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The pharmacology of second-generation chimeric antigen receptors

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Abstract

Second generation chimeric antigen receptors (CARs) retarget and reprogram T lymphocytes to augment their anti-tumour efficacy. The combined activating and costimulatory domains incorporated in these CARs critically determine the function, differentiation, metabolism and persistence of engineered T cells. CD19 CARs that incorporate CD28 or 4–1BB signalling domains are the best known to date. Both have shown remarkable complete remission rates in patients with refractory B cell malignancies. Recent data indicate that CD28-based CARs direct a brisk proliferative response and boost effector functions, while 4–1BB-based CARs, direct a gradual T cell accumulation that may eventually overcome lesser functional potency. These distinct kinetic features can be exploited to further develop CAR T cell therapies for a variety of cancers. A new field of immuno-pharmacology is emerging.

Introduction

The goal of T cell engineering (TCE) is to rapidly generate potent and specific immune responses.[1, 2] TCE is predicated on methods to safely and effectively genetically modify human T lymphocytes, which became available in the mid 1990's. TCE allows to instruct T cells to recognize any antigen of interest, for example a tumour antigen. Targeting may be achieved through the transfer of a physiological receptor for antigen, which is known as the T cell receptor (TCR),[3, 4] or artificial, T cell-activating receptors, which encompass a diverse set of fusion receptors that we eventually grouped under the general term of chimeric antigen receptor (CAR).[5] Emulating the TCR, the first chimeric receptors were designed to mimic its T cell activation, a function that is sufficient to specify cytolysis, but not sufficient to direct a sustained, effective T cell response. Such activating receptors are now referred to as first generation CARs.[5] To augment T cell function and extend T cell life span, a different kind of receptor had to be designed. Receptors providing both activating and costimulatory signals were designed to augment T cell function as well as redirect T cell specificity.[5] These second generation CARs have recently yielded impressive clinical results in patients with B cell malignancies, especially acute lymphoblastic leukaemia[6–11] and B cell lymphomas.[12–15]

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Here we review the nature and biological effects of engineered costimulation in tumour-targeted T cells, focusing on the pharmacology of second generation CARs. The best know to date are those that utilize the signalling domains of the CD28 or 4–1BB costimulatory receptors, fused to the cytoplasmic domain of the T cell activating CD3ζ chain.[16, 17] Hereafter we refer to these two categories of receptors as 28ζ and BBζ CARs.

Principles of T cell activation and CAR design

Physiological T cell responses are initiated by the TCR, which imparts antigen specificity and acts as the gatekeeper of T cell activation.[18, 19] TCR signalling is amplified and modulated by a series of receptors known as costimulatory receptors, the prototype of which is CD28.[20] In the absence of costimulation, primary TCR stimulation may fail to drive T cell proliferation beyond the G_0/G_1 phase of the cell cycle and render the T cell unresponsive, a state known as anergy.[21] Costimulation prevents anergy, as well as some forms of activation-induced cell death (AICD) and T cell exhaustion, although senescence will eventually limit T cell function and/or survival.

The first chimeric T cell activating receptors were generated in the early 90's when the ζ chain of the CD3 complex was cloned.[22] As this T cell-specific chain lacks an extracellular domain, the groups of Weiss, Seed and Klausner fused it to the extracellular domain of CD8, CD4 or CD25 (Fig. 1A) to study its function in leukemic T cells.[23–25] The cross-linking of these chimeric receptors induced calcium influx and other hallmarks of early T cell activation, thus establishing the physiological function of the ζ chain and providing a road map for the design of T cell activating receptors. The addition of a single chain variable fragment (scFv) to these receptors, as first reported by Eshhar et al,[26] afforded binding to the hapten TNP and antigen-induced T cell activation (Fig 1B). These fusion receptors were shown to redirect specificity and induce proliferation in T cell hybridomas and pre-activated T cells, but not in naïve cells.[27, 28] Transgenic mouse models later revealed that T cells expressing ζ chain-based CARs only modestly delayed tumour progression in vivo, as they only produced low amounts of interferon-γ (IFNγ) and rapidly anergised.[28, 29] Having established methods for transduction of human T cells (based on γ-retroviral mediated gene transfer[30]), we were able, for the first time, to test ζ chain-based CARs in primary T lymphocytes. Although these receptors effectively mediated cytotoxicity, we found them to be unable to direct T cell expansion upon repeated exposure to antigen.[31]

In earlier studies, we had demonstrated that costimulation can be provided to T cells through antigen-specific receptors termed chimeric costimulatory receptors (CCRs, Fig 1C). A CD28-based CCR was shown to direct Interleukin (IL)-2 synthesis and offset activation-induced apoptosis in human primary T cells.[32] Having thus validated the costimulatory signal in a relevant cell type, we then fused our CCR construct with a ζ chain-based CAR (Fig. 1D). For the first time, we were able to engineer human primary T cells such that they can expand upon repeated exposure to antigen.[16] Finney et al and Hombach et al reported on similar dual domain receptors, showing increased cytokine secretion induced by CARs containing a costimulatory signalling domain.[33, 34] Multiple costimulatory domains have since been introduced into CARs, the best known of which are those incorporating CD28 or 4–1BB signalling elements.[16, 17] These have both been utilized in patients with B cell

malignancies. Here we focus on the pharmacological properties afforded by these receptors, and also briefly summarize other second generation CAR designs.

CD28 costimulation

The TCR alone is not sufficient to prime naïve T cells to clonally expand and differentiate into effector and memory T cells. For a productive immune response to develop, T cell activation requires a concomitant costimulatory signal provided by CD28.[35, 36] CD28 is a member of the immunoglobulin superfamily of costimulatory/inhibitory receptors, which also includes ICOS (inducible costimulator), BTLA (B- and T-lymphocyte attenuator), CTLA-4 (cytotoxic T lymphocyte-associated antigen 4) and PD-1 (programmed death receptor-1).[37–40] CD28 is a 44kD type I transmembrane protein, expressed as a glycosylated, disulfide-linked homodimer.[41, 42] It is expressed in 95% of human $CD4^+$ T cells and 50% $CD8^+$ T cells[43] (unlike in the mouse where it is homogenously expressed on $CD4^+$ as well as $CD8^+$ T cells[44]) as well as regulatory T cells (T_{REGs}), plasma cells, neutrophils, eosinophils, and Natural Killer T (NKT) cells.[45–49]

Together with the TCR, CD28 is recruited to microclusters in the immunological synapse that forms between T cells and antigen presenting cells (APCs). The main function of CD28 is to augment TCR signalling, resulting in increased cytokine production, clonal proliferation, differentiation and survival. In doing so, CD28 increases TCR sensitivity by lowering the threshold TCR engagement that is required for efficient T cell activation[50] and enables T cell responses against weak agonist peptides.[51] Although it was thought early on that CD28 may have its own signalling pathway, it is now known that CD28 essentially amplifies TCR signalling,[52, 53] affecting cytokine production,[54, 55] cell cycle progression,[56] apoptosis,[57], epigenetic structure[58] and metabolism.[59, 60]

The cytoplasmic tail recruits the PI3K/Akt pathway, as well as the kinases PKCθ, Lck and RAS.[20] Lck and PKCθ recruitment induces Ca^{2+} influx and activation of the transcription factor (TF) NFAT, resulting in upregulation of T-bet, a TF that induces Th1 cytokine production.[55] Increased expression of the cytokines IL-2, IFNγ, IL-4 and the macrophage inflammatory protein MIP1α, as well as cytokine and chemokine receptors, is induced.[52] CD28 costimulation is critical for the induction of IL-2 secretion.[54] IL-2 secretion is induced through GRB2, AP-1 dependent RAS signalling[61] as well as via NFAT.[20] Additionally, CD28 induces chromatin remodelling via c-Rel (an NF-κB-family TF)[62], rendering the *IL-2* locus accessible, and thereby allowing for a more rapid secondary response.[63, 64] Similar changes are induced to other cytokine loci including *IL-4* and *IFNγ*.[65] Cell-cycle progression is enhanced via upregulation of Cyclin-D, facilitating progression to late G_1 and S phases.[56] The recruitment of the PI3K/Akt pathway enhances the expression of Glut-1, mTor and c-Myc, as well as NFAT and the canonical NF-κB pathway.[66] Glut-1, mTor and c-Myc facilitate the required metabolic switch from fatty acid, pyruvate and glutamine oxidation[67–69] to aerobic glycolysis and glutaminolysis to meet the energy and biosynthesis requirements associated with T cell activation.[70–72] PI3K/Akt also induces the upregulation of Bcl-X_L and suppression of p73 (both in an NF-κB dependent manner[73, 74]) which, combined with inhibition of FasL expression and reduction of caspase 8 activation, enhances cell survival.[75, 76] Through ERK/JNK dependent upregulation of the co-stimulatory

receptors ICOS, CD40L and 4–1BB, CD28 facilitates a sustained secondary response.[77–79] Importantly, CD28 costimulation is not only required during T cell priming, but also contributes to the expansion, cytolytic function, IL-2 production and cell cycle completion of memory T cells.[80, 81]

CD28 signalling is regulated in multiple ways. First, activation of CD28 requires binding with its ligands CD80 or CD86. Low levels of CD86 are constitutively expressed on B-cells, dendritic cells (DCs), Langerhans cells, monocytes and activated $CD4^+$ T-cells, and are upregulated upon activation, as is CD80.[82, 83] Both ligands induce the same signalling cascades, although it has been suggested that CD86 plays a greater role in initiation and CD80 in maintenance of immune responses.[66, 82] Second, after repeated TCR and CD28:CD80 stimulation, CTLA-4 and TNF (tumour necrosis factor) down-regulate CD28.[84–86] However, stimulation through 4–1BB can reconstitute CD28 expression and promote T cell reactivation and IL-2 production.[87, 88] Third, CD28 signalling is inhibited via CTLA-4 and PD-1. Cell surface CTLA-4 is induced upon T-cell activation and is a high affinity receptor for CD80/86. It inhibits CD28 signalling by competing for receptor binding as well as enhancing the internalization and degradation of CD28 and trans-endocytosis of CD80/86.[86, 89–91] PD-1 expression is induced within 24hrs after TCR stimulation and induces T cell exhaustion through blockade of CD28-induced PI3K activity.[92–94]

Due to the importance of CD28 in both T cell priming and memory responses, incorporation of CD28 signals into a second generation CAR is thus a fundamental and logical way to amplify CAR signalling, promote T cell proliferation and persistence, and offset anergy,

4–1BB costimulation

Following T cell priming, a number of costimulatory receptors appear on the surface of T cells in order to sustain activation. This includes members of the tumour necrosis factor receptor superfamily (TNFRSF), such as OX40, CD27, CD30, HVEM and 4–1BB, also known as CD137 or TNFRSF9.[95]

4–1BB is a type II transmembrane protein that was first found in activated lymphocytes.[96–99] It is expressed on a variety of lymphoid cells,[100–103] as well as some non-haematopoietic tissues.[99, 104, 105] Unbound 4–1BB is expressed as a 30kD monomer on the T cell surface, which forms a trimeric complex upon binding to its ligand. 4–1BBL is expressed on activated APCs as well as on activated T-cells.[106–110] 4–1BBL is distinct from the other members of the TNFR family by its three-bladed propeller like structure.[111] Formation of the trimeric complex results in both signalling downstream of 4–1BB as well as downstream of 4–1BBL.[112]

4–1BB is transiently induced by TCR and CD28 signalling via the ERK and JNK signalling pathways in both $CD4^+$ and $CD8^+$ T-cells, faster and more durably in the latter.[113–115] In memory cells, 4–1BB can be induced by IL-15 in the absence of antigen stimulation.[116]

4–1BB signalling enhances T-cell proliferation, cell cycle progression, cytokine secretion and cytolytic potential as well as prevention of clonal deletion and AICD, and reduces sensitivity to Transforming Growth Factor (TGF)β suppression.[117, 118] 4–1BB-mediated

costimulation increases IFNγ and IL-2 secretion by $CD8^+$ T cells and IL-2 and IL-4 secretion by $CD4^+$ T cells.[119, 120] It enhances TCR signalling through tyrosine phosphorylation of SLP-76, CD3ε, CD3ζ and Lck as well as the recruitment of PKCθ and increase in intracellular Ca^{2+} levels.[117] Signalling is mediated by TNF Receptor Associated Factor (TRAF)−1, −2 and −3.[121–124] TRAF1 is essential for the activation of ERK, the upregulation of Bcl-X_L and downregulation of Bim.[125] Additionally, it restricts the activation of the non-canonical NF-κB pathway in the absence of costimulation by preventing NIK (NF-κB inducing kinase) activation, but enhances the canonical NF-κB pathway upon costimulation.[126] TRAF2 is essential for the activation of p38 and the induction of NF-κB dependent activation of Bcl-X_L and Bfl-1, and also induces cytokine production via the MAPK pathway, promoting Th1 differentiation and cytokine production in $CD4^+$ T cells.[123, 127, 128] TRAF3 moderates activation through suppression of the non-canonical NF-κB pathway.[129] In both $CD4^+$ and $CD8^+$ T-cells, 4–1BB induces cell cycle progression in an IL-2 independent manner via ERK1/2 and PI3K and in an IL-2 dependent manner via the PI3K/Akt pathway.[130] 4–1BB signals are potentiated in the presence of CD28 signalling, but 4–1BB can still be upregulated in $CD28^{null}$ $CD8^+$ memory T cells wherein it exerts CD28-independent costimulation.[131–134] Similar to CD28 signalling, 4–1BB is able to induce telomerase activity, enhancing its levels after CD3/CD28 stimulation and induce re-activation of CD28 unresponsive T cells.[135]

4–1BB signalling affects the size, quality and maintenance of the memory $CD8^+$ T cell pool, and T cell expansion upon secondary challenge.[136–138] 4–1BB can amplify T cell proliferation in both $CD8^+$ and $CD4^+$ T cells.[114, 137] Although 4–$1BB^{-/-}$ and 4–$1BBL^{-/-}$ mice do not show a defect in CD4:CD8 T-cell ratios, 4–$1BB^{-/-}$ mice have a reduced number of primary $CD8^+$ T-cells due to reduced proliferation of naïve cells,[139, 140] a reduced $CD8^+$ T cell response and a reduced CD8 memory pool.[141, 142]

4–1BB stimulation can rescue T cells from anergy and exhaustion, even after down-regulation of CD28.[143, 144] However, the timing of 4–1BB signalling is of great importance. 4–1BB activation early after viral infection can have a detrimental effect by inducing AICD through prolonged up-regulation of TNF and Fas.[102, 145]

The incorporation of 4–1BB signalling domains in second generation CARs is therefore a logical choice to prevent anergy and to promote T cell proliferation and memory, with the anticipation of a greater effect on T cell maintenance than on functional activation relative to CD28.

28ζ and BBζ CARs–structure and signalling functions

Whereas a large amount of biological data on CD28, 4–1BB and some other costimulatory receptors is available, less is known about the function of their costimulatory domains within CARs. It would be mistaken to extrapolate all of the physiological functions of natural receptors to CARs for a number of reasons. First, the recruitment of these domains does not follow interactions with a single or sometimes two physiological ligands but varies, depending on the level of antigen on the target cell, the level of expression of the CAR in the T cell and the affinity of the CAR. There also is a temporal and spatial difference with the

expression pattern of the natural receptors, due to the constitutive expression of the vector-encoded CAR and the covalent linkage of the costimulatory and activating domains. Furthermore, receptors such as 4–1BB are monomers that normally trimerize upon activation, but they are forced dimers in most CAR designs. Additionally, the nature of the synapse that second generation CARs form with antigen presenting cells may not be the same as TCR-centred synapses. Finally, CAR functionality is not solely determined by the cytoplasmic signalling domains, as other structural features may affect its overall function (Box 1). All in all, the pharmacology of recombinant costimulatory receptors is an emerging field in need of more experimentation. There are presently few data on CAR signalling or comprehensive comparisons between CARs. Functionality

The initial characterization of any new CAR typically consists of functional *in vitro* assays, including measurements of antigen-specific cytotoxicity, antigen-induced proliferation and cytokine production. The most informative reports utilize primary cells, an experimental setting that requires efficient and non-toxic T cell transduction, such as that afforded by γ-retroviral or lentiviral vectors. Studies in leukaemic cells or hybridomas are less dependable, given the profound genetic and functional alterations that affect the proliferation, apoptosis, differentiation and function of these cells. It should also be appreciated that CAR studies in primary T cell studies are most commonly restimulation studies, in so far that T cells are activated and go through S phase prior to transduction,[146, 147] such that the first activation through the CAR (in vitro as well as in vivo) is *de facto* a second stimulation.

Both 28ζ and BBζ CARs augment cytokine secretion relative to first generation CARs.[16, 17, 148] Both induce Th1 cytokines including IL-2, IFNγ, TNF, and GM-CSF, although more briskly for 28ζ CARs and more slowly for BBζ CARs.[149–156] Th2 cytokines including IL-4 and IL-10 are also produced, with markedly lower levels induced by BBζ constructs.[150, 156, 157] 28ζ CARs elicit more IL-2, although it should be noted that the level of IL-2 production elicited by different 28ζ CARs varies, potentially exceeding or under-achieving that afforded by endogenous CD28.[16, 148] IL-2 is a critical cytokine for T cell function and adoptive cell therapy.[158] The impact of CAR-induced IL-2 secretion is complex. IL-2 promotes CAR T cell proliferation and sustains effector function, and may also affect neighbouring cells such as NK cells or T_{REG}s. While T_{REG} support, if it occurs, is undesirable, 28ζ CAR T cells are less sensitive to T_{REG} inhibition via IL-10 and TGFβ than first generation CAR T cells.[159] Furthermore, a modified CD28 domain reducing IL-2 production can partially restore cytolytic function of 28ζ CAR T cells in the presence of T_{REG}s without affecting IFNγ secretion and T cell proliferation.[160] T_{REG}s resistance can also be achieved by providing IL-12 to 28ζ CAR T cells[161] or through constitutive Akt activity.[162]

Limited information is available on the signalling cascades induced by CARs. In leukaemia cell lines, Finney et al showed that second generation constructs containing either CD28, ICOS, OX40 or 4–1BB domains, do not show increased tyrosine phosphorylation compared to ζ-chain only constructs.[155] Consistent with the known CD28 biology, 28ζ CARs induce signalling via NF-κB, Akt, ERK and NFAT and induce T-bet, EOMES and GATA3.[163] 28ζ CARs have been shown to activate the PI3K pathway, more consistently than BBζ CARs.[152, 155, 164]

A key factor in the specificity and safety of CAR T cells is their dependence on antigen stimulation to induce signalling. Some second generation CARs have been reported to possess constitutive or tonic activity. CD19-specific BBζ T cells show increased proliferation *in vitro* and enhanced *in vivo* survival in the absence of antigen[150, 165] This may be due in part to the unnatural dimeric 4–1BB structure, as TRAF recruitment is highly dependent on 4–1BB conformational changes.[166] Tonic signalling has not been observed with CD28 CARs,[16, 150] although it may occur when the scFv's oligomerise and induce CAR clustering and downstream signalling.[163] Antigen independent activation due to scFv crosslinking can be reduced by selection of an appropriate Heavy/Light chain orientation, hinge region or use of ligand-derived binding moieties instead of an scFv.

28ζ and BBζ CARs–anti-tumour efficacy in preclinical models

Over a decade ago, we provided the first demonstration that CD19 CAR therapy utilizing human peripheral blood T lymphocytes can eradicate established lymphoma and leukaemia in immunodeficient mice.[167] In this study, the use of a first generation CAR required a high T cell dose and optimized T cell expansion in the presence of CD28 costimulation and IL-15.[167] We later showed that a 28ζ CAR vastly outperformed a first generation CAR in an aggressive acute lymphoblastic leukaemia (ALL) model[148] (and likewise in a syngeneic CD19 mouse model[168]). An initial comparison of a 28ζ and a BBζ CAR showed similar anti-tumour activity, albeit with higher accumulation of BBζ T cells, even after the tumour had been eradicated.[149]

Little detail is known about CAR-mediated tumour elimination. The anti-tumour effect of CAR T cells may be mediated by direct tumour cytolysis as well as cytokine secretion. Interestingly, direct cytolysis is not confined to $CD8^+$ T cells, as $CD4^+$ CAR T cells can also acquire strong cytolytic potential.[167, 169] IFNγ and TNF produced by $CD4^+$ and $CD8^+$ T cells are able to damage the tumour microvasculature and induce tumour cell cycle arrest and senescence.[170–172]

Complementing functionality, T cell persistence is another major determinant of anti-tumour activity. Both CD28 and 4–1BB costimulatory domains extend T cell survival compared to first generation CARs,[148, 150, 173] albeit with different characteristics. 28ζ CARs program higher functionality while BBζ CAR direct greater longevity. The mechanisms for the latter remain to be fully elucidated. Two studies showed a higher induction of the antiapoptotic factor Bcl-X_L by 28ζ compared to BBζ CARs, accompanied by reduced AICD,[152, 154] but *in vitro* Bcl-X_L induction may not directly correlate with *in vivo* persistence. Telomerase activity might also contribute to the differences in persistence. One study showed that a 28ζ CAR induced peak levels of telomerase 2 days after antigen stimulation, which declined by day 4, whereas a BBζ CAR induced more persistent activity.[174]

Increased *in vivo* persistence of BBζ CAR T cells may also result from reduced exhaustion and apoptosis upon repeated antigen stimulation. For example, PD-1 upregulation in T cells following CAR stimulation, combined with the induction of PD-L1 on tumour cells by IFNγ, could reduce T cell function and ultimately T cell longevity. Combined 28ζ CAR T cells and PD-1 blocking antibody thus enhances proliferative and functional T cell capacity

in vitro and anti-tumour functionality in vivo, associated with reduced infiltration of myeloid derived suppressor cells.[175] It was also shown that engineered production of IL-15 by 28ζ CAR T-cells can lead to a reduction in PD-1 expression and enhanced functionality.[176] The significance of PD-1 expression, however, needs to be interpreted with caution. In tumour-infiltrating T cells, PD-1$^+$ CD8$^+$ T cells are potent tumour-reactive T cells that produce copious amounts of IFNγ production and up-regulate 4–1BB expression.[177] Nonetheless, the combination of PD-1/PD-L1 blockade with CAR T cell therapy, especially 28ζ CARs, is an attractive perspective.

CAR T cell functionality is commonly studied in bulk CD4$^+$ and CD8$^+$ T cells. Recent reports have shown that, in the context of 28ζ constructs targeting Mesothelin[169] or CD19,[178] CD4$^+$ CAR T cells have a stronger expansion and higher cytokine secretion compared to CD8$^+$ CAR T cells, and are more resistant to AICD. CD4$^+$ CAR T cells alone may achieve tumour control and establish long-term persistence, and their presence enhances the anti-tumour activity of CD8$^+$ CAR T cells.[169]

The engineering of T cells at different maturational stages is another important determinant of T cell anti-tumour activity, a topic that is beyond the scope of this review and has been recently reviewed elsewhere.[179, 180]

28ζ and BBζ CARs in the clinic: The CD19 paradigm

The most investigated target for CARs to date is CD19. We initially chose CD19 as a target for CAR-based therapy because of its common expression in most B cell leukaemias and lymphomas and absence in all normal tissues other than the B cell lineage.[181, 182] We provided the first proof-of-principle that CAR-modified human peripheral blood T cells targeted to CD19 can eradicate a broad range of B cell malignancies.[167] Successful B cell tumour eradication was eventually obtained with different CD19 CARs,[148, 150, 173, 183] paving the way for numerous on-going clinical trials. The targeting of CD19 eventually became a paradigm for evaluating CAR technology.[184] CD19 malignancies are so far the only setting where both 28ζ and BBζ CARs have been studied in the clinic.

While the first clinical reports on CD19 CAR trial focused on non-Hodgkin lymphoma (NHL)[185–187] and chronic lymphocytic leukaemia (CLL),[6, 13–15, 188, 189] the most dramatic results have been obtained in ALL.[7–11] Three centres made seminal contributions to these clinical studies – Memorial Sloan Kettering Cancer Center (MSKCC) for adult ALL, and the Children's Hospital of Philadelphia (CHOP) and the National Cancer Institute (NCI) for childhood ALL. The first clinical results obtained with CAR therapy utilized the 19–28z CAR (Fig. 2), which we transduced in autologous peripheral blood T cells collected by apheresis.[7, 190] Adult patients with relapsed, chemorefractory disease were infused with 3 million CAR$^+$ T cells/kg following a single infusion of cyclophosphamide (3 g/m^2) as conditioning. Four out of four patients with measurable disease went into molecular remission within 4 weeks.[7] The groups of Stephen Grupp, Crystal Mackall, and ours subsequently published follow-up studies in adult and paediatric disease.[8–11] All three centres reported a highly remarkable complete remission rate – a rare occurrence for phase I studies in oncology and especially for relapsed ALL. All reported the same two main

toxicities: B cell aplasia, which is expected when targeting CD19,[148, 168] and cytokine responses, sometimes causing severe cytokine release syndrome.[8, 9, 191] Similar toxicities have been seen with antibody therapies targeting costimulatory pathways (Box 2).

While these studies follow overall similar steps (apheresis, retroviral CAR transduction, T cell infusion following chemotherapy conditioning), they differ in several regards, including the CAR design (28ζ dual-signalling domain[16] utilized at the NCI and MSKCC, BBζ[17] utilized at CHOP Fig 2), T cell manufacturing, conditioning chemotherapy, patient age, tumour burden, tumour chemo-sensitivity, and T cell dosage.[192] It is striking that irrespective of these variances, the outcomes have been similar, which speaks to the extraordinary robustness of CD19 CAR therapy in ALL. The differences in procedures and patient characteristics, as well as the still low number of patients (approximately 74 published and an estimated 150–200 infused at the time of this writing), makes it difficult to yet draw mechanistic conclusions. Some observations nonetheless emerge. CAR T cells traffic very effectively to bone marrow, whether they express a 28ζ or BBζ CAR.[9, 14] They occasionally cause severe cytokine release, especially in patients with higher tumour burden.[7, 11] A fever tends to start early on in recipients of 28ζ CAR T cells, later in recipient of BBζ CAR T cells. BBζ CAR T cells may persist many months in paediatric ALL patients,[8, 11] but less so in adults, where persistence of 28ζ or BBζ CAR T cells is on the order of 1–3 months.[9, 11] More patients need to be studied in detail to better apprehend the function and persistence of CAR T cells in different patient populations.

One next frontier to explore is the activity of second generation CARs against solid tumours. Such clinical studies are just starting in a handful of centres. Expectations notwithstanding, patience will be needed – several years – before sufficient data are gathered to evaluate the therapeutic potential of second generation CARs in multiple tumour types.

Beyond 28ζ and BBζ CARs

It is unlikely that there is one universally optimal costimulatory domain for CAR-induced T cell activation. Optimal signals may differ in different T cell subsets or T cell differention stages or tumour microenvironments. Costimulation is a dynamic process to which multiple receptors contribute. Several of these have already been incorporated into CARs.

ICOS

Like CD28, ICOS (Inducible Costimulator) is a member of the B7 family, although it is not expressed constitutively but upregulated upon TCR/CD28 signalling. ICOS activates the PI3K/AKT pathway, but is unable to recruit GRB2 and Lck and therefore fails to induce strong IL-2 production. However, through activation of C-MAF, it induces higher secretion of other cytokines such as IL-4 and IL-21.[36] ICOS is required for Th17 development[156, 193, 194] and plays a critical role in the induction of Bcl-6, which is required for the transition from effector to memory phenotype.[36, 195] ICOS deficiency affects both T cell responses, resulting in reduced levels of T_{EFF}, T_{MEM} and TREGs, and cytokine deficits.[196–198] ICOS has been incorporated in multiple second generation CAR constructs, targeting either CD33, EGFRvIII or mesothelin.[155, 156, 199] ICOSζ CARs have higher PI3K activation and IFNγ production than BBζ CARs.[155] In Th17 cells, ICOSζ CARs maintain a bipolar

Th17/Th1 phenotype and secrete a distinct cytokine profile, including IL17A, IL-17F and IL-22. In one study, the anti-tumour effect of one ICOSζ CAR was not greater than a 28ζ and BBζ CAR, although it resulted in greater $CD4^+$ T cells persistence.[156]

OX40

Like 4–1BB, OX40 is a member of the TNFRSF, expressed by activated T cells after TCR/CD28 stimulation. OX40 deficiency affects CD8 T cell responses and reduces CD4 T cell proliferation and effector differentiation.[200–202] Its signalling is mediated by TRAF2, TRAF3 as well as TRAF5, inducing the canonical and non-canonical NF-κB pathways.[203, 204] OX40-based CARs targeting CD33 have been developed and their characteristics appear to be overall similar to BBζ constructs in terms of proliferative support and cytokine secretion.[155] Numerous third generation constructs incorporating OX40, targeting G_{D2}, MUC1 or CEA have been assessed.[157, 205, 206]

CD27

CD27 is also a member of the TNFRSF, but one that is constitutively expressed by naïve and memory T cells. TRAF2 or TRAF5 binding induces the (non)canonical NF-κB pathways, as well as AP-1 downstream of the JNK/MAPK pathway.[203] CD27 stimulation results in IL-2, IFNγ and TNF production, as well as the upregulation of Bcl-X_L and memory formation. However, chronic CD27 signalling induces T cell apoptosis.[203] CD27 is able to induce T cell expansion in an IL-2 independent manner[207] and stimulates glycolysis, aiding transition of naïve and memory to effector T cell.[208] Absence of CD27 results in reduced T cell expansion and impaired memory responses.[136, 202] The incorporation of CD27 into second generation CARs targeting folate receptor-α induces T cell proliferation, Bcl-X_L expression and IFNγ production, displaying comparable *in vivo* efficacy with 28ζ and BBζ CARs in one study.[153]

NKG2D

In a CAR design that does not utilize an scFv for ligand (antigen) binding, NKG2D CARs utilize the NK-cell receptor NKG2D to provide costimulation via interaction with the endogenous DAP10 co-signalling domain, providing PI3K/AKT stimulation and promoting a Th1 response.[209] NKG2D ligands are expressed in a variety of tumour cells as well as normal tissues, especially in the context of inflammation.[209, 210] Different types of NKG2D-based CARs have been developed, using either the full length or the extracellular domain of NKG2D, combined with CD3ζ, CD28 and/or 4–1BB domains.[211, 212] *In vitro*, expression of NKG2D-based CARs in T or NK cells induces lysis of multiple tumour targets and displays a high capacity to secrete cytokines including INFγ, TNF and GM-CSF. *In vivo*, full length NKG2D-CD3ζ CARs provide a better immune response than T cells transduced with NKG2D alone.[213, 214]

Other combinatorial strategies

Costimulatory signalling domains may also be combined, either in *cis* (third generation CARs) or in *trans* (combined CAR and CCR expression). Briefly, third generation CARs incorporate two costimulatory domains within their cytoplasmic tail. Most commonly, CD28

and 4–1BB[149–152, 154, 215, 216] or CD28 and OX-40[157, 205, 206] domains have been combined Third generation CARs showed similar or improved *in vitro* and *in vivo* cytolytic function compared to other CARs. Cytokine production levels vary in comparison to second-generation CARs, with both increases and decreases reported.[150, 152] T cell proliferation and *in vivo* persistence may be increased in some cases.[149, 152, 215] Few clinical trials have utilized third generation CARs to date. These did not suggest a clinical advantage over second generation CARs.[217, 218] When combining costimulation in *trans* through two independent antigen-specific receptors such as a CCR and a CAR,[32, 219] two antigens are targeted, e.g., ErbB2 and Mucin-1,[220] PSMA and PSCA,[219] and mesothelin and α–folate receptor.[221] Dual antigen targeting may be used to enhance tumour targeting,[220, 221] or reinforce tumour selectivity.[219]

Conclusions

CARs that incorporate costimulatory domains are conceptually distinct from those that only elicit T cell activation. The latter effectively redirect cytotoxicity, but dual-signalling CARs further reprogram T cell function and T cell persistence. These second generation CARs have recently yielded exciting clinical results in patients with B cell malignancies. While more is to be learned about the molecular functions of these CARs, both 28ζ and BBζ CARs have yielded high complete remission rates in B cell malignancies, especially ALL. Emerging data, albeit still incomplete, suggest that 28ζ CARs provide a brisker proliferative response and more vigorous activation of effector functions than BBζ CARs. The latter however persist longer, and achieve comparable anti-tumour responses over a more protracted time period. This suggests that BBζ CAR T cells compensate for their lesser functional potency by gradually building up and sustaining their numbers. These different kinetics may be exploited in different ways to further enhance the efficacy of CAR T cells and tailor CAR therapy to the requirements of different tumour burdens and tumour microenvironments. More studies are needed to better understand CAR function in different T cell types and to adjust CAR signalling to balance its effects on T cell function and persistence. A new field of immune-pharmacology is born.

Glossary Terms

Anergy
Rapidly acquired state of T cell unresponsiveness occurring after suboptimal activation via the TCR in the absence of costimulation or in the presence of co-inhibitory molecules

Activation-induced Cell Death (AICD)
apoptosis occurring following T cell activation

Exhaustion
progressive hyporesponsiveness developed by T cells after repeated exposure to antigen

T cell Engineering
cell modification intended to alter T cell antigen specificity, proliferation, persistence and/or function

Chimeric Antigen Receptor
Fusion receptor combining antigen-recognition and T cell activating properties. CARs consist of an antigen binding moiety (scFv or ligand), a hinge, a transmembrane domain and a cytoplasmic signaling domain, which includes a costimulatory domain in second generation CARs

1st Gen CAR
CAR with an endodomain consisting of the cytoplasmic signalling domain of CD3ζ or the Fc-γ receptor

2nd Gen CAR
CAR design including a single costimulatory element within the endoplasmic domain in trans with the activating CD3z domain

3rd Gen CAR
CAR design containing two costimulatory elements within the cytoplasmic domain in trans with the activating CD3z domain

scFv
single-chain variable fragment; fusion protein consisting of the variable fragments of the immunoglobulin heavy- and light chains, connected via a glycine/serine linker

Hinge
also referred to as spacer domain, it is an extracellular component connecting the binding moiety to the trans-membrane element

Chimeric Costimulatory Receptor
Fusion molecule coupling antigen-specificity to T cell costimulatory signalling, without activating domains

Primary T cell response
response upon the first encounter with foreign antigen, in which naive lymphocytes are primed, resulting in differentiation and clonal expansion

Secondary T cell response
response upon a repeat encounter with a foreign antigen, in which memory lymphocytes are activated resulting in stronger clonal expansion and enhanced antigen eradication compared to the primary response

Molecular remission
complete remission and absence of disease based on PCR analysis

Complete remission
absence of disease based on imaging or biopsy analysis

Severe cytokine release syndrome
A clinical syndrome comprising fever, hypotension, hypoxia and/or neurologic symptoms, associated with cytokine release occurring following *in vivo* T cell activation

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Box 1 –

Structural features of CARs

The functional properties of CARs are primarily determined by the signalling domains that are arrayed in the cytoplasmic tail. However, other structural components influence the overall signalling properties.

Binding Moiety

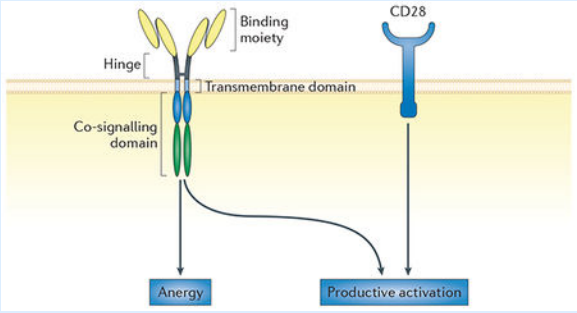
The binding moiety enables HLA-independent antigen recognition. The affinity of the CAR for the antigen and the location of the epitope on the targeted antigen affect overall CAR function.[26] CAR affinity, along with CAR expression levels, will determine the antigen-binding characteristics of the CAR and the effectiveness of target cell recognition.

Hinge

The hinge or spacer domain provides a separation between the binding moiety and the T cell membrane. The requirement for a hinge is target dependent.[222] To establish an optimal effector:target inter-membrane distance, membrane-distal epitopes do not require a large hinge within the CAR; in contrast, membrane-proximal epitopes elicit suboptimal T cell activation in the absence of a hinge.[223] Longer flexible hinges also allow for better binding to relatively inaccessible epitopes.[206, 224] The hinge can also have detrimental effects. Cross-activation can occur between IgG1 Fc-hinge containing CAR T cells and $Fc\gamma R^{+}$ cells, resulting in unspecific innate immune activation and AICD.[222, 225]

Transmembrane domain

The transmembrane domain (TM) is considered to be a purely structural requirement. Within first generation CARs, CD4 and CD8 TM domains allowed for similar cytokine secretion.[226] However, the CD3ζ TM domain was shown to facilitate interaction between the CAR and endogenous CD3, allowing for hetero-dimerisation with the endogenous TCR, resulting in enhanced anti-tumour function and cytokine production.[227]

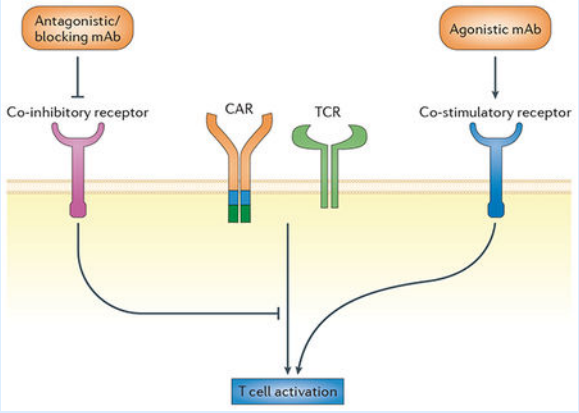


Box 2

Costimulation targeted by monoclonal antibodies.

Costimulatory pathways may be recruited to support T cell function via second generation CARs or by using monoclonal antibodies (mAbs) that target costimulatory receptors.[228] These mAbs, which may exert agonistic or antagonistic effects, are potent modulators of tumour immunity. Like CAR T cells, they may also induce immune toxicities. The **CD28** superagonist TGN1412 mAb activates T cells bypassing TCR stimulation and other costimulatory signals. It may trigger massive cytokine release, known as a cytokine storm, associated with pulmonary infiltrates, acute lung injury, acute renal failure followed by disseminated intravascular coagulation.[229] The **4–1BB** agonist mAb BMS-663513 has been tested in patients with solid tumours. Objective responses were observed in 17% of melanoma patients and 14% of renal cell carcinoma patients at low doses, but high doses may cause liver toxicity or fatalities.[230–232] Agonists of **OX40** and **CD40** are under investigation for multiple tumours, inducing potent responses, with occasional grade 3–4 toxicity.[232–237] Trials investigating targeting of **CD27** and **GITR** are on-going.

Immune checkpoint blockade using antagonists against **CTLA-4** (Ipilimumab) and **PD-1** (Pembrolizumab) has been approved by the US FDA. The best results have been seen in metastatic melanoma, where anti-CTLA-4 treatment increases 3-year survival rates to 20%, and anti-PD-1 results in 32–38% overall responses.[238, 239] Targeting of the PD1/PD-L1 axis can also be achieved through blocking of **PD-L1**. In a variety of solid tumours, 6–17% OR was achieved.[240] Several additional anti-CTLA-4, anti-PD-1 and anti-PD-L1 trials are on-going. Immune checkpoint blockade is also associated with occasional immune related adverse events. This includes fatigue, rash, dermatitis, uveitis, neurotoxicity, hepatotoxicity, pancreatitis, nephritis and pneumonitis. Rare fatalities due to anti-CTLA-4 induced enterocolitis, colonic perforation, hepatotoxicity and cardiac arrests have been reported.[239, 241] Fatal pneumonitis has also resulted from anti-PD-1 treatment.[242] These outcomes underscore the potency and enormous potential of targeting costimulatory pathways to augment tumour immunity and the need, whether using CAR T cells or mAbs, to tame and carefully manage these powerful drugs.



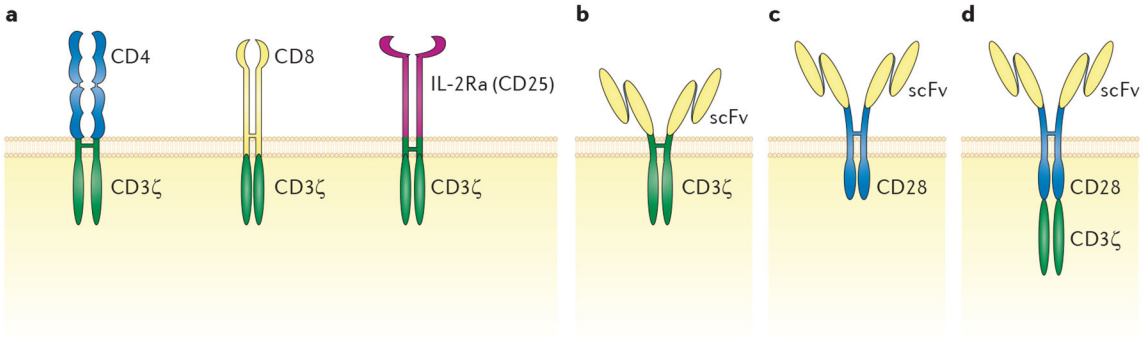


Figure 1: Chimeric Antigen Receptor evolution.

A: First fusion receptors as developed by the Weiss,[23] Seed[24] and Klausner[25] teams, coupling CD3ζ to the CD4, CD8 or CD25 extracellular domains. **B**: First generation CAR (or T body), allowing for antigen recognition through addition of an scFv.[26] **C**: Chimeric cytokine receptor, coupling an scFv to the intracellular domain of CD28 to induce antigen-specific costimulation.[32] **D:** Second generation CARs, facilitating T cell reprogramming by incorporating a costimulatory-signalling domain in tandem with the CD3ζ chain.[16]

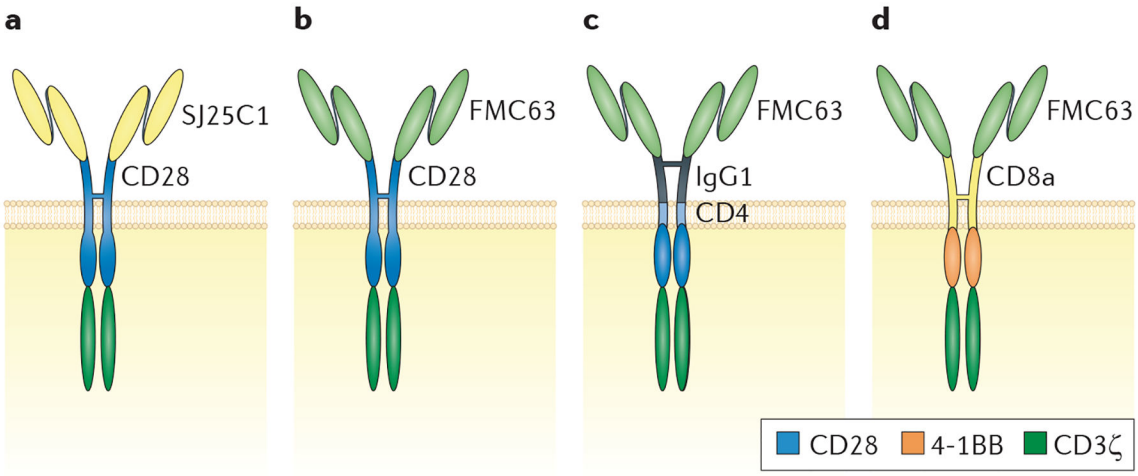


Figure 2: Second generation anti-CD19-CARs used in clinical trials to treat Acute Lymphoblastic Leukaemia (ALL).

A. CAR tested in MKSCC-trials,[6, 7, 9] consisting of SJ25C1 scFv, CD28 spacer, transmembrane and signalling domain and CD3ζ chain[16] **B**. CAR used in clinical trials at NCI.[10] This CAR contains the FMC63 scFv but is otherwise the same as the MSKCC CAR shown in A. **C**. CAR used and developed by the Baylor College of Medicine team.[188] This CAR utilizes the FMC63 scFv, IgG1 hinge, CD4 transmembrane domain, CD28 signalling domain and CD3ζ chain. **D**. Construct developed at St Jude Children's Research Hospital[17] and used by the CHOP and UPenn teams.[8, 11] This CAR contains the FMC63 scFv, CD8α hinge, and 4–1BB signalling domain in tandem with the CD3ζ chain.