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Armored CAR T-cells: utilizing cytokines and pro-inflammatory ligands to enhance CAR T-cell anti-tumour efficacy

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Abstract

Chimaeric antigen receptor (CAR) T-cells are T-cells that have been genetically modified to express an artificial construct consisting of a synthetic T-cell receptor (TCR) targeted to a predetermined antigen expressed on a tumour. Coupling the T-cell receptor to a CD3ζ signalling domain paved the way for first generation CAR T-cells that were efficacious against cluster of differentiation (CD)19-expressing B-cell malignancies. Optimization with additional signalling domains such as CD28 or 4-1BB in addition to CD3ζ provided T-cell activation signal 2 and further improved the efficacy and persistence of these second generation CAR T-cells. Third generation CAR T-cells which utilize two tandem costimulatory domains have also been reported. In this review, we discuss a different approach to optimization of CAR T-cells. Through additional genetic modifications, these resultant armored CAR T-cells are typically modified second generation CAR T-cells that have been further optimized to inducibly or constitutively secrete active cytokines or express ligands that further armor CAR T-cells to improve efficacy and persistence. The choice of the 'armor' agent is based on knowledge of the tumour microenvironment and the roles of other elements of the innate and adaptive immune system. Although there are several variants of armored CAR T-cells under investigation, here we focus on three unique approaches using interleukin-12 (IL-12), CD40L and 4-1BBL. These agents have been shown to further enhance CAR T-cell efficacy and persistence in the face of a hostile tumour microenvironment via different mechanisms.

Keywords

adoptive immunotherapy; armored chimaeric antigen receptor T-cell (CAR T-cell); chimaeric antigen receptor

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Conflicts of interest

R.J. Brentjens is a scientific co-founder of, reports receiving a commercial research grant from, has ownership interest (including patents) in and is a consultant/advisory board member for JUNO Therapeutics. No potential conflicts of interest were disclosed by the other author.

Introduction

Chimaeric antigen receptor (CAR) T-cell therapy represents an exciting new paradigm in the treatment of patients with cancer [1]. Adoptive transfer of CAR T-cells has shown significant promise in the treatment of haematologic malignancies and to a lesser extent, solid tumours. The CAR construct typically consists of a single chain variable fragment (scFv) directed against a known tumour antigen [2]. This is followed by a signalling domain, typically cluster of differentiation 3ζ (CD3ζ), which provides the so called 'signal 1', necessary for T-cell activation [2]. Engagement and signalling via the ζ chain is required for T-cell stimulation and proliferation but is not often sufficient for sustained proliferation and activity in the absence of a second signal or 'signal 2'. Preclinical studies using first generation CAR T-cells were promising when directed against cluster of differentiation (CD)19 [3] and HER2/Neu [4]. In both cases, there was robust activation of the CAR T-cells when exposed to cells expressing the target antigen followed by effective target cell killing *in vitro* and in preclinical *in vivo* tumour models [3]. Unfortunately, anti-tumour efficacy was not seen in subsequent clinical trials. For example, in a phase I study of patients with metastatic renal cell carcinoma using first generation CAR T-cells directed against an epitope on carbonic anhydrase IX (CAIX), there were no objective clinical responses [5]. Unfortunately, patients treated on this trial developed acute liver toxicity attributed to CAR T-cell therapy [6]. Furthermore, the authors found induction of a human anti-chimaera response (HACA) and limited peripheral persistence of the infused CAR T-cells *in vivo* [5]. In another report, Till et al. [7] treated patients with indolent non-Hodgkin lymphoma with a first generation CAR against CD20, an antigen commonly expressed on normal and malignant B-cells. Of the eight patients treated, two patients who had already achieved a complete response (CR) after cytoreductive therapy remained in CR and only one other patient achieved a partial response. Notably, there was no host-generated immunoreactivity to the CAR T-cells in these patients. In order to address some of the shortcomings of first generation CAR T-cells, further genetic modifications were made to include a CD28 costimulatory domain that functioned independently of its ligand B7. These CD28/CD3ζ (CD28ζ) domains provide both 'signal 1' and 'signal 2' for T-cell activation upon antigen recognition on the target cell and mitigate the anergy and activation-induced cell death seen with first generation CAR T-cells [8]. To test the efficacy of second generation CAR T-cells, Brentjens et al. [9] used CAR T-cells directed against CD19-expressing NALM-6 cells in an ALL (acute lymphoblastic leukaemia) xenograft tumour model and reported significantly enhanced anti-tumour efficacy. Similarly, enhanced activation and efficacy was reported in a preclinical model of prostate cancer using PSMA (prostate specific membrane antigen) directed CD28ζ second generation CAR T-cells [10]. Savoldo et al. [11] compared first and second generation CAR T-cells (CD19ζ compared with CD19–28ζ) targeted against CD19, an antigen commonly expressed on normal and malignant B-cells in patients with non-Hodgkin lymphoma. After infusing patients with both anti-CD19ζ and anti-CD19-28ζ CAR T-cells simultaneously, anti-CD19–28ζ CAR T-cells showed vastly superior expansion, persistence and infiltration of tumour sites compared with anti-CD19ζ CAR T-cells in the same patients. Patients with relapsed B-cell ALL treated with anti-CD19-28ζ CAR T-cells had a rapid response to therapy in all five patients treated [12]. In another study, Davila et al. [13] reported an 88% CR rate in patients with relapsed/refractory B-cell malignancies

treated with anti-CD19-28ζ CAR T-cell therapy [13]. Second generation CAR T-cell therapy utilizing 4-1BB, another commonly used costimulatory molecule, has also shown efficacy in the treatment of haematologic malignancies including chronic lymphocytic leukaemia (CLL) [14,15]. Further optimization has led to the development of 'third generation' CAR T-cells which utilize two distinct costimulatory domains (e.g. CD28/4-1BB/CD3ζ or CD28/OX-40/CD3ζ). These constructs have shown varying degrees of *in vitro* and *in vivo* levels of activation, proliferation and interleukin-2 (IL-2) production [16–18].

This review focuses on the optimization of CAR T-cell efficacy via additional genetic modifications designed to secrete cytokines, or express ligands that are known to enhance or interact with endogenous immune cells such as dendritic cells (DCs), macrophages or regulatory T-cells (Treg cells) [19]. These so-called armored CAR T-cells have been specifically designed to survive, disrupt and/or favourably modulate an otherwise immunosuppressive tumour microenvironment. In solid tumour malignancies where exciting preclinical CAR T therapy has not translated in clinical gains, these armored CAR T-cells represent a potential advancement in CAR T-cell therapy. Here we focus on three distinct armored CAR T-cell approaches utilizing IL-12, CD40L and 4-1BBL.

IL-12

IL-12 is a potent inflammatory cytokine consisting of a heterodimeric p35 and p40 subunit which constitutes the active IL-12 p70 protein [20]. IL-12 is not secreted by T-cells but is produced by DCs, macrophages and neutrophils and has been shown to induce polarization of CD8 + T-cells to a pro-inflammatory TH1 ($CD62L^{hi}$, $IL\text{-}7R\alpha^{hi}$, $IL\text{-}2R\alpha^{hi}$, $Sca1^{hi}$) phenotype, enhance antigen cross presentation and reprogramme myeloid derived suppressor cells [21]. Furthermore, IL-12 enhances the cytotoxic ability of CD8 + T-cells and leads to increases in interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and granulocyte macrophage colony stimulating factor (GM-CSF) secretion [22]. Taken together, introduction of IL-12 into the tumour microenvironment is predicted to improve CAR T-cell cytotoxic function, mitigate Treg-suppression and reprogramme tumour-associated macrophages and DCs (Figure 1). Multiple preclinical studies demonstrate anti-tumour efficacy of intra-tumoural injection of IL-12 expressing vectors [23,24] or IL-12 expressing bone marrow derived DCs [25] without any significant toxicity in mice. Pegram et al. [26] showed that treatment with anti-CD19–28ζ/IL-12 CAR T-cells in a syngeneic mouse EL4 (expressing human CD19) tumour model led to improved tumour eradication without the need for cytotoxic chemotherapy preconditioning [26]. Furthermore, IL-12 was reported to function in an autocrine manner via IL-12 receptors on the CAR T-cells and significantly mediate resistance to Treg inhibition [26]. A study by Kerkar et al. [27] reported IL-12 toxicity in mice characterized by weight loss and decreased survival. In this study, the authors speculated that this was due to the dose of adoptively transferred IL-12 producing T-cells. In clinical trials, attempts at systemic IL-12 treatment have not been as successful. For instance, systemic IL-12 treatment in patients with metastatic renal cell carcinoma led to severe side effects and treatment-related mortality [28]. The phase II trial had to be halted after a single dose of 500 mg/kg of IL-12, given the development of grade 3/4 fatigue, dyspnoea, stomatitis, leukopenia, elevated transaminases and two treatment-related mortalities. Although these toxicities were felt to be due to the schedule of IL-12

administration, regardless, there has been reluctance to further clinical development of systemic IL-12 therapy. If IL-12 is targeted to the tumour microenvironment, less IL-12 could be needed to provide anti-tumour efficacy while mitigating systemic side effects. Since CAR T-cells traffic to the tumour site, modifying CAR T-cells to secrete IL-12 could serve as a mechanism to deliver IL-12 to the tumour microenvironment. By engineering the IL-12 gene under the control of an IRES (internal ribosomal entry site) promoter, we were able to generate constitutive IL-12 producing CAR T-cells that secreted less IL-12 than would be needed for systemic administration and we did not observe any adverse effects in mice [26]. As mentioned above, we were able to improve tumour eradication and the requirement for preconditioning using this approach [26]. In a SCID Beige mouse model of ovarian cancer, we were also able to show superior efficacy of IL-12 armored CAR T-cells directed against the ectoplasmic domain of MUC16 ($MUC16^{ecto}$) [29]. In a syngeneic mouse model of ovarian cancer, we observed increased CAR T-cell expansion, cytokine production, cytotoxic effect and reprogramming of tumour-associated macrophages using our $MUC16^{ecto}$-directed CAR T-cells (unpublished work; Chekmasova, A. Renier Brentjens Laboratory). Most importantly, none of the previously described toxicities were seen in mice treated with our IL-12 armored CAR T-cell. However, it should be noted that mouse models do not completely recapitulate IL-12 toxicity in humans. Clinical trials utilizing IL-12 armored CAR T-cells are currently underway [30].

CD40L

CD40L (CD154) is a type II transmembrane protein that binds to its cognate receptor, CD40, a member of the TNF-α receptor superfamily [31]. Expression of CD40L is induced upon activation of CD4 + T-lymphocytes, but its expression has also been described on DCs, macrophages and B-cells [32]. Upon binding to CD40 (CD138) on DCs, CD40L mediates CD8 + T-cell immunity via secretion of inflammatory cytokines such as IL-12 and IFN-γ, increases downstream antigen presentation and DC antigen licensing [33]. In a murine experimental model, animals lacking CD40L expression showed a deficiency in antigen-dependent T-cell priming and activation [34]. Engagement of CD40L/CD40 also increases T-cell proliferation and CD8 + T-cell immunity and memory [35]. Furthermore, CD40 engagement by CD40L on CD40-expressing tumour cells has been shown to change the tumour phenotype [36] and induce direct tumour apoptosis [37]. Due to the prominent role of immunosuppressive macrophages and DCs in the tumour microenvironment (Figure 2), several preclinical studies have attempted to utilize CD40L expression as a means of improving T-cell efficacy. In one study, infusion of adenovirus encoding murine CD40L in CLL patients led to an increase in inflammatory cytokines (IL-12 and IFN-γ) and an objective anti-tumour response [38]. Other studies have also utilized agonistic CD40 antibodies with evidence of DC activation and anti-tumour efficacy [39,40]. Curran et al. [36] generated a CAR vector with an additional CD40L gene designed to constitutively express CD40L. *In vitro*, CD40L-expressing CAR T-cells co-cultured with DoHH2 tumour cells altered the tumour phenotype to increase susceptibility to apoptosis and increase immunogenicity via up-regulation of costimulatory molecules (CD80, CD86), HLA molecules and Fas-death receptor on tumour cells. Furthermore, CD19-directed CAR T-cells with constitutive CD40L expression showed increased *in vitro* cytotoxicity compared with

CD19-directed second generation CAR T-cells. This enhanced efficacy resulted in a significant survival benefit in DoHH2 tumour bearing mice treated with CD40L armored CAR T-cells compared with control second generation CAR T-cells. CD40L armored CAR T-cells represent a promising approach to improving CAR T-cell therapy and are currently under consideration for clinical trials.

4-1BBL

4-1BB and its ligand, 4-1BBL, belong to the tumour necrosis factor receptor and tumour necrosis factor superfamily respectively [41]. 4-1BB is expressed on T-cells and is known to facilitate cell activation, survival and proliferation upon binding to its ligand, 4-1BBL [41]. 4-1BB has long been described as a stimulatory receptor on T-cells [42]. Additionally, 4-1BB expression is present on monocytes, DC, Tregs, neutrophils and natural killer cells (NK cells) [43]. Of note, 4-1BB expression has been reported on tumour vasculature but not on tumour cells [44]. 4-1BB signalling has also been shown to increase the expansion of CD8 + T-cells [45] and play key roles in central memory T-cell maintenance [46]. Binding of 4-1BB on DCs by 4-1BBL enhances secretion of IL-12, IL-6 and other inflammatory cytokines necessary for T-cell activation [47]. Taken together, these studies provided the rationale for the incorporation of constitutively expressed 4-1BBL on engineered CAR T-cells due to the predicted effect of enhanced CAR T-cell activity, cytotoxic function and tumour microenvironment modulation (Figure 3). Preclinical studies by Stephan et al. [48] showed T-cells co-transduced with CD80 and 4-1BBL showed robust proliferation and increased cytokine production compared with T-cells transduced with either construct alone. Furthermore, they were able to show successful eradication of disseminated prostate cancer (LNCap) cells in a SCID Beige model using PSMA directed 4-1BBL armored CAR T-cells. They also showed evidence for transcostimulation of tumour-specific bystander T-cells, opening the possibility of broadening anti-tumour activity via antigen spreading. In a recent study, Zhao et al. [49] compared the persistence and functionality of first generation, second generation and 4-1BBL armored CD-19 targeted CAR T-cells. Compared with first (19ζ1) and second (1928ζ and 19BBζ) generation CAR T-cells, 4-1BBL armored CAR T-cells showed enhanced *in vitro* efficacy. *In vivo*, mice treated with 1928ζ/4-1BBL CAR T-cells had the highest survival and tumour eradication rates. Furthermore, this approach provided a comparably increased tumoricidal profile and increased CAR T-cell persistence. The authors were further able to show that this anti-tumour efficacy was mediated by IRF7 (interferon regulatory factor 7), a transcription factor regulating the interferon type I (IFN-1)/interferon-β (IFN-β) pathway. The authors postulated that IFN-β could inhibit Treg activation [50], augment tumour antigen cross presentation [51] and disrupt tumour microvasculature [52]. All these factors are proposed to synergistically amplify the efficacy of CAR T-cells and produce ongoing anti-tumour activity even after the infused CAR T-cells are no longer detectable. The ability of 4-1BBL to not only induce intrinsic proliferation and activation of CAR T-cells, but also modify the landscape of the tumour microenvironment by recruiting endogenous T-cells has made it a promising candidate currently under clinical development.

Conclusions

CAR T-cells are an exciting new development in cancer immunotherapy. The ability to target CAR T-cells to virtually any tumour-associated antigen (TAA) provides an unparalleled flexibility that has yet to be fully exploited. With each new generation of CAR T-cells, there has been step-wise improvement in anti-tumour efficacy and CAR T-cell persistence. However, despite these improvements, solid tumour malignancies remain an unconquered frontier in CAR T-cell immunotherapy. Despite impressive preclinical results, there have not been any significant clinical gains. One plausible explanation for this discrepancy is the immunosuppressive effect of the tumour microenvironment that cannot be fully recapitulated in current *in vitro* and *in vivo* animal models.

Armored CAR T-cells represent the next generation of optimization that focuses on reinforcing modified CAR T-cells against the influences of a hostile tumour microenvironment. By carefully optimizing second or third generation CAR T-cells with additional genetic modifications such as IL-12, CD40L or 4-1BBL, CAR T-cell efficacy and persistence could be further improved, especially for hard-to-treat haematologic or solid tumour malignancies.

Although the opportunities and benefits of armored CAR T-cells are apparent, there are also unexpected on-tumour off-target side effects. In the case of IL-12, the toxicities of elevated systemic levels have been well described [27,28]. In the case of interleukin-15 (IL-15), another promising cytokine for application in armored CAR T-cells, concerns of T-cell leukemogenicity has tempered some of the excitement surrounding this candidate [53]. No such concerns have been reported for CD40L or 4-1BBL armored CAR T-cells but as previously alluded to, our murine models might not always be accurate and complete predictors of toxicity or response.

As we glean better understanding of the tumour microenvironment and how it overcomes endogenous and adoptively transferred T-cells, we can better design CAR T-cells that are armored against these inhibitory influences. Research efforts directed at understanding how armored CAR T-cells interact with endogenous T-cells and other elements of the immune system are urgently needed. Additionally, armored CAR T-cells with other cytokines, combination of cytokines or novel ligands need to be explored. It is not difficult to envision rationally designed armored CAR T-cells to different types of haematologic and solid tumour malignancies based on what is known about the nature of the inhibitory microenvironment. In fact, combination of different types of armored CAR T-cells such as cytokine secreting CAR T-cells and ligand-expressing CAR T-cells can be explored based on the niche of the tumour.

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Abbreviations

ALL	acute lymphoblastic leukaemia
CAR T-cell	chimaeric antigen receptor T-cell
CD19ζ	cluster of differentiation 19ζ
CD28ζ	cluster of differentiation 28ζ
CLL	chronic lymphocytic leukaemia
CR	complete response
DC	dendritic cell
IFN-β	interferon-β
IFN-γ	interferon-γ
IL-12	interleukin-12
IL-15	interleukin-15
PSMA	prostate specific membrane antigen
TNF-α	tumour necrosis factor-α
Treg	regulator T-cells

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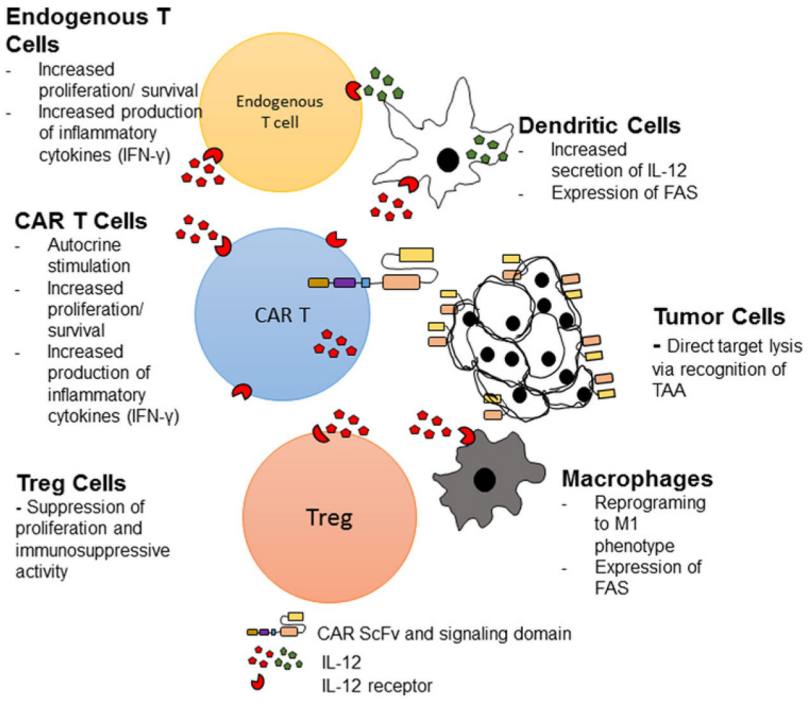

Figure 1. Armored CAR T-cells genetically engineered to constitutively secrete IL-12

CAR T-cells modified to secrete IL-12 improve CAR T anti-tumour efficacy by potentially modulating the tumour microenvironment via autocrine activity on CAR T-cells, suppression of Treg cells, reprogramming of tumour-associated macrophages and enhanced IL-12 production by DCs.

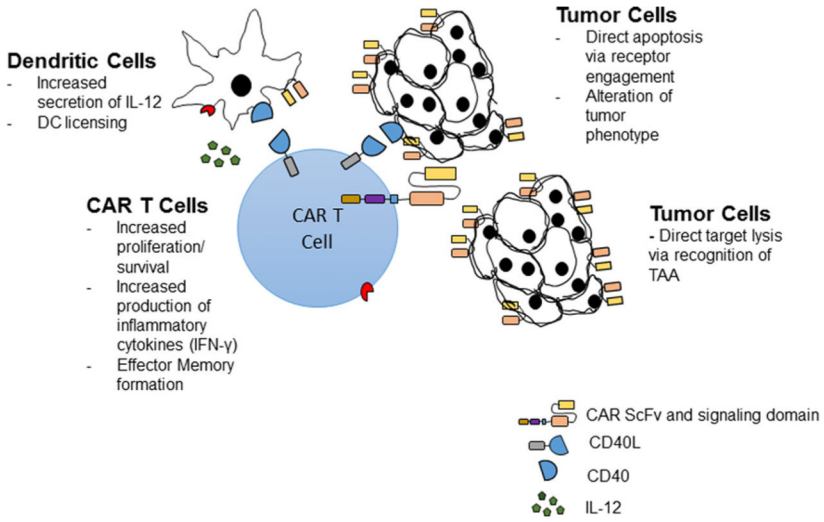

Figure 2. Armored CAR T-cells genetically engineered to constitutively express CD40L

CAR T-cells constitutively expressing CD40L improve CAR T anti-tumour efficacy by enhancing IL-12 secretion by DCs, maintaining effector T-cell memory and mediating direct tumour apoptosis upon CD40 engagement.

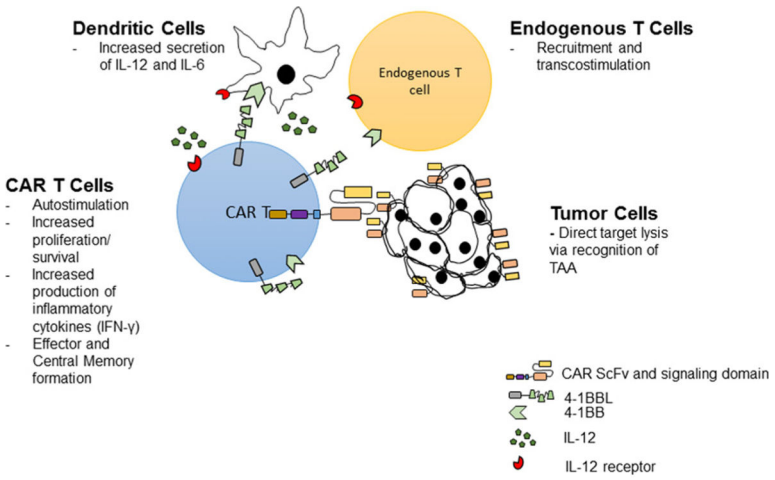

Figure 3. Armored CAR T-cells genetically engineered to constitutively express 4-1BBL

CAR T-cells constitutively expressing 4-1BBL improve CAR T anti-tumour efficacy by potentially modulating the tumour microenvironment via enhancement of DC IL-12 secretion, transcostimulation of endogenous T-cells and central memory maintenance.