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

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List of 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; NDV: Newcastle disease virus ; OV: oncolytic virus; PAMP: pathogen associated molecular pattern; scFV: single chain fragment variable; TAA: tumour associated antigen; Th₁: T helper type 1; TME: tumour microenvironment; TLR: toll like receptor; TIL: tumour infiltrating lymphocyte; NK: natural killer; VSV: vesicular stomatitis virus.
Abstract

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.

Key Words: Chimeric Antigen Receptor, Immunotherapy, T cell Immunology, Co-stimulation
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 thirty 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 1st 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 (see 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 are 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] (See Figure 1-1). CARs including one co-stimulatory domain are classified as 2nd generation, while those including two co-stimulatory domains are classified as 3rd generation. 3rd generation CARs demonstrated superior anti-tumour responses and magnitude of in vivo expansion compared to 2nd generation CARs in some studies. Ramos et al demonstrated that 3rd generation CAR T cells persisted longer and with superior in vivo expansion compared to 2nd 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 2nd generation anti-PSCA-CD28 CAR with the 3rd generation anti-PSCA-CD28-4-1BB CAR indicated that the 2nd generation CAR was superior in their anti-tumour effect in a human pancreatic cancer xenograft model [19]. The
superiority of 3rd 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 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 receptor (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 CAR T 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] (See Figure 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-PD1), 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 IL4 with an intracellular signalling domain of a pro-survival cytokine receptor such as IL7. These receptors deliver pro-survival cytokine signals (signal 3) in the presence of suppressive cytokines in the TME (See Figure 1-2). For example, CAR T cells expressing a GM-CSF/IL18 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 IL2 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] (see Figure 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] (see Figure 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 IFN\textgamma; 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-36\gamma; also mediated tumour regression but with different kinetics to previously tested IL-1 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 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 and have demonstrated to enhance CAR T cell efficacy 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] (see Figure 1-4). However these agonist antibodies have not progressed beyond clinical trials due to systemic toxicity and requirement of FcyRIII 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, IL2 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 being 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 OV 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 (see Figure 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 (NDV) 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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Figure 1: Strategies enhancing co-stimulation of CAR T cells. 1-1: Co-stimulation and synthetic signaling domains can be integrated directly within the CAR receptor. These domains provide co-stimulatory signals when the CAR is activated. 1-2: Switch receptors (for example PDL1-CD28) and inverted cytokine receptors (for example GM-CSF-IL18) 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 IL18 or IL12 license APCs or act on T cells to drive an antitumor response.
Table 1: FDA approved CAR T 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 |
| **ABECMA**  
| (Idecabtagene vilcleucel) | Celgene Corporation | BCMA | Relapsed/refractory multiple myeloma | 4-1BB | NCT03435796  
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| | | | | |  
| **TESCARTUS**  
| (brexucabtagene autoleucel) | Kite Pharma | CD19 | Relapsed/refractory mantle cell lymphoma | CD28 | NCT02601313  
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* **TESCARTUS** employs the identical retroviral vector to **YESCARTA** however is manufactured using a distinct protocol which enriches for T cells.
