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Review

Review

Enhancing co-stimulation of CAR T cells to improve treatment outcomes in solid cancers

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Summary

Co-stimulation is a fundamental component of T cell biology and plays a key role in determining the quality of T cell proliferation, differentiation, and memory formation. T cell-based immunotherapies, such as chimeric antigen receptor (CAR) T cell immunotherapy, are no exception. Solid tumours have largely been refractory to CAR T cell therapy owing to an immunosuppressive microenvironment which limits CAR T cell persistence and effector function. In order to eradicate solid cancers, increasingly sophisticated strategies are being developed to deliver these vital co-stimulatory signals to CAR T cells, often specifically within the tumour microenvironment. These include designing novel co-stimulatory domains within the CAR or other synthetic receptors, arming CAR T cells with cytokines or using CAR T cells in combination with agonist antibodies. This review discusses the evolving role of co-stimulation in CAR T cell therapies and the strategies employed to target co-stimulatory pathways in CAR T cells, with a view to improve responses in solid tumours.

Keywords: chimeric antigen receptor, immunotherapy, T cell immunology, co-stimulation

Abbreviations:APC: Antigen presenting cell; CAR: Chimeric antigen receptor; CD: Cluster of differentiation; DAMP: Danger-associated molecular pattern; DC: Dendritic cell; FDA: American Food and Drug Administration; HSP: Heat shock protein; ICR: Inverted cytokine receptor; OV: Oncolytic virus; PAMP: Pathogen-associated molecular pattern; scFV: Single-chain fragment variable; Th_1: T helper type 1; TME: Tumour microenvironment; TLR: Toll-like receptor; TIL: Tumour-infiltrating lymphocyte; NK: Natural killer; VSV: Vesicular stomatitis virus.

Introduction

Immunotherapies are an increasingly prevalent therapeutic option for patients with cancer. Chimeric antigen receptor (CAR) T cell immunotherapy is a strategy to genetically engineer patient T cells with a synthetic receptor targeting a specific antigen [1]. The CARs are composed of an antigen binding single-chain fragment variable (scFV) extracellular domain, transmembrane domain, and the intracellular CD3ζ and co-stimulation signalling domains. CAR T cells are currently FDA approved for the treatment of certain B cell malignancies [2]. However the overall responses are disappointing in solid cancers [3]. This is due to several factors such as an immunosuppressive tumour microenvironment (TME), poor trafficking into the tumour and limited persistence of CAR T cells [4].

Optimal T cell activation results from cognate antigen recognition (signal 1), co-stimulation (signal 2), and cytokine support (signal 3). The precise timing and context of co-stimulation signals are understood to ultimately define the effectiveness of the T cell response [5]. Integrating this understanding with CAR T cell design will lead to more robust CAR T cell therapies for solid cancers. This review will summarize the role of co-stimulation in CAR T cell therapies with a focus on strategies to improve responses in solid cancers.

Importance of co-stimulation in CAR design

The first generation of CARs were developed more than 30 years ago. These CARs contained a single CD3ζ chain but did not include any co-stimulation intracellular domain, thus had limited anti-tumour function due to the lack of co-stimulation. In an early phase I study using the first-generation CAR against alpha-folate receptor (FR) in metastatic ovarian cancer, none of the treated patients developed any anti-tumour response, demonstrating the importance in incorporating co-stimulation in the CAR design [6]. The first studies exploring the use of co-stimulation in CAR T cells included a CD28 co-stimulation intracellular domain into the CAR receptor [7]. CD28 co-stimulation domain greatly enhanced CAR T cell function leading to early clinical responses to CAR T cell therapy, highlighting the importance of co-stimulation signalling [8, 9]. In an early trial, a patient with advanced follicular lymphoma was treated with a CD19-CAR that contained a CD28 co-stimulation domain. This patient's cancerous B cells were eliminated and absent for at least 39 weeks after CAR T cell transfusion. Inspired by the success, other co-stimulatory domains have been included in CARs and some trials have demonstrated great success [8, 9].

Until now only a limited number of co-stimulatory domains have been thoroughly investigated [10]. CD28 and 4-1BB (CD137) are the best characterized domains and the only two included in current FDA approved CAR T cell formulations (Table 1). These domains trigger distinct downstream signalling pathways resulting in either increased persistence or enhanced effector function of CAR T cells [11]. The selection of co-stimulatory domains within the CAR is believed to be key to overcoming barriers imposed by solid tumours. Screening approaches have demonstrated a wide range of novel candidate co-stimulatory domains which can be incorporated into CARs [12]. To this end, many groups are exploring additional domains such as OX40 (CD134), CD27, GITR (CD357), and ICOS (CD278) [13–17] (Fig. 1-1). CARs including one co-stimulatory domain are classified as second generation, while those including two co-stimulatory domains are classified as third generation. Third-generation CARs demonstrated superior anti-tumour responses and magnitude of *in vivo* expansion compared to second-generation CARs in some studies. Ramos *et al.* demonstrated that third-generation CAR T cells persisted longer and with superior *in vivo* expansion compared to second-generation CAR T cells in relapsed/refractory non-Hodgkin lymphoma patients [18]. However, other studies have demonstrated opposing results. For example, a study comparing the second-generation anti-PSCA-CD28 CAR with the third-generation anti-PSCA-CD28-4-1BB CAR indicated that the second-generation CAR was superior in their anti-tumour effect in a human pancreatic cancer xenograft model [19]. The superiority of third-generation CARs is therefore still debatable. Collectively, these studies demonstrated that co-stimulation within the CAR receptor is a key factor determining CAR T cell efficacy.

Co-stimulation delivered intrinsically within the CAR can be coupled with other methods of co-stimulation to overcome the key barriers imposed by solid cancers. Some novel designs include co-stimulatory domains from certain signalling pathways. CARs incorporating MyD88 domains along with intracellular domains of CD40 demonstrated improved efficacy. The incorporation of these 'MC' co-stimulatory domains resulted in increased long-lived central memory CAR T cells associated with improved clinical outcomes [20, 21]. Coupling co-stimulation and CAR engagement affords precise control over when and how co-stimulation is delivered. Other strategies may include transducing additional genes that code for cytokines, synthetic signalling domains and receptors into the CAR T cells. For example, a study included a JAK-STAT signalling domain into a CAR to resemble γ-chain cytokine signalling and

Table 1. FDA-approved CART therapies and their associated co-stimulatory domains. Data collected from Clinicaltrials.gov and fda.gov

Product	Company	Target	Disease	Co-stimulatory domain	Clinical Trial
KYMRIAH (tisagenlecleucel)	Novartis	CD19	Diffuse large B cell lymphoma (DLBCL), high grade B-cell lymphoma and DLBCL arising from follicular lymphoma.	4-1BB	NCT02445248
YESCARTA (axicabtagene ciloleucel)	Kite Pharma	CD19	DLBCL not otherwise specified, primary mediastinal large B-cell lymphoma, high grade B-cell lymphoma, and DLBCL arising from follicular lymphoma.	CD28	NCT02348216
BREYANZI (lisocabtagene maraleucel)	Juno Therapeutics	CD19	DLBCL, high-grade B-cell lymphoma, primary mediastinal large B-cell lymphoma, and follicular lymphoma grade 3B	4-1BB	NCT02631044 NCT03484702 NCT03744676 NCT03310619 NCT03483103 NCT03331198 NCT03743246 NCT03435796
ABECMA (Idecabtagene vicleucel)	Celgene Corporation	BCMA	Relapsed/refractory multiple myeloma	4-1BB	NCT03361748 NCT02215967 NCT02658929
TESCARTUS[a] (brexucabtagene autoleucel)	Kite Pharma	CD19	Relapsed/refractory mantle cell lymphoma	CD28	NCT02601313

[a]TESCARTUS employs the identical retroviral vector to YESCARTA however is manufactured using a distinct protocol which enriches for T cells.

resulted in increased CAR T cell proliferation *in vivo* in a model of oesophageal cancer [22]. Including domains such as this within the CAR circumvents potential cytokine release syndrome (CRS) associated with non-specific secretion of cytokine and avoids administration of toxic cytokines. Toll-like receptors (TLR) are known co-receptors in T cells, and CARs incorporating TLR domains are being developed [23]. TLR2 is expressed on memory T cell subsets and detects pathogen-associated molecular pattern (PAMPs) and endogenous danger-associated molecular patterns (DAMPs), such as heat shock protein (HSPs) and amyloids [24, 25]. Unlike commonly used domains, TLR2 signals through MyD88 to improve cytokine secretion and effector function in T cells [26, 27]. The incorporation of TLR2 domains improved the efficacy of MUC1-CAR T cell function in a solid tumour model [28].

Synthetic and combinatorial co-stimulatory receptors enhance CART cell function

CAR T cells can be transduced to express additional synthetic receptors, which act in *trans* or parallel with CAR receptors to provide co-stimulation to CAR T cells. These receptors often target molecules overexpressed by the TME. Switch receptors link a checkpoint extracellular domain to a co-stimulatory intracellular domain, for example, PD-1 (CD279) and CD28 [29] (Fig. 1-2). This PD-1-CD28 receptor delivers CD28 co-stimulation to CAR T cells when ligating PD-L1 (CD274), which is overexpressed by solid tumours. The ligation leads to enhanced cytokine secretion and restimulation of the switch CAR cells. In two models of mesothelin and several $PSCA^+$ solid tumours, the switch CAR anti-tumour effect is stronger than the non-switch CAR cells used in combination with pembrolizumab (anti-PD-1), indicating that signalling through CD28 of the switch receptor is driving this effect [30]. Inverted cytokine receptors (ICR) function similarly to switch receptors but leverage the abundance of immunosuppressive cytokines in the TME [31, 32]. ICRs couple an extracellular domain of an immunosuppressive cytokine receptor such as IL-4 with an intracellular signalling domain of a pro-survival cytokine receptor such as IL-7. These receptors deliver pro-survival cytokine signals (signal 3) in the presence of suppressive cytokines in the TME (Fig. 1-2). For

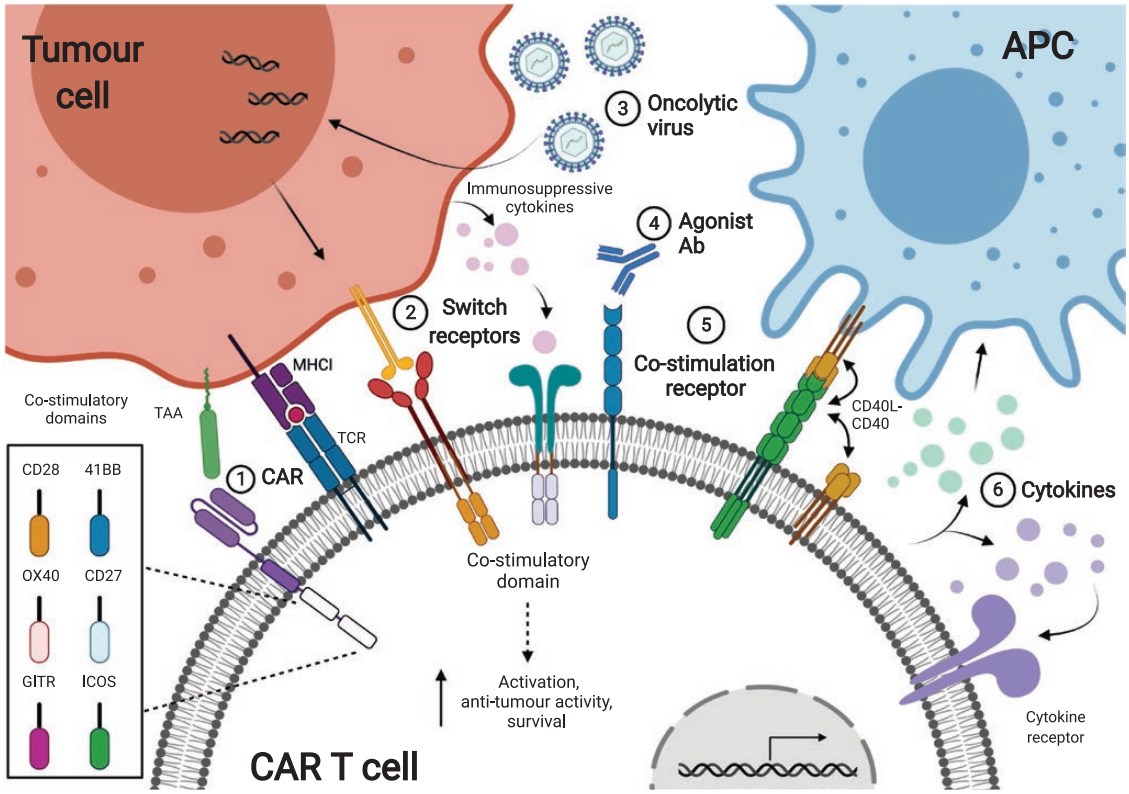

Figure 1. Strategies enhancing co-stimulation of CAR T cells. 1-1: Co-stimulation and synthetic signalling domains can be integrated directly within the CAR receptor. These domains provide co-stimulatory signals when the CAR is activated. 1-2: Switch receptors (e.g. PDL1-CD28) and inverted cytokine receptors (e.g. GM-CSF-IL-18) transduce a co-stimulation signal when ligating immunosuppressive cytokines such as PDL1 or GM-CSF. 1-3: Oncolytic viruses target tumor cells and remodel the TME with immunostimulatory molecules such as OX40. 1-4: CAR T cell secreting agonist antibodies against CD40 or 4-1BB activate CAR T or endogenous immune cells. 1-5: CD40 (represented by the yellow molecule) can act in *cis* and *trans* when expressed on CAR T cells. 1-6: CAR T cells secreting cytokines such as IL-18 or IL-12 license APCs or act on T cells to drive an antitumor response.

example, CAR T cells expressing a GM-CSF-IL-18 ICR were able to mediate tumour regression in HER2 and EphA2 solid tumour models. In this design, the ICR contained an extracellular domain of the GM-CSF receptor and the signalling domains of the IL-18 receptor (GM18). GM18 can be activated in the tumour by endogenous GM-CSF of the TME, leading to enhanced CAR T cell survival and tumour cell clearance [33]. GM-CSF has also been targeted with an IL-2-based ICR [34]. These additional co-stimulatory triggers may synergize with CAR signalling by including distinct domains, effectively augmenting CAR signalling localized within tumour tissues. Additional synthetic co-stimulatory receptors also flip key interactions within the TME to deliver additional pro-survival signals to CAR T cells.

CD40 is a receptor expressed on antigen presenting cells (APCs) and is central to developing tumour-specific T cell responses [35]. When expressed on T cells, CD40 is able to act in *cis* and *trans* by binding to CD40-L expressed on T cells, ultimately enhancing the survival of CAR T cells in tumours [36] (Fig. 1-5). Solid tumours evade the immune system through a number of mechanisms including a large degree of antigen heterogeneity, as well as their immunosuppressive microenvironment. Enhancing co-stimulation of both CAR T and endogenous T cells may boost endogenous immune responses to recognise neoantigens and reduce tumour immune escape. $CD40L^{+}$ CAR T cells are shown to be superior in their anti-tumour effect and provide a rational to incorporate CD40-CD40-L signal in CAR T design [36].

Cytokine co-stimulation is a crucial component of a CAR T cell response

Cytokines are secreted proteins with a range of effects on all sets of immune cells. In the context of CAR T cells, these proteins constitute the 'signal 3' checkpoint for activation. γ-chain cytokines such as IL-2 and

IL-15 have essential non-redundant roles in supporting the survival and differentiation of T cells, as well as CAR T cells [37]. *Ex vivo* production of CAR T cells using these γ-chain cytokines drives CAR T cell differentiation to effective subtypes for solid cancers, and these cytokines have also been used as direct therapies *in vivo* [38]. Cytokines such as IL-2 and IL-12 have been used to activate and expand tumour-infiltrating lymphocytes (TILs) in solid cancers resulting in some curative responses, but are associated with toxicity [39]. Therefore, 'armoured CAR' T cells have been developed to secrete such cytokines specifically within the TME to reduce toxicity as well as recruit endogenous T cells to overcome tumour heterogeneity [40] (Fig. 1-6). CAR T cells transduced to secrete IL-12 increased macrophage and innate cell-mediated clearance of TAA-negative cells, leading to enhanced control of tumours [41]. However, excessive cytokine co-stimulation with IL-12 has been documented to drive CART cell exhaustion [42]. IL-1 family cytokines are a group of proinflammatory cytokines including IL-1, IL-18, and IL-36γ [43]. These cytokines are generally proinflammatory and can act on both T cells and dendritic cells (DCs) to drive a Th_1 type response and increase IFNγ secretion by T cells [43]. IL-18, best known for inducing antigen-independent bystander T cell activation, can act synergistically with IL-12 to inhibit solid cancer progression [44, 45]. CAR T cells expressing IL-18 were able to mediate effective responses in a model of colon cancer while also activating endogenous TILs [46]. Similarly, CAR T cells expressing IL-36γ also mediated tumour regression but with different kinetics to previously tested IL-1 family cytokines, demonstrating non-redundant signalling within this cytokine family [47]. Chemotactic cytokines, or chemokines, can also be used to enhance trafficking of CAR T cells to solid tumours. CAR T cells secreting IL-7 and CCL19 provide both pro-survival signals to CAR T cells in the tumour as well as recruit and license intertumoral APCs in a model of lung cancer [48]. This resulted in increased immune cell infiltration and memory formation as cured mice were resistant to tumour re-challenge, and these results have now been extended to human xenograft models. Manipulation of the cytokine milieu by direct CAR T cell secretion has demonstrated effects directly on the function of CAR T cells and endogenous cells, remodelling the TME to a more permissive immune environment. Understanding the role of cytokines in sustaining, improving or hampering intra-tumoral immune response will facilitate their optimal incorporation into CAR T cell therapy regimes.

Antibody-based approaches utilizing co-stimulation in CAR T cell therapies

Checkpoint blockade therapies are an indirect method of modulating T cell co-stimulation by utilising antibodies to inhibit negative regulators of co-stimulatory molecules. These therapies have demonstrated to enhance CAR T cell efficacy and have been reviewed elsewhere [49, 50]. Antibodies directly targeting co-stimulatory molecules can also boost the immune response to cancer. CD40 antibodies are approved therapeutics for cancer and have both T cell intrinsic and pleiotropic effects [51]. When used in combination with IL-15, CD40 agonists were able to increase CD8 T cell and NK cell infiltration into pancreatic cancers, leading to establishment of immune memory response [52]. In a novel approach, CAR T cells were engineered to secrete CD40 agonist antibodies. Compared with traditional CAR T cells, these anti-CD40 secreting CAR T cells demonstrated elevated cytotoxic effect on cancer cells and increased proportion of central memory phenotype [53]. 4-1BB agonist antibodies have also been investigated in the context of solid cancers and were able to increase the cytokine secretion of CAR T cells as well as remodelling of endogenous T cells in a model of breast cancer [54] (Fig. 1-4). However, these agonist antibodies have not progressed beyond clinical trials due to systemic toxicity and requirement of FcγRIII to facilitate hyper clustering of 4-1BB [55].

Co-stimulatory bispecific antibodies have been developed which combine two antibody or ligand specificities [56]. This strategy allows for agonist antibodies being targeted to the TME by coupling with an antibody specific for a TAA [57, 58]. For example, a bispecific composed of 4-1BBL (CD137L) and fibroblast activator protein was able to provide co-stimulation to T cells [59]. Similarly, coupling antibodies to collagen factors in tumour-associated vasculature has been used to deliver checkpoint antibodies, IL-2 or chemokine factors to the TME, leading to APC recruitment [60, 61]. A CD27-PD-L1 bispecific was able to simultaneously deliver co-stimulation and checkpoint blockade, leading to increased T cell function [62]. These bispecific antibodies have great potential to be used together with CAR T cells to boost CAR T cell anti-tumour effect. For example, bispecific engager antibodies targeting CD40 and the c-Myc tag expressed within CAR was able to eliminate tumours in mouse models of breast cancer [63]. The eradication of tumour was due to enhanced co-stimulation of CAR T cells by APCs mediated by this bispecific antibody. Currently CD27, CD28, CD40, and 4-1BB co-stimulation have been tested in the form of a bispecific engagers.

Antibody-based therapies offer precise dose control and targeting to the TME to limit toxicity. Additionally, antibody therapies offer a high degree of flexibility for combination with many CAR T formats already in use and have pleiotropic effects to enhance both CAR T cell and endogenous immune responses.

Non-antibody-based approaches utilizing co-stimulation in CAR T cell therapies

Nanotechnology and biotechnology are increasingly utilized in health and medicine. In the context of CAR T cell therapies, these fields offer alternative methods of delivering co-stimulation to antibody-based methods. Nanoparticle vaccines have been demonstrated to engage the host APCs to activate T cells and can be used in cancer immunotherapy [64]. For example, a nanoparticle targeting CLEC-9A was able to effectively deliver antigen to host cross presenting DCs promoting the activation of CAR-TCR dual-specific cells [65]. Additionally, a nanoparticle RNA vaccine enabled claudin-presentation by APCs to claudin-specific CAR T cells, and enhanced CAR T cell trafficking to tumour tissues, leading to eradication of disease [66]. A similar technology utilised APC targeting 'amph ligands' to direct CAR T cell interactions with endogenous DCs. This platform utilises the CAR-specific ligand attached to a DC targeting phospholipid polymer, resulting in CAR T cell and DCs interactions [67]. The co-stimulatory signals delivered by DCs to CAR T cells leads to increased proliferation and tumour control [68].

Viruses can alter the TME to enhance CAR T cell infiltration, activation, and anti-tumour effects. Oncolytic viruses (OV) naturally infect malignant cells and are therefore good theoretical candidates for synergy with CAR T cell therapy. OV can remodel the TME, as well as cause tumour cell death and release of neoantigens [69, 70]. Some studies armed OVs with molecules such as cytokines or co-stimulatory ligands, which are expressed by tumours after OV infection. The expression of these immune modulatory molecules subsequently drives CAR T cell activation (Fig. 1-3). OV-mediated expression of a bispecific engager worked synergistically with CAR T cell activity in two tumour models [71]. In a tumour model of B16 melanoma, modified OV expressing IL-21 enhanced the survival of mice compared to a panel of co-stimulatory molecules including CD86 and 4-1BB [72]. Other therapies utilising OVs to express molecules such as OX40, IL-2, and CD40 have also been studied [73, 74]. OV therapies can be further refined to enhance tropism for tumour cells through the inclusion of tumour-specific promoters such as survivin or hTERT, or modification of OV capsid proteins [75]. For example, a chimeric OV created from vesicular stomatitis virus (VSV) and Newcastle disease virus generated potent anti-tumour effect with greatly reduced hepatotoxicity and neurotoxicity compared to wild-type VSV OV. [69].

Platforms for delivering co-stimulation specifically to the TME or specific subsets of APC within the immune system can be used to drive CAR T cell proliferation and persistence *in vivo*. These methods offer several advantages over antibody-based methods, including delivering flexible payloads or antigens. Therefore, these technologies should be developed further to deliver specific co-stimulatory payloads for each tumour type.

Conclusions and future directions

The understanding of the role of co-stimulation for the design of immunotherapies including CAR T cell therapies has expanded rapidly. Co-stimulatory pathways are demonstrating potential to overcome barriers specifically associated with the TME such as impeded cell trafficking, persistence and exhaustion. The identification and thorough characterisation of novel co-stimulatory pathways and their potential role in improving CAR T cell persistence and avoiding exhaustion in the solid tumour TME is one of the most pressing areas to develop for CAR T cell research. To date, the majority of CARs have incorporated CD28 or 4-1BB domains, but T cells are known to utilize a multitude of co-stimulatory signals to develop a potent immune response. Novel platforms such as OV, nano-emulsion vaccines and combination therapies with antibody therapeutics offer bespoke strategies for delivering such broad co-stimulatory signals to allow CAR T cells to overcome these barriers. Solid tumours continue to be a major human and economic toll in our society. Understanding and refining the use of co-stimulation in CAR T cell design is critical for the future application of CAR T cell therapy enabling all of us to live longer, healthier lives.

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Author contributions

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Conflict of interest

None declared.

Ethical approval information

Not applicable.

Data availability

Not applicable.

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