microenvironment have indicated as another advantages of the fourth generation of CAR T cells. Such TRUCKs can be used also to treat some diseases, including metabolic disorders, viral infection and autoimmune diseases [30]. Fig. 1 demonstrates the structure of different generations of the CAR T cell.

Overall, unlike various available choices for cancer therapy, application of these CAR T cells in cancer immunotherapy has unique advantages. Moreover, several mechanisms used by infectious and tumour agents in order to escape from the immune system are no longer effective against these cells [31]. Most of the CAR T cells can only recognize unprocessed antigens, which are presented naïvely on the cell surface and are therefore differed from the TCRs. They detect antigens only when it already has been processed inside the cell and presented by the MHC. On the other hand, since CAR T cells can recognize a broad spectrum of antigenic epitopes (e.g. proteins, carbohydrates, and glycolipids), an increased variety of potential target antigens is expected [32]. Additionally, aside from the restriction caused by the MHC class type proteins, both $CD8^+$ and $CD4^+$ T cells can be used to construct a CAR T cell [33].

2. Toxicities of CAR T cells and challenges in treating solid tumours

For many years researchers have been trying to promote tumour regression using strategies that target shared antigens either on the tumour cells or on normal tissues, with the aim of ignoring normal tissue expression by these T cells. However, they were mostly ineffective and induced different types of toxicities. The most common illustrated toxicities are:

2.1. On-target on-tumour toxicity

On-Target On-Tumour toxicity is associated with the administration of T cell itself, which is triggered by excessive production of cytokine in cytokine release syndrome (CRS) [34] or large quantity of tumour cell necrosis resulting in tumour lysis syndrome (TLS) [35]. According to the evidences, the severity of CRS and TLS depends on the burden of disease [36] and it can be controlled by high-dose corticosteroid administration [37].

2.2. On-target off-tumour toxicity

It results from a direct attack on healthy tissues that have expressed the shared antigen and has been reported in numerous. The selection of target antigen, which is strictly specific to the tumour but not presented in normal cells is probably the most acute determinant to overcome this barrier. However, it is difficult particularly in solid tumours, so designing a flexible tunable CAR-T cell which can react against a favourable amount of targeted antigens is the best solution [38].

2.3. Immunogenicity

The majority of antigen-recognising regions in engineered CAR-T cells are derived from mouse mAbs [39], which may lead to severe anaphylaxis due to its potential foreign immunogenicity [40]. Application of a humanised scFvs rather than the mouse mAbs is recommended to control this life-threatening toxicity [41].

2.4. Other toxicities

Neurotoxicity and genotoxicity have also been reported due to further perseverance of CAR T cells in the tissues [42,43].

Consequently, the designed CAR T cells for tumour treatment, exceptionally solid tumours need accurate targeting and control mechanisms to avoid off-tumour effects while preserving on-target effects.

Accordingly, the effective immunotherapy using T cells always need to overcome multiple operational challenges, simultaneously. Some other challenges especially those which are seen in solid tumours are reported by several studies as follows:

2.5. Migration, trafficking, and penetration

Although trafficking of CARs is not considered as a significant issue for hematopoietic malignancies, it remains a significant concern in solid tumours. It has been reported that CAR T cells trafficking can be ameliorated through the induction of chemokine receptors to these cells. However, there are limited related investigations to elaborate these strategies [44,45]. On the other hand, the trafficking and penetration of CARs are influenced by T cells delivery route and tumour microenvironment.

2.6. Tumour recognition and bystander discrimination

In most of the trials done on T cell therapies, some cross-reactions and killing of bystander non-tumoural cells have been reported, which can lead to lethal side effects.

2.7. Proliferation and persistence

It is reported that due to a suppressive tumour microenvironment, without adding co-stimulatory domains to the intracellular part of the receptor, T cells proliferation, persistence, and clinical efficacy can not be observed [46]. In recent preclinical studies, CARs incorporated with CD28 or 4-1BB co-stimulatory domains showed more IL-2 secretion, which leads to more T cell proliferation, long term persistence and rapid tumour elimination compared with the cell expressing only a zeta chain in the presence of co-stimulatory domains. Besides CD28 and 4-1BB, various co-stimulatory switch molecules have been reported to be used as split signalling domains [27,47].

2.8. Suppressive microenvironment

The immunosuppressive tumour microenvironment also can prevent effective infiltration of T cells, as well as suppressing their survival and function inside the tumour [48]. CAR T cells are generated by several groups, which secrete cytokines, such as IL-12 to stimulate T cell-mediated immune response in the tumour microenvironment [49–51]. Others support the combinatorial immunotherapy strategies, like PD-1 checkpoint or CTLA-4 as a promising approach to overcome the suppressive tumour microenvironment [52,53].

2.9. Tumour heterogeneity

For solid tumours, tumour heterogeneity and antigen escape are the primary causes of tumour evasion in cancer immunotherapy [54]. Several strategies aimed at improving the function of T Cells in the presence of tumour heterogeneity have been done on CAR-T cells design.

Accordingly, there is a vital need to develop overcoming strategies to tune the amplitude and length of CAR T cells activity, including modulation of their survival, strength, and where they affect.

3. Overcoming strategies

3.1. Co-expression of a suicide gene in CAR T cells (suicide switch)

A suicide gene is a genetically encoded molecule that allows the selective destruction of expressing cells. Although it can be introduced into the CAR T cells via HSV-tk/GCV [55], iCasp9/AP1903 [56,57] or mAbs, such as tEGFR/mAb [58] or CD20/mAb [59]; but, no therapeutic CAR T cells and as a result, no T cell therapy in the cancer recurrence can be observed via such approaches. Due to the insufficient therapeutic potential of suicide genes, there is a great necessity to develop CAR T cells with controllable activity.

3.2. Targeting two or more tumour-associated antigens

In order to enhance tumour specificity, CARs which are able to sense the combinatorial antigens and reduce antigen escape rate are developed by the following distinct strategies:

a) Dual targeting CAR T cell (CAR + CCR, OR-gate): it provides a suboptimal activation against the binding of an antigen to a chimeric co-stimulatory receptor (CCR) that recognizes a second antigen [60].
b) Bispecific tandem CAR T Cell (TanCAR): two distinct antigen recognition domains are present in one CAR and are linked to each other by Gly-Ser linker [61].
c) CAR + iCAR (NOT gate): T cells can be designed with an inhibitory receptor harbour intracellular domains of PD1 or CTLA4, that can be triggered by an antigen expressed on normal tissues, leading to blunted activation of CAR T Cells [62].
d) Syn-notch CAR T Cell (AND-gate): it requires a simultaneous

sensation of two antigens for activation. Upon ligand recognition by Syn-notch receptor, an orthogonal transcription factor is cleaved and regulates CAR T Cells activation. However, the immunogenicity of non-human transcription factor is still under investigation [63].

e) Pooled CAR T Cell: it consists of multiple CAR T cells with different antigen specificities [64].
f) Multi CAR T Cell: a unit T cell harbours several CAR molecules with different antigen specificity [65].
g) Conjugated CAR T cells: below they are comprehensively discussed.

4. Conjugated CAR T cells

As discussed above, human tumours have often heterogeneous cell-surface antigens. They are not only various among individuals, but are also different in the same patient. On the other hand, tumour relapsing is commonly due to a surface antigen loss even following a particular therapy that had been primarily effective. Moreover, targeting the shared tumour-associated antigens on normal tissues can result in severe toxicities leading to loss of costly vectors. Therefore, there is a crucial need to develop new advanced CAR T cells with different characteristics and specific for each patient. In the last few years, five different switchable conjugated CAR T cells have been proposed, which can be activated and proliferated depending on adaptor molecules that mediate the formation of immunological synapses between tumour-associated antigen and the target cell.

4.1. BBIR-avidin CAR T cells

Urbanska et al. in 2012 for the first time proposed a novel insight in CAR T cell therapy and improved its effectiveness. They developed a universal panel of CAR T cells targeting known as tumour-associated antigens by designing a new method of antigen recognition system, which allows bioengineered leukocytes to recognize and be redirected flexibly against new tumour-associated antigens. This flexible approach was achieved using Biotin-Binding Immune Receptor (BBIR) consisting of an extracellular modified monomeric (mcAv) or dimeric (dcAv) avidin molecule, attached to the CD3ζ and/or CD28 intracellular signalling domains via a CD8α transmembrane hinge. This structure was used as a chimeric receptor on T cell and formed the first and second generation of CAR T cells. They also designed some split specific biotinylated molecules composed of an scFv portion linked to biotin called as “Biobodies”, which could specifically recognize tumour cells and guide CAR T cells to the tumour site.

Urbanska et al. also reported that mcAv-CAR T cells do not show immune reactivity, whereas dimers and tetramers of avidin are effectively reactive, which is probably due to the low binding affinity between biotin and mcAv. It is worth mentioning that the affinity of dcAv was enough to bind to biotin and activate T cells, but it was not enough to establish a strong permanent connection and following activation of the CAR T cell biobodies can be separated. They also indicated that (dcAv), BBIR-CD28-CD3ζ CAR T cells are responsive to the same quantity of biobody as (dcAv), BBIR-CD3ζ CAR T cells; however, they secrete more IFN-γ.

In addition, the reactivity of CAR T cells to a soluble antigen-free form of biotin was measured, since the soluble form of biotin is available in plasma at 0.2–2 nM. As a result, CAR-T cells were even at nM irresponsive to the antigen-free biotin alone.

Furthermore, the anticancer activity of BBIR-CAR T cells was similar to the conventional CAR T cell against the same antigen (Table 1). Overall, such versatile system allowed them to target tumour cells sequentially and also to target different tumour-associated antigens *in vitro* and *in vivo,* simultaneously [7]. Fig. 2a demonstrates BBIR-avidin strategy of conjugated CAR T cell therapy.

4.2. Anti-FITC CAR T cells

In order to control CAR T cell proliferation, some previous studies have developed immunological synapses dependent on antibody-based switch molecules to control the activation, phenotype and antigen specificity of CAR T Cells. The activities of these switch CART (sCAR) T cells entirely are dependent on the presence and dosage of switch molecules.

Two types of sCAR platforms have recently been developed; one used semisynthetic switches, consisting of site-specific chemical conjugation of targeting antibody with small molecule fluorescein isothiocyanate (FITC), and another used a fully-recombinant switch, including genetic fusion of targeting antibody with a short peptide neoepitope (PNE) derived from a GCN4 peptide sequence. This type of conjugated CAR T cells has been evaluated to treat cancers by several researchers.

For example, Tamada et al. in 2012 proposed another conjugation approach for CAR T cells called “Anti-FITC CAR T cell”. They used anti-FITC as the cellular recogniser and FITC-scFv as the split extracellular structure. Studies on Anti-FITC CAR T cell revealed that they are able in recognition of various cancer cells, efficient specific lysis, T cell proliferation and secretion of cytokines and chemokines *in vitro* and *in vivo,* as well. They used antibodies approved by FDA, including Cetuximab (Ctx), Trastuzumab (Her2) and Rituximab (Rtx) as the recognition portion of split structures and linked them to a FITC molecule. Co-administrating of anti-FITC CAR T cells with split pieces resulted in successful eradication of advanced pancreatic cancer, expressing uniform EGFR.

Furthermore, anti-FITC CAR T cell showed considerable anti-tumour activity against syngeneic Her2-expressing breast cancer and $CD20^+$ B cell lymphoma, when co-administered with FITC-Her 2 and FITC-Rtx (Table 1). Despite the initial delay in the growth of colon cancer following administration of FITC-CAR and FITC-Ctx, an outgrowth of EGFR-negative tumour cell lines surprisingly occurred. They also reported that non-specific FITC-IgG can easily disrupt the activity of anti-FITC CAR T cells and has the potential to be of use to stop the cell function if needed. They finally concluded that anti-FITC CAR T cells can be a useful flexible candidate for the treatment of patients suffering fromdifferent types of cancer, since they are regulatable via various kinds of split molecules. In contrast to the Urbanka *et al.* who studied avidin-biotin CAR T cells, this technology avoids non-specific interactions between endogenous biotin or biotin-like molecules with avidin *in vivo* [8].

Moreover, Jennifer et al. in 2016 developed another switch CAR T cell using antibodies, in which anti-CD19 and anti-CD22 antibodies are conjugated with different sites of FITC molecule, leading to an evoked, potent, antigen-specific and dose-dependent effector function of the anti-FITC CAR T cell. They also achieved multiple effective pseudo-immunological synapses between the anti-FITC CAR T cell and targeted antigens as CD19-expressing cancer cell lines and *in vivo* anti-tumour activities in a xenograft model (Table 1) [66].

In another study, Kim et al. in 2015 designed another FITC for CAR T cell therapy, based on a conjugation approach, using small switch molecules consisting of the fab portion of an antibody linked to FITC. They chose the folate receptor (FR) as a marker for targeting FR-over-expressing B-cell precursor leukemias and made FR-FITC switch molecules. They produced six different types of switch molecules using site-specific protein conjugation strategy in order to find out the effect of conjugation site of FITC on the effectiveness of the intervention. The results implicated that when FITC is conjugated to the proximal portion of fab, it shows higher potency compared with distally conjugated FR-FITCs. In addition, bivalent switch molecules were produced and the effect of valency on the activity of CAR T cells was evaluated. Bivalent split molecules were more effective than monovalent switch molecules and had two to three folds higher affinity. The co-administration of these molecules along with anti-FITC CAR T cells showed specific

Table 1
Studies related to the production and characteristics of conjugated CAR T cells.

Author	Year	Cancer	Mice/Cell lines	*Type of study*	E:T ratio	Target antigens	Split structures	CAR
Wenqi Chu et al.	2018	Lung	HEK293T, A549, THP-1, Jurkat, HL-60	*in vitro*	02:01	FRa, FRb	FITC-Folate, FRa ,FRb	3rd gen, CD8,4-1BB, CD3V
Erhao Zhang et al.	2018	Chronic myelogenous leukemia, erythroleukemia type	CEA+ and MSLN+ K562 cells	*in vitro*	1:2 and	integrin αvβ3	FITC-HM-3 bifunctional molecule (FHBM)	MSLN, AVb3 AND gate
				in vivo	05:01	human mesothelin (MSLN)		CD8, 4-1BB
L.R. Lourerio et al	2018	Breast cancer adenocarcinoma	MDA-MB-231, MCR NC	*in vitro*	05:01	Sialyl Tn	UniCAR	Anti-La mAb E5B9 fused to the
							Anti E5B9	Transmembrane domain of human CD28. Intracellularly, signaling domains of CD28 and CD3ζ
				in vivo	–		TM (target module)	Anti-STn TM
Jang Hwan cho et al.	2018	Myelogenous leukemia	FreeStyle 293-F, K562 cells, K562 cells HER2+Luc+, K562 cells HER2+Axl+Luc+, Jurkat NFAT-GFP, SK-BR-3	*in vitro*	E:T = 8:1, 4:1, 2:1, or 1:1	α-Her2, α-Axl, α-Mesothelin	zip-Fv, RR leucine zipper	Zip-Fv, RR leucine zipper, CD28, 4-1BB, CD3ζ
				in vivo				
Anna Wing et al.	2018	Ovarian, colon, pancreas, lung cancers	SKOV3, HCT116, Panc-1, and NCI-H226	*in vitro*	03:01	FRα, EGFR	BiTE anti-CD3-anti-RGFR	CD8α leader, FR binding site, CD8a hinge, ICOS, TM, CD3ζ
				in vivo				
Susann Albert et al.	2017	Pharynx squamus cell carcinoma, Epidermoid Carcinoma	FaDu, A431	*in vitro*	1:1, 5:1	a-EGFR	eu and pro E-tag, vhh EFGR	Anti-La mAb E5B9, transmembrane domain of human CD28. Intracellularly, signaling domains of CD28 and CD3ζ, anti αCD33, αCD123TM
				in vivo				
Anja Feldmann	2017	Prostate cancer	PC3, LNCaP, CHO	*in vitro*	1:1, 2:1, 5:1	PSCA, PSMA	TMs against PSCA and PSMA, anti-La 5B9 single chain fragment variable	Anti-La mAb E5B9, transmembrane domain of human CD28. Intracellularly, signaling domains of CD28 and CD3ζ, anti αCD33, αCD123TM
				in vivo				
E.P. Bajestani et al.	2017	Prostate cancer	CHO, PC3wt, PC3psca	*in vitro*	1:1, 2:1, 5:1, 10:1	anti-PSCA TM	5B9, anti-PSCA TM	Second-generation CAR-T, transmembrane domain of human CD28. Intracellularly, signaling domains of CD28 and CD3ζ cell recognizing the epitope 5B9,
				in vivo				
Yu Cao et al.	2016	Breast cancer	SKBR3, HCC1954, MDA MB453, MDA MB361, BT-20, MDA MB231, and MDA MB468, MDA MB435 and its Her2-transfected cell line, MDA MB435/Her2	*in vitro*		Her2	FITC-anti HER2, anti-FITC scFv, GCN4, anti-GCN4 scFv with 3 different affinities	the hinge, CD8 transmembrane region, and the cytoplasmic regions of human 4-1BB and CD3z
				in vivo				
M. Cartellieri	2016	AML	CHO, MOLM-13, MV4-11, OCI-AML3	*in vitro*	05:01		αCD33, αCD123	Anti-La mAb E5B9, transmembrane domain of human CD28. Intracellularly, signaling domains of CD28 and CD3ζ, anti αCD33, αCD123TM
				in vivo				
Jennifer S. Y. Ma. et al.	2015	B cell precursor leukemia	Nalm-6, Raji cells	*in vitro*	5:1, 10:1	CD19, CD22	anti- CD19&CD22 FITC, anti FITC scFv with different affinities	Second generation CAR : hinge and transmembrane region of the human CD8, cytoplasmic domains of human 4-1BB and CD3ζ
				in vivo				
Min Soo Kim et al.	2015	KERATIN- forming tumor cell	KB (FR+) cell lines, A549 (FR-) cell linse	*in vitro*	10:01	FR	anti-FITC scFv -FITC	2nd gen,CD3ζ,4-1BB,CD8

(*continued on next page*)

Table 1 (*continued*)

Author	Year	Cancer	Mice/Cell lines	*Type of study*	E:T ratio	Target antigens	Split structures	CAR	
M. Cartellieri	2014	Prostate cancer	PC3, PC3-PSCA, RT4	*in vitro*	1:1, 3:1, 6:1, 10:1, 12:1/ ECAR 5:1	PSCA, CD33	E-CAR, Emab coated bead	E-Tag, scFv, CD28 ECD/TM, CD28 SD, CD3ζ	
				in vivo					
Koji Tamada et al.	2012	Colon, breast, pancreatic adenocarcinoma, B-Cell lymphoma, mammary tumor	NSG, DBA/2, C57BL/6, and C3H/HeN mice	human pancreatic adenocarcinoma cell line expres- sing both EGFR and Her2	*in vivo*	0-10:1	EGFR	FITC	4th gen, CD8a,CD28, 4-1BB, CD3V
				(EGFR)-positive colon cancer SW480				Anti-EGFR/	
				Her2-positive AU565 breast cancer cell lines			Her-2	FITC, Anti-Her-2	
				Her2-positive AU565 breast cancer cell lines					
				mouse B-cell lymphoma line 38C13			CD20	FITC, Anti-CD20	
Jennifer D. Stone et al. (comparison of CAR and BiTE)	2012	Fibrosarcoma	BiTE CAR	Ag104A, ACosmc, Neuro2A, Neuro2A + OTS8	*in vitro*	05:01	237 epitope	CD3e, Anti-237	CD8a, CD28, CD3z
		Neuroblastoma				05:01		–	
Katarzyna Urbanska, et al.	2012	Ovarian cancer, malignant mesothelioma	Human ovarian cancer cell lines A1847, and mouse malignant mesothelioma cell line, AE17	*in vitro*	1:1, 5:1, 10:1, 30:1	Mesothelin P4, EpCAM and Fra	Avidin, biotin	(BBIR) comprising extracellular avidin in monomeric (mcAv) or dimeric (dcAv) form, linked to the intracellular human CD3-z chain signaling domain alone or in tandem with CD28, via a CD8α hinge and transmembrane region	
				in vivo					

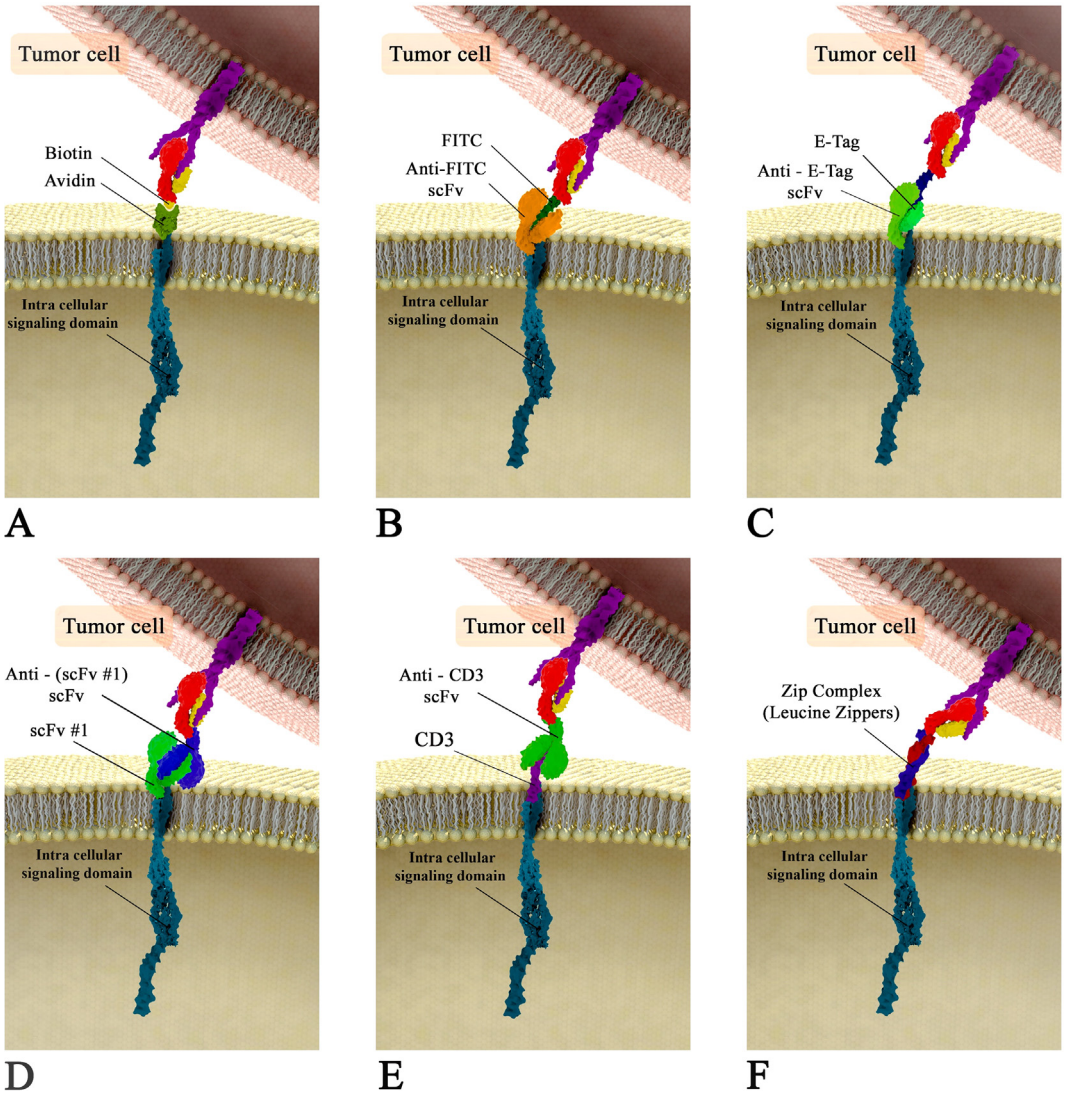

Fig. 2. A schematic demonstrating 6 strategies of conjugated CAR T cells; A) using avidin and biotin for the site of conjugation; B) Using FITC and anti-FITC scFv for formation of conjugation; C) using E-tag (LLa/SSb) and anti-E-tag scFv for formation of conjugation; D & E) using BiTe and scFv/CD3 for formation of conjugation; F) using leucine zippers for formation of conjugation.

activation, proliferation, and cytotoxicity against FR positive cells and had no effect on FR negative cells (Table 1). They also compared anti-FITC CAR T cell with conventional CAR T cell and reported that cytotoxic effect of the cells is comparable both *in vitro* and *in vivo*. Finally, they showed that the effect of anti-FITC CAR T cells can be terminated by the discontinuation of switch molecule administration [67].

Moreover, Cao et al. in 2016 compared two conjugation strategies for CAR T cell therapy. They produced anti-FITC and anti-GCN4 CAR T cells and designed split molecules using anti-Her2 fab portion bound to either FITC or Peptide NeoEpitope (PNE) chain to target breast cancer cells, respectively. They indicated that distal site to the antigen binding domain is the best conjugation site for FITC and PNE to switch molecules. The highest activity and cytotoxicity of anti-FITC CAR T cells were observed when bivalent switch molecules and CD8 hinge were used (Table 1), whereas for anti GCN4 CAR T cells the highest activation and cytotoxicity was achieved when IgG4m hinge structure was used. Accordingly, they concluded that in order to achieve the optimal function of the cell, immunological synapse needs to be formed at an optimal distance to the fab portion and cell membrane. This indicated that when using a split piece the hinge and cite of the conjugation should be accurately selected. They concluded that the numbers of immunological synapses need to be at an optimal quantity to target the cells effectively and achieve maximum cytotoxicity. Furthermore, they reported that cytotoxicity of the split CAR T cells is as an appropriate as conventional CAR T cells at any effector to target (E: T) ratios both *in vitro* and *in vivo* [68].

In order to improve the safety and flexibility of CAR T cell therapy in non-small-cell lung carcinoma (NSCLC), Wenqi et al. (2018) used a valid bispecific ligand folate-FITC CAR T cell, which targets both tumour cells and tumour-associated macrophages (TAMs). Consistent with other studies, they reported that its function relies on the coexistence of folate-FITC and FR-expressing target cells. They proposed the effectiveness of folate-FITC as a switch, due to its high affinity and specificity of folate to FR alpha and beta isoforms. So, by directing a single anti-folate-FITC CAR T cell, it would react against not only FRalpha-positive tumour cells, but also FRbeta expressing TAMs in a dose-dependent manner, which improves tumour microenvironment and CAR T cells activation. The plasma-clearance half-life of folate-FITC and the NSCLCs with no overexpression of FRalpha, in which they do not benefit folate-FITC as an adaptor can be considered as a limitation of this CAR T cell [69].

In a recent study, Zhang et al. (2018) improved CAR T cell activity through the simultaneous actions of dual-receptor CAR (sdCAR) specific for FITC and mesothelin (MSLN), and a bifunctional molecule (FHBM),

which are composed from a switch molecule (FITC) and a cognate tumour cell expressing avb3 and MSLN to control CAR T cells cytotoxicity. They showed a significant anticancer activity *in vitro* and *in vivo*, as well. In addition, they also compared the number of cytokines released from sdCAR T cell with the conventional CAR T cell and reported that following using conventional CAR T cells, the cytokine levels were twice as high as sdCAR T cells, and the mean peak concentration of cytokines was achieved after 20 hr and was gradually diminished. They also measured the half-life of FITC-HM molecules, which obtained 20.06 hr in mice (Table 1) [70]. The structure of FITC CAR T cell is shown in Fig. 2b.

4.3. E-CAR/Uni CAR T cell

Another conjugation strategy proposed by Cartelliery et al. to overcome the limitations of previously described strategies is to use a modular antibody-based platform technology called UniCAR. In this technique, the cross-linkage between effector cell and the target molecule is mediated by a complex of two components: 1) UniCAR T cell with a recognition domain against a peptide epitope (E5B9, UniCAR tag). This domain is derived from La/SS-B nuclear protein (E-Tag parts); 2) a target module (TM) consisting of a binding site against the targeted tumour-associated antigen, so the epitope which is recognised by UniCAR infused to TM redirects UniCAR T cells. This UniCAR can be inactivated by stopping TM infusion, when severe side effects are occurred or upon eradication of all tumour cells. Moreover, this CAR T cell can reversibly be equipped with multiple TMs and reduce the risk of the development of Ag loss tumours, which can adjust potential adverse effects. A series of UniCAR-TMs have recently been described based on this approach (See Table 2).

Cartelliery et al. (2016) used LA/SS-B protein and linked it to the anti CD33 and CD123 scFvs to target acute myeloid leukemia (AML). According to the studies on the necessity of additional co-stimulatory signals for the activation and expansion of CAR T cells, they constructed their UniCARs as a second generation by adding CD28 to the intracellular domain, which enables CAR T cell to produce and release IL-2 and consequently stimulate proliferation. Moreover, this CD28 signalling enhances the persistence of engineered T cells by preventing activated T cells from cell death. They also showed a great anti-cancer activity either *in vitro* and *in vivo* (Table 1). They proposed using multiple TMs and compared their effectiveness with bispecific TMs. As a result, they demonstrated that the administration of dual-specific TMs can be more efficient than using multiple monospecific TMs. They also determined the half-life of TMs in the body, which was approximately 1 and 6–8 h following I.V. and I.P. injections, respectively [71].

In another study, Feldmann et al. (2016) used UniCAR/TMs to treat metastatic prostate cancer as a solid tumour by targeting prostate stem cell (PSCA) and/or prostate specific membrane antigen (PSMA) both *in vitro* and *in vivo*. They also used second generation UniCAR T cell, which showed a significant anti-cancer effect in contrast with modular bispecific T cell engager (BiTE) approaches, as they had lower EC50 amounts compared with BiTEs (Table 1). Moreover, they indicated that the administration of multiple TMs leads to a significant rapid tumour lysis, whereas the total mean of cytokine secretion did not change compared with the control groups. Overall, they proposed a ranking: UniCAR = Conventional CAR > BiTE = modular BiTE [71].

Pishali et al. (2017) developed application of UniCAR T cell *in vivo*. They reported that upregulation of immune-inhibitory molecules, such as programmed death ligands (PDL) is responsible for the immunoevasion mechanism of solid tumours, induced by UniCAR T cell activation. Furthermore, they evaluated the half-life of TMs after I.V. and I.P. injection, which was obtained 16 and 96 min, respectively. They also calculated the half-life of TMs in tumour tissues 20.8 h. TMs cleared rapidly from the mice hearts. The half-life of alpha and beta phases calculated 7 and 2.6 h min, respectively. The half-life of UniCAR T cells in the bloodstream was also examined. The maximum levels of UniCARs was reported achievable after 4–6 weeks and began to drop after eight weeks. They also introduced peripheral blood cytokine levels as a reliable index to assess UniCAR activity, whereas circulating UniCARs could not be monitored as an anticancer activity of cells, since no significant correlation was observed between the amounts of cytokine secretion and intratumoural UniCAR T cells [9].

Albert et al. (2017) developed a novel TM against EGFR *in vitro* and *in vivo*, as well. They constructed TMs for the first time based on single-domain Abs (nanobody-based TMs), however in other studies it is based on scFvs from mAbs. In other words, they created alpha-EGFR TM by fusing the peptide epitope E5B9 to its C-terminus, which is recognized by the extracellular domain of UniCAR. They showed that both prokaryote and eukaryote alpha-EGFR TM redirect UniCAR T cells to EGFR-positive tumour cells, but the binding affinity of alpha-EGFR TM (prokaryote) was higher, and its EC50 value was about ten-fold lower compared with eukaryote -type. Moreover, they showed that TMs is rapidly removed by kidneys and its half-life was about 4–20 min, which provides a safety aspect for switching UniCAR T cells on and off. Moreover, they claimed that these nanobody-based TMs can not only be used for redirecting UniCAR T cells, but also they can be used as a tool for PET-imaging [72].

Loureiro et al. (2018) established a UniCAR approach for retargeting STn-positive cancerous cells. Sialyl-Tn (STn) modifies a malignant phenotype associated with poor tumour prognosis. Studies have shown it is overexpressed in more than 80% of carcinomas, but it has rarely found in healthy tissues. They have tried to develop UniCAR T cells, due to unsuccessful cancer therapies results by targeting STn. They reported that the function of UniCAR T cells depends on TMs. Cytokine release through retargeting tumour cells was also analyzed and it was demonstrated that none of the cytokines at a significant concentration can be used as an index to monitor UniCAR function. They also evaluated the clearance and half-life of TMs and showed that TMs are equally cleared by kidneys and liver, however kidneys were more active than the liver. The half-life of TMs in different tissues was 3.1, 7.8, 11.7, and 110 min in kidneys, liver, muscles and tumour sites, respectively. In total, they showed the effective eradication of STn-expressing breast- and bladder-associated cancerous cells both *in vitro* and *in vivo* and its potential as a diagnostic tool in PET-imaging for cancer [73]. Fig. 2c demonstrates the structure and function of E CAR T cell.

4.4. BiTE CAR T cell

Wing et al. (2018) proposed a new approach in the treatment of solid tumours. They used a conventional anti-FRα CAR T cell and loaded the cell with an Oncolytic Virus (OV) encoding BiTE consisting of anti-EGFR scFv linked to an anti-CD3 scFv. They reported a significant enhancement in anti-cancer activity of conventional CAR T cell indicating lytic effect of OV and retargeting effect of BiTE. They also reported an increase in the amount of cytokine secretion than the conventional CAR T cell, which shows higher cytotoxicity of OAd-CAR T cell, when compared with conventional CAR (Table 1). However, this increase in cytokine secretion may have a higher risk for CRS. Their biodistribution studies also confirmed that OAdBiTE significantly enhances the accumulation of CAR T cell in tumour site either in the presence or absence of targeting Ag (FR in this study) (Fig. 2c). Therefore, they overcame the main challenges facing CAR T cell therapy for solid tumours [10].

Another approach using BiTE against T cell CD3 and tumour-specific scFv (Fig. 2d) has been used and reported previously, but as the split structure was not based on the CAR design, it is not further discussed in this article [74].

4.5. SUPRA CAR T cell

Although all described CAR T cells help to ensure the safety and efficacy of CAR T cell, all features are not included. Hwan et al. (2018)

Table 2
Studies related to function of conjugated CAR T cells.

Author	Cytotoxicity	EC50	%Lysis	Concentration of cytokines chemokines	Extended survival days	Extended tumor free days	Concentration of split structure
Wenqi Chu, et al.	20–50%	0.094 nM	–	IFN-g (80000 pg/ml)	–	–	
Erhao Zhang , et al	77 ± 5%	~10 pM	Survival rate: less than 20% (% lysis = > 80%)	IFN-g (15–20 ng/ml), IL-2 (600–800 pg/ml)	–	–	100 pM
			Survival rate: 30–40% (% lysis = 60–70%)	IFN-g (30 ng/ml), IL-2 (1300 pg/ml), IL-6 (1160 pg/ml), TNFα (380 pg/ml)			0.5 mg/kg
L.R. Lourerio et al.	–	25 nM	40–50%	GM-CSF (200–300 pg/ml), IFN-g (200–300 pg/ml/ml), IL-2 (100–200 pg/ml), TNFα (200–300 pg/ml), IL-6 (400–600 pg/ml),			80 nM
Jang Hwan cho et al.	40–60–80% according to the tuned affinity	EE = 1000 pm, SYN3 = 8000, SYN5 = 20000	–	E:T ratio of 2:1 (IFN-g (EE = 2000, SYN3 = 1000, SYN5 = 500 ng/ml), IL-2 (1300 pg/ml))			EE = 5000 pm, SYN3 = 100000, SYN5 = 200000
				IFN-g (500–1000 pg/ml)	more than 40 days (prevents xenograft tumor)		
Anna Wing. Et al							
Susann Albert et al.		eu = 1.7 nM, pro = 0.1 nM	5:1 eu = 20%, pro = 40% 5:1 eu = pro = 40–60%; 1:1 eu = pro = 20–40%	GM-CSF (eu = 1000-1500, pro = 1500 - 2000 pg/ml), IFN-g (eu = 1000, pro = 1500 pg/ml), IL-2 (eu = 500 – 1000, pro = 1500 – 2000 pg/ml), IL-4 (eu = not detectable, pro = 200–250 pg/ml), IL-9 (150–200 pg/ml), TNF-a (400–800 pg/ml)			
			eu – 20–40%, pro = 40–60%				50 nM
Anja Feldmann		Unicar = 12.7 pM = αPSCA TM, 12.4 pM = αPSMA TM, modular BiTE : αPSCA TM = 378 pM, αPSMA TM = 629 pM	Unicar = 50, BiTE = 60	GM-CSF (1500 pg/ml), IFN-g (600–700 pg/ml/ml), IL-2 (500 pg/ml), TNFα (200–300 pg/ml), IL-6 (less than 20 pg/ml), IL-4 (600 pg/ml), IL-17A (less than 20 pg/ml)IL-10 (less than 20 pg/ml)			
					at day 5, no more luciferase activity can be detected in all the fifteen treated animals		300 pM per mouse of each or both
E.P.Bajestani et al.	e:t = 1:1, 55%	0.4 ng/ml	1:1 = 15%, 2:1 = 20%, 5:1 = 30%, 10:1 = 40%				1 nM
				IFN-g (750 pg/ml), TNF-α (25–30 pg/ml),IL-18 (1200 pg/ml), IL-4 (15–20 pg/ml)	80% survival after 13 weeks	7 days extended days of survival	250 ng 7KATMPSCA per gram body weight
Yu Cao et al.							
M. Cartellieri		αCD33 = 131.6, αCD123 = 44.9 pM, αCD33 + αCD123 = 80.2, αCD33-αCD123 = 11.7	αCD33 + αCD123 = 60–80, αCD33 − αCD123 = 50–70	GM-CSF (3000 pg/ml), IFN-g (4000 pg/ml), IL-2 (300 pg/ml), IL-13: (700 pg/ml)			αCD33 + αCD123 = 5.5 log pM, αCD33- αCD123 = 4.5 log pM
					2–3 weeks		250 ng/g

(*continued on next page*)

Table 2 (*continued*)

Author	Cytotoxicity	EC50	%Lysis	Concentration of cytokines chemokines	Extended survival days	Extended tumor free days	Concentration of split structure
Jennifer S. Y. Ma. et al.	60–80%	5 different affinities, from 0.9 to 2.3 PM		IFN-g (1000 pg/ml/ml), IL-2 (1500 pg/ml), TNFα (500–700 pg/ml), IL-6 (less than 20 pg/ml), IL-4 (less than 500 pg/ml), IL-10 (less than 100			0.5 mg/kg or PBS every other day for a total of six doses (intravenously)
Min Soo Kim et al.	77 ± 7.2%	8.7 pM	–	IFN-g (1500–2000 pg/ml), IL-2 (800–1000 pg/ml)	-	-	
M. Cartellieri	60–80%		60–80%	Emab coated bead = IFN-g (1* ECAR28/ζ pg/ml), IL-2 (0.8 * ECAR28/ζ pg/ml),TNF a (1* ECAR28/ζ pg/ml), ECAR = IFN-g (4000–6000 pg/ml), IL-2 (4000–6000 pg/ml),TNF a (1000–2000 pg/ml)			
		20 μg/ml			more than 125 days (prevents xenograft tumor)		40 μg/ml
Koji Tamada et al.	–	–	40%	IFN-g (1000–10000 pg/ml), IL-1 (100–1000 pg/ml) ,IL-2 (100 pg/ml) , IL-3 (1000–10000 pg/ml), IL-4 (10000–100000 pg/ml), IL-5 (1000–10000 pg/ml), IL-6 (10–100 pg/ml), IL-9 (100–1000 pg/ml), IL-10 (1000–10000 pg/ml), IL-12 (100–1000pg/ml), IL-13 (10000–100000 pg/ml), IL-17 (10–100 pg/ml), Eotaxin (1000–10000 pg/ml), G-CSF (100–1000 pg/ml), GM-CSF (1000–10000 pg/ml), CXCL1 (10–100 pg/ml), MCP-1 (100–1000pg/ml), MIP-1a (100000–1000000 pg/ml), MIP-1b (10000–100000pg/ml), RANTES (100–1000pg/ml), TNF-a (100–1000 pg/ml)	15–30 (pancreatic & sw480), 60 (38C13)	15 (SW480)	
Jennifer D. Stone et al. (comparison of CAR and BiTE)	Comparison of BiTE and CAR T-cell						
Katarzyna Urbanska et al.			1:1 = 20%, 10:1 = 40%, 30:1 = 80%	IFN-g (8000–10000 pg/ml), TNF-α (25–30 pg/ml),IL-18 (1200 pg/ml), IL-4 (15–20 pg/ml)			

designed a new split universal and programmable CAR T cell platform named SUPRA-CAR consisting of two components, including zipCAR and zipFv. zipCAR is an adaptable receptor expressed on T cells, which has a leucine zipper as an extracellular domain fused to intracellular signalling domains, whereas zipFv are switch molecules containing scFv and a cognate leucine zipper. Binding scFv to tumour antigens, leucine zipper pairs can bind to each other and consequently activate SUPRA CAR against target cells. Accordingly, by designing different zipFvs they could target multiple cancerous antigens with a single SUPRA-CAR without further genetic manipulations. They suggested tunable CAR T cell engineered by different affinities between leucine zipper pairs, binding affinity between tumour-associated antigen and scFv, the concentration of zipFv, and expression level of zipCAR. Fig. 2e illustrates the structure of SUPRA CAR T cells and zipFvs.

They generated multiple zipFvs with the same scFv but different affinity to zipCARs, resulting in an inverse correlation between the amounts of zipFv needed to activate T cell and affinity between leucine zipper pairs. They showed the affinity and dosage of each zipFv can be varied to personalised-defined T cell activation. It has also showen that there is a direct correlation between the amount of IFN-ɣ secretion as a killing efficacy and the affinity between leucine zipper pairs. Furthermore, they reported a direct relationship between the expression level of zipCAR and activated T cells induce cytokine. Moreover, a similar direct correlation was found between the affinity between leucine zipper pairs and cytokine production by activated T cells. Therefore, they concluded that T cell toxicity can be decreased by controlling zipFv concentration, the affinity between leucine zipper pairs and expression level of zipCAR *in vitro* and *in vivo*. SUPRA-CAR is a cell sorter, which is regarded as another critical aspect, since it contains two types of cells, expressing either Her2 or Her2 and Axl, in which Axl acts as a safety marker and prevents SUPRA-CAR T cell activity. They also showed OFF switch and OR-gate implementation of SUPRA-CAR T cell to reduce cytokine secretion and combat antigen escape, respectively. Leucine zipper domains derived from human transcription factor were used to humanize SUPRA-CAR components in order to reduce their potential immunogenicity and test their system against two different tumour models to demonstrate it is widely used. Eventually, in order to regulate different signalling pathways, they used multiple orthogonal SUPRA CARs, which leads to independent control of different immune cell types, resulting in a base to a prosthetic immune system [11].

5. Conclusion

CAR T cell therapy has been introduced as a promising practical therapy for patients suffering from haematological malignancies. All the options into account, CAR T cell therapy is the most effective method in cancer treatment, since it is resulted in improvement and recovery Controlling cellular activity, both chronological and functional, using On/Off, and/or switches and adjustable affinity of different parts of the receptor is another outstanding characteristic of these cells. As a result, the therapist is provided with a spectrum of effectiveness and is able to adjust these parameters to achieve the best results on the specificity and cytotoxicity in both hematologic and solid tumors.

Nevertheless, several side effects have been reported as the activity of the conventional CAR T cells *in vivo* can not be adjusted. Currently, the significant challenges facing CAR T cell therapies include the limited tumor-specific antigens, the inhibitory microenvironment of solid tumors, off-target toxicity, definite longevity of T cells, immunologic response of the host to the administered cells or split structures and cytokine release syndrome (CRS), which is currently being monitored and controlled for every regimen of CAR T cell therapy.

Furthermore, according to the various studies, tumor recurrences are mostly due to cancer stem cells (CSC) with different antigens, which are resistant to conventional therapies. This fact is not considered in the mentioned CAR T cell therapies.

There is a vital need to discriminate between normal tissues and tumor microenvironment in order to redirect the CAR T cells to the desired site. Therefore, More research is needed to find tumor-specific antigens in order to accurately target tumor cells and minimize their off-target activity. Moreover, the immunologic response against the CAR T cell itself, can be controlled through autograft T cells or deleting allogeneic TCR and MHC of the cells, whereas there are few studies to conclude that these manipulations are enough to avoid side effects.

In addition, in order to overcome the inhibitory microenvironment of the tumour, the therapist can alter this microenvironment, using agents like monoclonal antibodies targeting active inhibitory factors, like PD1/PD-L1. Another strategy is to manipulate the cells to be desensitized to inhibitory factors, genetically.

Regarding the longevity of effector cells, different approaches are made, including either genetically manipulation of the cell to hinder the ageing effect, or preconditioning the cells via some factors, like L-Arginine.

In order to adjust the effectiveness of the CAR T cell and overcome abovementioned challenges and improve patient safety, soluble switch molecules have been suggested by several researchers in *in vitro* and *in vivo* preclinical studies.

According to the mentioned studies, the therapist can adjust the characteristics of the cells in split approach, by manipulating split structure, which minimises genetic manipulation on CAR T cell and significantly enhances the effectiveness of therapy, whereas the conventional CAR T cell is entirely based on genetic manipulation. In this respect, manipulations are greatly convenient using conjugated CAR T cell compared with the conventional cells. The therapist can change the target of the cell by easily changing the soluble fragments. It is also possible to target multiple antigens at the same time. Another possibility is to code the targeting ability of the cell using multiple soluble fragment in a way that different combinations of soluble fragments result in different functions, which also enables a more effective discrimination of target cells from normal cells and therefore, reduction of off-target toxicity. Termination of the therapy is another aspect which is now easier using these fragments. This can be made either chronologically or actively. Since the soluble fragments show limited half-lives in the body, after a particular period, there will be no more soluble fragments to activate the cell. The other way is to actively add the antidote of the fragments and terminate the function when needed.

Regarding its flexible nature, the adjusted conjugated CAR T cells are the excellent opportunity for personalized medicine to be involved in this field and to determine the optimum concentrations, regimen, and method of cell therapy for each subject. CAR T cell therapy is newly developed and there is a need to carry out more investigations on their effectiveness.

The split approach in CAR T cell therapy impacts the current knowledge and outcomes CAR T cell therapy in different aspects, including tumour diagnosis, treatment and the total cost of cancer cell therapy. This split CAR uses a universal receptor as the basis, allows covering tumour heterogeneity by targeting multiple TAAs without the necessity to regenerate new CAR T cell.

Using immunological marked split structures alone, we will be able to evaluate different parameters in cancer and evaluate forcoming cell therapy strategies. This includes the diagnosis of tumour by the administration of marked split structures targeting suspected tumour antigens, evaluation of distal metastasis and staging the cancer, distribution of different antigens in tumour mass/masses, evaluation of the optimum ratio for application of split structures, evaluation of the off-target effect of CAR T cell therapy and calibrating the optimum number of effector cells needed to deal with cancer prior administration of effector CAR T cells.

Implicating conjugated CAR T cells in cancer immunotherapy seems promising, since it can be combined with different cancer treatments, including immunotherapy, chemotherapy, and radiation.

Regarding the flexible nature of conjugated CAR T cell therapy, the fact that designing CAR T cell can be done only once for each patient, and the changes are made in split structures when needed, the cost for each therapy regimen is decreased significantly. On the other hand, since conjugated cells are more effective than conventional cells, it is comprehensible that the total cost of therapy also decreases significantly, since the number of regimens needed declines using split CAR T cells. This indicates that the maximum cost-effectiveness can be achieved in CAR T cell therapy using conjugated strategy.

There are still some critical limitations, which are needed to be more investigated. An important issue is finding specific cancer antigens that are not expressed in human healthy tissues or have a minimal expression, which can be resolved possibly by the advance of bio-molecular methods to detect new antigen in the future. Another issue is the limited capacity of CAR T cell to create genetic manipulations without affecting their survival and activation in *in vitro* and *in vivo* conditions, that can be eliminated by identifying useful factors in longevity and durability while maintaining cell function and increasing the accuracy of gene expression modalities.

The non-specific release of oncolytic viruses by CAR-T cell, despite their specific function, is another safety concern that can be solved by designing smart intracellular synthetic compartments that only release the oncolytic virus in the tumor environment.

The similarity of CSC antigens with other healthy stem cells of the human body is another limitation to use CAR T cells, which can be resolved by investigating new cancerous features that are exclusively specific for tumor masses.

In addition, mathematical modelling was not possible due to the limited and heterogeneous information extracted from the studied articles. It is strongly recommended that the exact amounts of EC50, cytotoxicity, cell lysis and released cytokine be reported in future studies on conjugated CAR T cells, so a universal formula for the application of these cells can be obtained. Furthermore, by mathematical modelling for each conjugated CAR T cell strategy, the therapist will be able to determine the exact needed amount of each split structure to induce the expected cytotoxicity, cell lysis, and cytokine release.

Although studies have shown the possibility to redirect engineered conjugated CAR T cells activity with switch molecules, some types of switch molecules are semi-synthetic or fully-recombinant, which may cause immunogenicity reactions in patients under treatment. In addition, Dose- titratable control is also vital to ensure the safety of CAR-T cells. It means that this type of CAR T cell therapy should begin using low doses. However, in several cases, these switch molecules do not have short half-life, making it challenging to bypass in emergencies.

No clinical trial has yet been registered using conjugated CAR T cell. Through the conjugated CAR T cells in clinical stage, new guidelines and regimens for cancer cell therapy using these cells will be introduced and used clinically. Moreover, designing a comprehensive new artificial immune system is considerable through this approach for each aspect of the immune system. It is also predictable that CAR T cell therapy will be generalized to treat other diseases, like hepatitis, HIV and other systemic diseases, by targeting specific factor for each condition. On the other hand, by manipulating and using inhibitory and regulatory immune cells, the therapist will be able to affect hyperinflammatory states, like rheumatologic disease, age-related macular degeneration (AMD), and other non-cancerous diseases and control them.

Overall, conjugated CAR T cells have a bright future ahead for cancer therapy, and it would definitely promote public health in the next ten years.

Declaration of Competing Interest

There is no conflict of interest.

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Appendix A. Supplementary data

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