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REVIEW

Targeted immunotherapy of cancer with CAR T cells: achievements and challenges

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Abstract The adoptive transfer of chimeric antigen receptor (CAR)-expressing T cells is a relatively new but promising approach in the field of cancer immunotherapy. This therapeutic strategy is based on the genetic reprogramming of T cells with an artificial immune receptor that redirects them against targets on malignant cells and enables their destruction by exerting T cell effector functions. There has been an explosion of interest in the use of CAR T cells as an immunotherapy for cancer. In the pre-clinical setting, there has been a considerable focus upon optimizing the structural and signaling potency of the CAR while advances in bio-processing technology now mean that the clinical testing of these gene-modified T cells has become a reality. This review will summarize the concept of CAR-based immunotherapy and recent clinical trial activity and will further discuss some of the likely future challenges facing CAR-modified T cell therapies.

Keywords T cell · Gene modification · Chimeric antigen receptor · Cancer · Immunotherapy

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Introduction

The concept of the chimeric antigen receptor (CAR; also known as T-bodies or chimeric immune receptors) was originally described over 15 years ago by Zelig Eshhar and colleagues working at the Weissman Institute in Israel [1]. The approach was based upon the idea of expressing novel receptors on the T cell surface that would enable the T cell to identify intact protein antigens present on the surface of a target cell. T cells generally recognize peptide antigens that are presented in association with major histocompatibility complex (MHC) proteins by the target cell. However, one well-documented tumor escape mechanism is the modulation or down-regulation of MHC on the surface of the tumor cell [2] which thereby effectively renders the tumor "invisible" to T cells, since binding of the T cell receptor to peptide-MHC is a pre-requisite for T cell effector function. However, the direct recognition of protein antigens through a CAR would then make the tumor cell "visible" to T cell immune surveillance once more. Moreover, the use of a targeting system that functions independently of MHC means that the CAR can be used in a generic manner rather than having the restrictions that are imposed by the use of T cell receptor (TCR) approaches where the specific receptor is only suitable for patients expressing a specific MHC. In addition, CARs can substantially broadened the range of antigens recognizable by T cells to include carbohydrate [3] and glycolipid tumor antigens that are not within the scope of TCR-based recognition [4, 5]. Consequently, these factors make the use of CARs highly attractive for adoptive gene-modified T cell therapy. The reader is also directed to other highly relevant and recent reviews of CAR T cell biology [6–8] and clinical application and focused reviews detailing novel directions of CAR T cell therapy approaches [9].

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The basic CAR structure: extracellular and transmembrane protein domains

The basic CAR consists of an extracellular antigen-recognition domain attached to an extracellular spacer domain, a trans-membrane region that anchors the receptor to the cell surface and a signaling endodomain. Single-chain antibody fragments (scFv) consisting of the variable heavy (V_H) and a variable light (V_L) chain isolated from an antibody linked by a flexible linker have been used extensively as the antigen-recognition domain in many CARs due to their small size, which facilitates both the genetic manipulation and expression of the CAR (Fig. 1). The scFv determines the CAR antigen specificity and binds the target protein in a MHC-independent, non-restricted manner, most commonly with the specificity and affinity similar to that of the antibody from which it was derived [10]. More recently, non-scFv-based ligand-binding domains have also been successfully utilized in a CAR format including endothelial growth factor polypeptide, an integrin binding peptide, heregulin, IL-13 mutein, and NK cell receptor NKG2D [11–14].

In most CARs, the antigen-recognition domain is connected to the transmembrane region by means of an extracellular spacer domain. The rationale for this is to distance the recognition domain from the membrane and to potentially make it more "accessible" to bind target. Spacer regions used tend to be comprised of common Ig-like domains due to the stability of the protein domain and have included immunoglobulin Fc or an extracellular fragment derived from CD28, TCRβ chain, CD8α, or NKG2D [13–19]. However, the absolute requirement for an extracellular spacer domain most likely depends upon the position of the target epitope with respect to the target cell surface. CARs targeting epitopes toward the proximal end of protein antigens tend to function well without spacer domains while CARs targeting epitopes buried closer to the target cell membrane appear to demonstrate improved function when a flexible spacer region is included [20, 21]. These observations suggest that there is still a requirement for the empirical testing of CARs with varying extracellular domains in order to clearly identify the optimal receptor for each target antigen.

In a similar manner, various transmembrane regions have also been employed in CARs including those derived from CD28, CD3ζ, CD8, CD4, or FcεRIγ [15, 17, 19, 22, 23]. While CARs bearing any of these protein domains have been shown to function in terms of down-stream signaling after antigen ligation, the structural and biochemical impact of the transmembrane domain upon the CAR remains largely unknown. Recent studies by Bridgeman et al. [24] investigating CARs employing the CD3ζ transmembrane domain indicate that the biochemical interactions that occur between the wild-type CD3ζ transmembrane domain and other components of the endogenous TCR/CD3 complex are important for the optimal activity of the CD3ζ CAR. These studies suggest that the over-expression of the CD3ζ containing CAR permits an increased level of endogenous TCR expression on the transduced T cell since the availability of CD3ζ protein represents the rate-limiting factor controlling cell surface TCR/CD3 complex expression in T cells; this translates to an increased responsiveness to mitogenic anti-CD3 antibody stimulation. Whether this confers an advantageous or deleterious effect upon T cells engrafted with CD3ζ transmembrane domain containing CARs in vivo remains unknown. However, there is a general lack of understanding of the specific structural and biochemical nature of the majority of CARs and, in particular, the specific effects that the range of extracellular and transmembrane domains impart upon the endogenously expressed TCR in the CAR-expressing T cell.

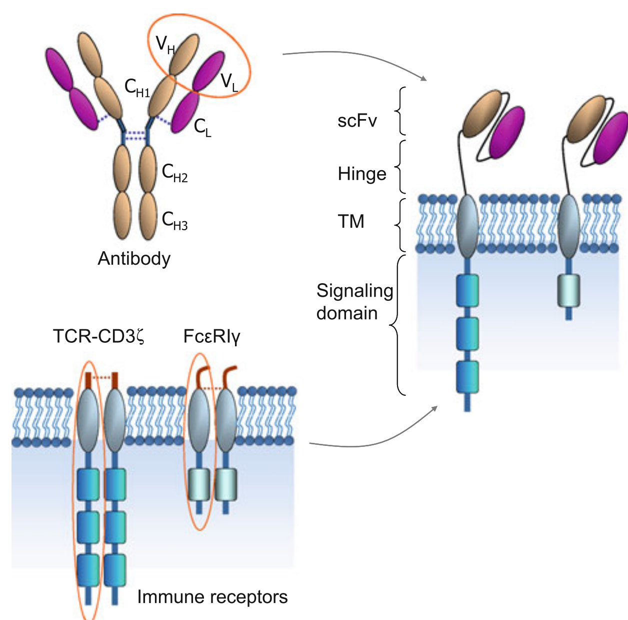

Fig. 1 The structure of a CAR. CARs consist of a scFv derived from an antibody variable heavy (V_H) and variable light (V_L) fragments, a hinge region linking the scFv with a transmembrane (TM) domain and signaling domain, most often CD3ζ or FcεRIγ. C_L—constant region of light immunoglobulin chain, C_{H1}–C_{H3}—constant region of heavy immunoglobulin chain, *dotted line* corresponds to disulfide bound

Basic CAR structure: signaling domains

In keeping with the plethora of options available for the extracellular domains of the CAR, there has been an ever-increasing range of intracellular signaling domains shown to function in the context of a CAR. Initial studies employed a single signaling domain, most commonly

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derived from the CD3ζ chain or the γ chain of the high-affinity IgE Fc receptor (FcεRI), referred to as FcRγ (Fig. 1). CARs consisting of either signaling domain proved capable of activating T cell effector function including target cell cytotoxicity and cytokine release in response to target antigen binding [25]. However, head to head in vivo studies suggested that mouse T cells engrafted with CD3ζ CARs demonstrated improved anti-tumor activity as compared to those bearing the FcRγ signaling domain [26].

CARs bearing signaling domains from a single receptor have been subsequently termed "first-generation" receptors. The modular nature of the CAR lends itself to further engineering involving combining intracellular signaling domains that can potentially increase the potency of the CAR. The full activation of T cells requires multiple signals, and it is clear that signaling from these first-generation CARs only supplied the so-called "signal one" that could drive T cell effector functions, but in the absence of further signals (signal two or co-stimulation), the T cells were unable to fully engage its effector machinery and therefore undergoes apoptosis.

The most studied co-stimulatory pathway has been ligation of T cell CD28 receptors with the B7 family of ligands. Ligation of CD28 on CAR T cells through the expression of B7 co-stimulatory ligands on target cells [15, 27, 28] or co-expression of the CD28 molecule together with the scFv and CD3ζ domains of the CAR [29] was shown to lead to proliferation of CAR-modified T cells and enhanced anti-tumor activity. Exploiting the modular nature of the CAR enabled the engineering of "second-generation" CARs where the signaling domains of two receptors were expressed within one CAR (Fig. 2). The fusion of CD28 and CD3ζ into a single CAR has proven to be effective in permitting the repeated antigen stimulation and proliferation of CAR-expressing T cells in the absence of exogenous co-stimulatory ligands, maintaining the ability to demonstrate both antigen-specific cytolysis and to secrete IL-2 in vitro [16, 28, 30, 31]. Recent findings have also shown that CARs containing the CD28 domain could also rescue activated T cells from antigen-induced cell death (AICD) [32] and enhance the resistance of CAR-modified T cells to regulatory T cells (Treg) activity [33].

Experiments in animal models confirmed these in vitro observations and demonstrated that T cells engrafted with CARs containing the CD28 signaling domain had prolonged survival, produced higher levels of cytokines, proliferated vigorously, and mediated enhanced anti-tumor effect compared to T cells expressing the conventional CARs [34–36]. These reprogrammed T cells have also been observed to proliferate robustly, even without administration of exogenous IL-2 [37].

Several other co-stimulatory receptors have been studied including OX40 (CD134) [38], 4-1BB (CD137) [39], DAP10, and ICOS [31, 40]. Although all these constructs showed antigen-dependent cytotolysis in vitro, only 4-1BB CARs showed enhanced persistence and anti-tumor activity in vivo [39, 41]. All other second-generation CARs failed to produce sufficient amounts of IL-2 to promote T cell proliferation in the absence of exogenous co-stimulation. However, when the exogenous B7 co-stimulation was delivered,

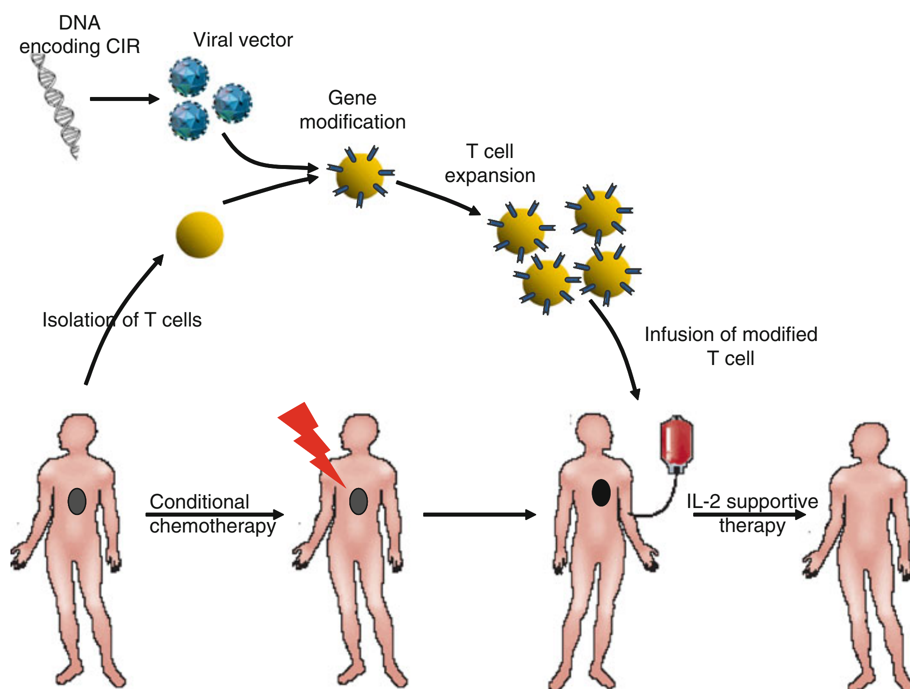

Fig. 2 Clinical protocol of adoptive transfer of CAR-expressing T lymphocytes. T lymphocytes are collected from a cancer patient and retrovirally transduced with CAR genes, then expanded ex vivo to large numbers and infused back into the patient. To facilitate engraftment of CAR-modified T lymphocytes in vivo, the patient is given a lymphodepletive chemotherapy regimen prior to infusion and cytokine support post-infusion

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transgenic T cell expressing these CARs produced IL-2 and showed improved proliferation as compared to T cells engrafted with CD3ζ alone CARs under the same conditions [31].

This finding paved the way for the engineering of "third-generation" CARs that contain CD28 and one other co-stimulatory domain, most often OX40 or 4-1BB, fused with the activation domain (Fig. 2). These CARs were found to drive equivalent levels of cytolysis as observed in second-generation CARs [38, 39, 42] but drove higher and more prolonged levels of cytokine production and cell proliferation [38].

The hierarchy of CAR T cell co-stimulation, reflected in the sequence of CAR co-stimulatory and activation domains, appears to directly translate into in vivo anti-tumor efficacy with second-generation CARs being superior to first-generation and third-generation outperforming the second-generation. However, the latest finding by Cheadle and colleagues suggests that functional activity of CAR-expressing T cells may be dependent not only upon the optimal combination of CAR signaling moieties but also upon the endogenous physiological receptor interactions provided by target cells [43]. Murine T cells engrafted with a first-generation CD19-specific CAR were found to produce IL-2 following co-culture with $CD19^+$ B-cell lymphoma cells independently of CD28 receptor ligation, with the production of IL-2 driven by endogenous CD2 receptor activity. This finding suggests that the optimization of CAR signaling domains according to target cells could possibly be the way forward in achieving the best anti-tumor effect rather than use of a universal CAR against all target cells.

As previously mentioned, the majority of engineered CARs contain the CD3ζ signaling moiety that exploits TCR proximal kinases signaling pathway. CARs based on alternative signaling domains utilizing TCR distal signaling events have also been engineered. These CARs include signaling domains derived mainly from the protein tyrosine kinase (PTK) Syk family and are capable of triggering antigen-dependent T cell activation with IL-2 production and target cell lysis [44]. Although the efficiency of Syk-based CARs is not superior to CD3ζ-based CARs, their main potential advantage is the ability to bypass TCR proximal signaling events, which are often defective in cancer patients, and directly trigger downstream signal transduction machinery.

Initial clinical trials with CAR-modified T cells

Clinical protocols for CAR T cell therapy usually involve the isolation of autologous T lymphocytes from cancer patients, their ex vivo modification with the CAR genes followed by large-scale expansion. These genetically modified T lymphocytes are then infused back into the patient, usually with administration of IL-2 to support their viability and function. To facilitate the engraftment and persistence of CAR-modified T cells, cancer patients are also pre-conditioned prior to the cell transfer (Fig. 2).

A number of first-generation CARs showed some success in pre-clinical models and subsequently entered phase I clinical trials. The tumors targeted included ovarian cancer [45], renal cell carcinoma [46, 47], neuroblastoma [48, 49], B-cell non-Hodgkin lymphoma (NHL), and mantle cell lymphoma (MCL) [50]. Unfortunately, despite promising preclinical results, the majority of these initial CAR T cell trials showed little evidence of anti-tumor activity with limited activation, persistence, and homing to tumor sites being the main barriers. However, responses were seen in a small number of trials with some anti-tumor responses being reported in B-cell lymphoma patients treated with αCD20-CD3ζ T cells, in which two patients were reported to maintain a previous complete response, one patient achieved a partial response and four patients achieved stable disease [50]. Further anti-tumor responses were also seen in patients with neuroblastoma treated either with CAR T cells targeting L1-cell adhesion molecule (L1CAM) [48] or diasialo-ganglioside (GD2) antigen [49]. To date, though, the GD2 targeting trail is the one in which first-generation CAR T cells have mediated a durable, complete response in cancer patients [51].

Although clinical studies of first-generation CARs produced rather modest clinical outcomes, they established the feasibility and safety of CAR-adoptive transfer therapy. This in turn paved the way for further improvements of CAR signaling capacity and resulted in the engineering of second- and third-generation CARs. These improved CARs are capable of delivering superior strength and quality activation signal, resulting in increased proliferation, cytokine release, and effector functions of CAR-modified T cells in vitro and in pre-clinical models. Both the second- and the third-generation CARs are now entering the clinical arena, and their therapeutic potential is under intense investigation (Table 1). Encouragingly, the first clinical reports from these studies have been published and have shown some promising results [52–56].

In a pilot clinical study with αCD19.4-1BB.CD3ζ T cells in three patients with advanced chronic lymphocytic leukemia (CLL), two complete remissions and one partial response that has been ongoing 10 months after the treatment has been achieved [52, 54]. The engineered T cells were found to expand over 3-logs in these patients, infiltrated and lysed tumor cells, and persisted at high levels for over 6 months. Interestingly, a fraction of these cells displayed a memory T cell phenotype, suggesting the potential for preventing tumor relapses.

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Table 1 Representative list of CAR T cell clinical trials (generated from a review of the clinical trials.gov database)

Antigen targeted[a]	Disease[b]	CAR generation	CAR endodomain[c]	CAR gene transfer[d]	Phase	Clinical trial. gov identifier	Center[e]
CD19	CLL	Second	CD3ζ/CD28	RV	I	NCT00466531	MSKCC
CD19	B-ALL	Second	CD3ζ/CD28	RV	I	NCT01044069	MSKCC
CD19	Leukemia	Second	CD3ζ/CD28	RV	I	NCT01416974	MSKCC
CD19	Lymphoma/leukemia (B-NHL)/CLL	First and second	CD3ζ/CD28 versus CD3ζ	RV	I	NCT00586391	BCM
CD19	B-NHL/CLL	First and second	CD3ζ/CD28 versus CD3ζ	RV	I	NCT00608270	BCM
CD19	Advanced B-NHL/CLL	First and second	CD3ζ/CD28 versus CD3ζ (EBV)	RV	I	NCT00709033	BCM
CD19	ALL	First	CD3ζ EBV	RV	I/II	NCT01195480*	UCL
CD19	Lymphoma/leukemia	Second	CD3ζ/CD28	RV	I/II	NCT00924326	NCI
CD19	ALL post-HSCT	Second	CD3ζ/CD28	RV	I	NCT00840853	BCM
CD19	Lymphoma/leukemia	First and second	CD3ζ/4-1BB versus CD3ζ	LV	I	NCT00891215	UP
CD19	B-cell leukemia/CLL/B-NHL	Second	CD3ζ/CD28	RV	I	NCT01087294	NCI
CD19	B-lymphoid malignancies post-HSCT	Second	CD3ζ/CD28	Electroporation/SB plasmid	I	NCT00968760	MDACC
CD19	B-lineage lymphoid malignancies post-UCBT	Second	CD3ζ/CD28	Electroporation/SB plasmid	I	NCT01362452*	MDACC
CD19	B-NHL	First	CD3ζ	RV	I	NCT01493453+	CHMAN
CD19	B-NHL	First	CD3ζ	LV	I/II	NCT01318317*	COH
CD20	Mantle cell lymphoma/indolent B-NHL	Third	CD3ζ/CD137/CD28	Electroporation	I	NCT00621452	FHCRC
GD2	Neuroblastoma	First	CD3ζ (EBV)	RV	I	NCT00085930	BCM
PSMA	Prostate cancer	First	CD3ζ	RV	I	NCT00664196	RWMC
PSMA	Prostate cancer	Second	CD3ζ/CD28	RV	I	NCT01140373	MSKCC
CEA	Breast cancer	Second	CD3ζ/CD28	RV	I	NCT00673829	RWMC
CEA	Colorectal carcinoma	Second	CD3ζ/CD28	RV	I	NCT00673322	RWMC
Her2/neu	Lung malignancy	Second	CD3ζ/CD28	RV	I	NCT00889954	BCM
Her2/neu	Advanced osteosarcoma	Second	CD3ζ/CD28	RV	I	NCT00902044	BCM
Her2/neu	Glioblastoma	Second	CD3ζ/CD28 (EBV)	RV	I/II	NCT01109095	BCM
Kappa light chain	B-NHL and B-CLL	First and second	CD3ζ/CD28 versus CD3ζ	RV	I	NCT00881920	BCM

[a] *CEA* carcinoembryonic antigen, *GD2* diasialoganglioside, *Her2* human epidermal growth factor receptor, *PSMA* prostate-specific membrane antigen

[b] *B-ALL* B-lineage acute lymphoblastic leukemia, *B-NHL* B-lineage non-Hodgkin's lymphoma, *CLL* chronic lymphocytic leukemia, *HSCT* hematopoietic stem cell transplantation, *UCBT* umbilical cord blood transplantation

[c] *EBV* Epstein-barr virus-specific CAR-modified T cells

[d] *LV* lentiviral, *RV* γ-retroviral, *SB* sleeping beauty transposon/transposase DNA plasmid system

[e] *BCM* Baylor College of Medicine, Houston, Texas, USA, *COH* City of Hope Medical Center, Duarte, California, USA, *FHCRC* Fred Hutchinson Cancer Research Center, Seattle, Washington, USA, *MDACC* M.D. Anderson Cancer Center, Houston, Texas, USA, *MSKCC* Memorial Sloan-Kettering Cancer Center, New York, USA, *NCI* National Cancer Institute, Bethesda, USA, *RWMC* Roger Williams Medical Center, Providence, Rhode Island, USA, *UCL* University College London, UK, *UP* University of Pennsylvania, Philadelphia, USA, *CHMAN* Christie Hospital, Manchester, UK

* Trials due to open

+ Trial suspended temporarily due to re-location of cell processing facility

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