The impact of accessory receptors on T cell activation by chimeric antigen receptors

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Abstract

T-cells can be redirected against novel targets, such as cancer cells, by engineering them to express alternative antigen recognition machinery in the form of chimeric antigen receptors (CARs). Despite promising results in treating blood cancers, their efficacy is limited and this has partly been traced to their poor antigen sensitivity. The reason for this is unclear. We show that CARs have a 46–2800 fold lower sensitivity than the T-cell receptor (TCR) when antigen is presented by antigen presenting cells (APCs) but have a similar 0.83–3.5 fold change in sensitivity when antigen is presented as purified protein in isolation. By systematically adding purified ligands to other, accessory, receptors expressed on CD8+ T cells, we identify that the CD2 and LFA-1 co-signalling receptors dramatically improve TCR antigen sensitivity (125 and 22-fold respectively) but not CAR antigen sensitivity (<5-fold). We found that CAR antigen sensitivity can be improved by fusing the CAR variable domains to the CD3ε subunit of the TCR (a TRuC), and restored to TCR levels by exchanging the variable regions of the TCR$\alpha\beta$ chains with those of a CAR (a STAR). These improvements are predicted by the improved ability of these receptors to exploit CD2 and LFA-1. The binding of CD2 to its ligand CD58 is thought to improve antigen recognition by precisely aligning membranes to the ~14 nm spanned by the TCR/pMHC interaction. We hypothesised that the CAR/antigen interaction may have a size incompatible with CD2/CD58 and therefore, engineered a panel of elongated CD2 receptors. We find that the antigen sensitivity increases by elongating CD2, with a different elongated CD2 being optimal for different antigen receptors. Taken together, CARs display a large defect in antigen sensitivity by their inefficient exploitation of the CD2 and LFA-1 interaction and engineered CD2 molecules can rescue their antigen sensitivity.

Explanation for the novice reader

T cells are the body's 'crime fighters'. They are the cells tasked with detecting and eliminating abnormal, infected, or cancerous cells from the body. They are able to do this because they have a special molecular sensor on their surface which can recognise markers on abnormal cells. This molecular sensor is called the 'T cell receptor'. Since a single T cell receptor is only able to detect a limited set of infection markers, the body produces an estimated 100 million different variations of this sensor. Despite this large number, T cells are unable to recognise all infections or diseases. Consequently, new molecular sensors called 'chimeric antigen receptors' (CARs) have been developed. When they are placed into T cells, they enable them to detect abnormal cells, such as specific cancer cells, and this allows them to directly kill these cells. Whilst the T cell receptor sensor can detect very small numbers of markers on abnormal cells, the engineered CAR sensors require large numbers of markers. This means that some abnormal cells are missed resulting in relapse in some patients. In this thesis, we identify the reason for why CAR sensors require a larger number of markers and using this information, we engineer new sensors that allow CARs to recognise much lower levels of markers. This may reduce relapses using existing therapies and may allow for new therapies.

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Chapter 0

Introduction

The immune system & T cells

There are two main components of the immune system, adaptive immunity and innate immunity. The innate immune system looks for defined molecular patterns and provides a general, nonspecific, defence against pathogens. These patterns can be 'pathogen-associated' (PAMPs) like lipopolysaccharide from the cell wall of certain bacteria, or 'damage associated' (DAMPs) such as cell products released during hypoxia. Adaptive immunity on the other hand provides a specific and specialised defence against a wide variety of pathogens. The adaptive immune system also provides 'immunological memory', allowing the immune system to respond more rapidly when it encounters pathogens to which it has previously been exposed.

The adaptive immune response can be further divided into two classes. Antibody responses, and cell-mediated responses. A specific kind of white blood cell is responsible for each of these responses. B cells are the cells which carry out the antibody response. Upon stimulation by an 'antigen', which is anything that elicits an immune response, and typically markers of infection or disease, B cells secrete antibodies. These are small Y-shaped proteins which circulate around the body binding to their corresponding antigen. When antibodies bind to viruses and toxins they inactive them by blocking their ability to bind to receptors on host cells. Antibody binding also marks pathogens for removal by phagocytic cells like macrophages.

The cell-mediated immune response is carried out by T cells. T cells are white blood cells capable of detecting and responding to specific antigens, which are those presented in a specific format on the surface of other cells. These cells that present antigen are generically referred to as 'antigen presenting cells' (APCs). In response to these antigens, not only can T cells kill abnormal cells, but they can also produce chemical messengers called cytokines, which are able to activate other cells of the immune system (Figure 1).

The antigens recognised by T cells are short peptides loaded on the carrier molecule MHC (for 'major histocompatibility complex') on the surface of other cells. The source of these peptide antigens is dependent on the cell which is doing the presentation. 'Professional' APCs, such as dendritic cells, primarily present antigen from external sources, these are taken up by the cell into vesicles which form as the result of endocytosis or phagocytosis. Though a fraction of the antigens presented by these cells may be derived from endogenous proteins [1]. The contents of these vesicles are broken down enzymatically resulting in peptides which are then loaded

onto class II MHC in the endoplasmic reticulum (ER) before being exported to the cell surface (Figure 2, right).

Non-professional APCs, indeed all nucleated cells, on the other hand, continuously load short peptides from the result of continued protein degradation within the cell, onto MHC class I molecules which are then exported to the surface (Figure 2, left). Because these peptides are loaded indiscriminately the majority of peptides arise from the cells own proteins, so-called 'self' peptides. However in the case of, for instance, a viral infection, some of these peptides will be derived from foreign proteins, like viral proteins. These are 'non-self' peptides. Notably, the size of peptides which can be presented by class I and II MHC are different. MHC class I can only bind short, 8-10 amino acid, peptides because of its fixed size groove [2]. MHC class II on the other hand can bind larger, 13-20 amino acid, peptides [2].

The number of 'non-self' peptides which end up being presented in this way is small. Both because these peptides are effectively 'competing' for presentation with all the self peptides, but also because many viruses have developed means of interfering with the peptide presentation pathway to downregulate the copy-number of MHCs. One study found that *in vitro* infection with HIV resulted in 8-46 pMHCs [3] and infection with cytomegalovirus (CMV) resulted in around 100 pMHCs [4].

The heart of what the immune system does is differentiate between *self* and *non-self*; the cells that are *you*, and everything else[1]. To achieve this task the T cell is equipped with a molecular 'sensor', the T cell receptor (TCR). By sampling information about the biophysical interaction between this receptor and antigen on the target cell the T cell is able to distinguish between self and non-self antigens. T cells may scan the surface of other cells using microvilli, small finger-like projections of the cell membrane, the tips of which have been reported to be rich in clusters of TCRs [7]. In this thesis the focus will be on T cells, of which there are a range; although all are equipped with a TCR, and scan for antigen on other cells they differ in their function and migration and consequently have differing roles in the immune response.

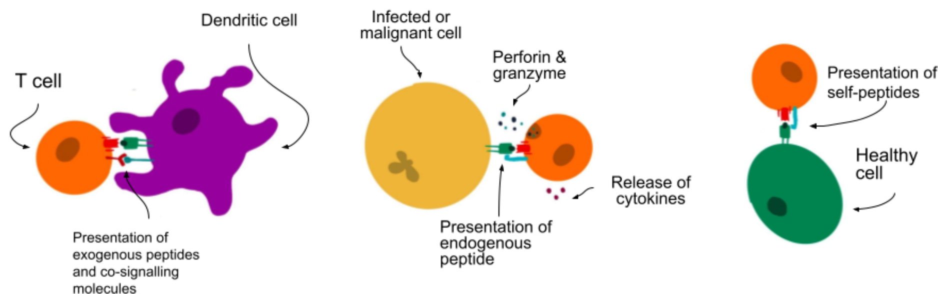


Figure 1: **T cells interact with a variety of cells**. T cells interact with professional antigen presenting cells (APCs) as well as with other cells presenting endogenous peptides. These can be self-peptides or those which arise from infection or malignancy. On encountering malignant or infected cells $CD8^{+}$ T cells produce cytokines and release granzyme and perforin to kill the target cell.

[1]The immune system is, by necessity, complex and only a fraction of it can be covered here. The reader in search of more information might try *How the immune system works* [5] or *Janeway's immunobiology* [6] for a broader, and more in depth view, respectively.

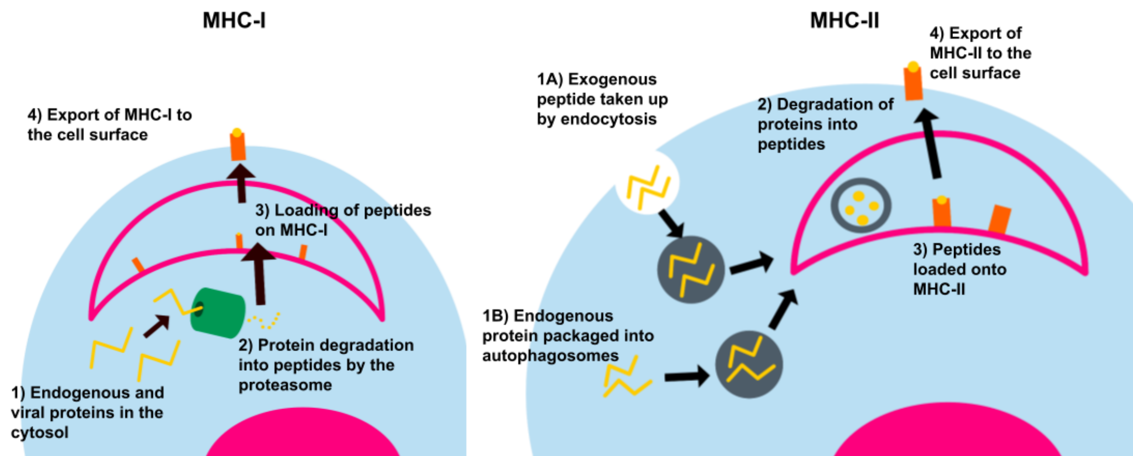


Figure 2: **Class I and class II MHC**. Professional and non-professional antigen presenting cells present peptides from different sources on different carrier molecules. Whilst all nucleated cells degrade endogenous protein and load it onto class I MHC, professional antigen presenting cells can take-up exogenous peptide to be presented on class II MHC.

T cell subsets

T cells can be grouped according to combinations of molecules on the cell surface which correspond to differences in the function and migration of the cells. The main distinction is between $CD8^+$ and $CD4^+$ T cells. These can be distinguished by the presence of either the CD4 or CD8 co-receptor on the cell surface. These co-receptors also bind MHC, but at a site distinct from the TCR, and can serve to recruit kinases to the TCR–pMHC binding site, as well as stabilising the TCR–pMHC interaction. $CD8^+$ T cells can carry out direct cytotoxic activity, whilst $CD4^+$ T cells signal other types of immune cell and regulate the immune response.

Both groups of T cell can be further divided into subgroups. The $CD8^+$ group includes naïve T cells, which are those which have not yet interacted with their cognate antigen. When they do encounter their cognate antigen naïve T cells start to proliferate and differentiate into memory or effector T cells. Effector T cells have improved cytotoxicity compared to memory subtypes. Memory T cells however respond rapidly to repeat antigen encounter [8]. Central memory T cells circulate through the secondary lymphoid organs and can rapidly produce cytokine in response to stimulation, they can also produce further central memory, effector, or effector memory cells [9]. Effector memory cells differ in that they mainly circulate in non-lymphoid tissues [9]. Some T cells do not circulate at all, but are instead tissue resident — these are tissue-resident memory T cells.

$CD4^+$ cells can differentiate into a variety of T helper cells, follicular helper cells and regulatory T cells. T helper cells help to activate cytotoxic T cells and B cells [10], T follicular helper cells play an important role in B cell differentiation, and regulatory T cells have an immunosupressive function and work to prevent autoimmunity and allergies [10, 11].

The TCR

T cells develop in the thymus, from which they take their name. There, via a process of genetic recombination they generate their T cell receptor. This is the molecular 'sensor' that allows T cells to detect markers of infection, disease or malignancy on other cells, in the form of peptide fragments presented on carrier molecules (MHCs).

The peptides which are most often associated with class I MHCs are 8 or 9 residues long. For just this length of peptide there is a possible $1.3 \cdot 10^{10}$ peptides that can bind to MHC. Since the body maintains far fewer T cell clones than there are possible peptides it must be the case that the TCR is cross-reactive. Indeed, one TCR is estimated to be capable of recognising around $3 \cdot 10^5$ peptides [12] and, experimentally, TCRs have been identified that can recognise more than 10^6 peptides [13].

The TCR is a multimeric protein which consists of an antigen binding region formed from the α and β chains which assemble with the associated $\zeta\zeta$, CD3$\varepsilon\gamma$ and CD3$\varepsilon\delta$ dimers. Only the $\zeta\zeta$ dimer can be expressed independently on the cell surface [14], the remaining CD3 chains must assemble correctly with the α and β chains in order to be expressed and contain ER retention signals that prevent the expression of non-functional TCR-CD3 complexes [14]. There is a 1:1 stoichiometry between all the chains of the TCR-CD3 complex, as confirmed by its crystal structure [15].

Binding of the TCR to MHC takes place through 3 complimentarity determining regions (CDRs) in each of the α and β chains. CDR3 is the product of the aforementioned genetic recombination process and is the region which is in direct contact with the peptide upon binding [16]. These interactions take place in so-called ‘close contact’ regions formed by the interactions between adhesion receptors on the T cell and their ligands on APCs. The adhesion receptors produce a higher effective on-rate for TCR–pMHC binding by creating a higher local concentration of TCR/pMHC [17].

The affinity of the TCR to non-self pMHC is weak, with a K_D of 1 µM to 100 µM [18]. This is the result of a fast off-rate. Affinity to self peptides is harder to determine because it is dramatically lower compared to affinity for non-self. Despite this lower affinity these interactions with self peptide are still important and provide tonic signals which are necessary for T cell maintenance [19]. The affinity of the TCR is not limited by its structure, as evidenced by the fact that ‘affinity matured’ TCRs can be produced *in vitro* with 5x higher affinities [20]. Though higher affinity does not necessarily translate to improved T cell functionality [21].

TCR triggering

The means by which pMHC binding is translated into an intracellular event is known as TCR triggering. Although the precise mechanism remains the subject of active research, a number of models for TCR triggering have been proposed.

Many proteins, such as G protein-coupled receptors (GPCRs), work on a principle of conformational change whereby ligand binding induces structural changes in the protein or stabilises a rare equilibrium state which causes downstream signalling within the cell. The same mechanism has been proposed for the TCR. However crystal structures of free TCR$\alpha\beta$ bound to pMHC superimposed with those of the intact TCR-CD3 complex show no large conformational changes [15]. In addition to recognising peptide on MHC the TCR is also able to recognise lipid antigens presented on CD1 [22] and bacterial metabolites presented on MR-1 [23]. Given such a diversity of targets it is unlikely that conformational change is the cause of TCR signal transduction.

A related, but distinct, model is that of ‘mechanosensing’. In this model, rather than binding inducing conformational changes, forces applied to the TCR–pMHC bond induce conformational changes in the TCR-CD3 complex. These changes are proposed to cause the immunoreceptor tyrosine-based activation motif (ITAM) containing cytoplasmic tails of the TCR-CD3 complex to disassociate, allowing for spontaneous phosphorylation and subsequent activation. T cells

have been shown to have the ability to exert force on the TCR–pMHC bond [24] and a further study [25] found differences in the TCR–pMHC bond between agonistic and non-agonistic pMHCs. That is, they found a catch bond (one which strengthens under force) with agonistic pMHCs but not with non-agonistic pMHC. This study found that the TCR elongated under force by 8 nm to 15 nm [25]. At the largest extent this is twice the size of the TCR (~7 nm [26]) so it is unclear how physiological this extension is.

A simpler model is that of induced receptor aggregation. This model proposes that clustering of the TCR would create a high local density of ITAMs which would permit efficient phosphorylation by (Src family) kinases and subsequent signalling. Early reports showed that cross linking of the TCR can cause triggering [27, 28] in cells, and a more recent study reconstituting the T cell signalling network on lipid bilayers has shown that clustering of the ζ chain and *Lck* is sufficient for TCR triggering [29]. These reports are however difficult to reconcile with evidence that few or even a single TCR binding event can lead to activation [30], including single-molecule tracking studies that find neither constitutive nor ligand-induced TCR oligomers to be important for TCR signalling [31].

The kinetic segregation (KS) model proposes that TCR triggering is instead controlled by a balance of kinase and phosphatase activities. This model holds that the TCR is constitutively phosphorylated by kinases such as *Lck* and dephosphorylated by phosphatases such as CD45 and CD148. At rest the phosphatase activity is dominant, preventing TCR signalling. Upon pMHC binding however a close-contact region is created between the TCR and the APC. The extent of this region is determined by the size of the TCR and pMHC, which is approximately 14 nm [32]. Formation of close-contacts is also promoted by interactions between other T cell surface receptors and their ligands that are of a similar size, like CD2–CD58 and CD28–CD80 [33].

By constraining the intermembrane distance the bulky extracellular domain of CD45 is excluded from the close contact area, reducing the amount of phosphatases in this region. Ordinarily TCR would rapidly diffuse in and out of this region and so would still be dephosphorylated by CD45. However binding of the pMHC is suggested to sufficiently slow the diffusion of the TCR, allowing it to become phosphorylated and to trigger downstream signalling. The theorised exclusion of CD45 from the close-contact region has been observed by microscopy [34] and can be abrogated by altering the size of the pMHC ligand [35].

TCR proximal signalling

The TCR proximal signalling cascade (Figure 3) consists of a series of binding and phosphorylation events. Initially when the TCR is stimulated with pMHC this leads to the phosphorylation of the tyrosine residues within the ITAMs of the TCR-CD3 complex by the Src family kinases Lck and Fyn [36]. Lck is anchored to the membrane and associates with CD4 and CD8 [37]. Consequently it has been theorised that binding of the CD4/8 co-receptor brings Lck into close proximity to the TCR-CD3 ITAMs allowing for efficient phosphorylation [38].

Phosphorylation of the CD3 ITAMs is followed by the binding of the kinase ZAP-70 [39] which in turn phosphorylates the scaffolding protein LAT [40]. This leads to signal amplification. LAT continues to propagate the TCR signal via the production of the secondary messengers diaglycerol (DAG) and lipid inositol triphosphate (IP3) by phospholipase C [36]. This eventually results in the activation of 3 transcription factors: NFκB, AP-1 and NFAT and leads to T cell effector function [36] and a broad range of phenotypic changes which include upregulation of CD69 and 41BB, cytokine production, cell division, and, for CD8$^+$ T cells, direct cell killing

through secretion of perforin and granzymes. The actual effector function displayed depends on the state of the T cell and any additional contextual signals that the cell might receive.

The importance of size

The size of the TCR is important for its function. Studies have found that placing peptide-MHC in the format of a single chain trimer at various spacings shows that the longer the TCR–pMHC interaction the worse T cells are at producing IL2 and IFNγ [35, 41]. Imaging studies suggest that ZAP70 recruitment was reduced at long TCR–pMHC interaction distances, which is in turn suggestive of poor TCR–CD3 ITAM phosphorylation. This also applies to other T cell surface proteins involved in signalling, for example elongation of the ligand to the T cell co-signalling molecule CD28, CD80, has also been shown to reduce IL2 production and to reduce segregation between CD28/CD80 and the inhibitory phosphatase CD45 [42]. The inverse also appears true of inhibitory molecules on the T cell surface. Increasing the size of the inhibitory molecules CTLA4 and PD1 prevents them from co-localising with the TCR and CD28 reducing their inhibitory activity [43, 44].

Size also appears to play a key role in other receptor–ligand interactions. Antibody-dependent phagocytosis by macrophages mediated by the Fc receptor for example [45], as well as the NKG2D receptor on natural killer (NK) T cells [46]. Synthetic receptors have also been shown to exhibit different behaviours as a result of differences in the interaction distance between the receptor and its ligand. For example chimeric antigen receptors have been shown to exhibit different levels of performance depending on their epitope on the target antigen [47, 48].

Thymic selection

The problem with generating the TCR ‘at random’ is that the process is likely to result in receptors that also recognise self. Indeed the theoretical number of TCRs that can be produced by genetic recombination is approximately 10^{18} [49]. To prevent the immune system from attacking the rest of the body, these self-recognising T cells have to be eliminated. This is

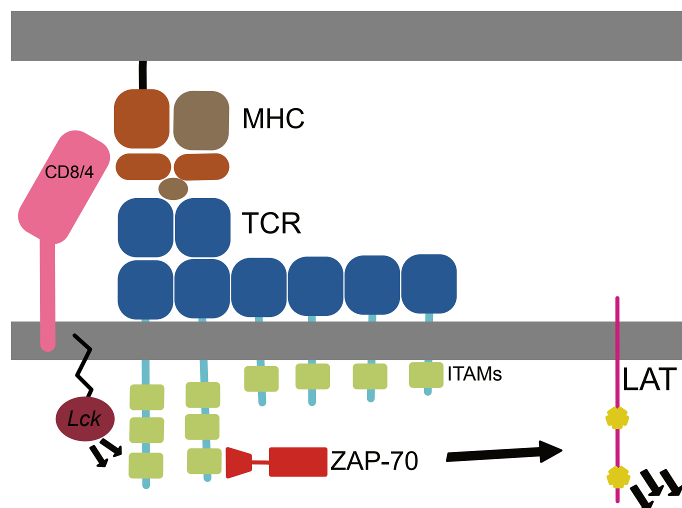


Figure 3: **The TCR proximal signalling cascade consists of a series of binding and phosphorylation events.** TCR binding leads to phosphorylation of ITAMs in the TCR–CD3 complex by *Lck* followed by the binding of ZAP-70 which in turn phosphorylates the scaffolding protein LAT and leads to the continued propagation of the TCR signal by second messengers.

done through a process called thymic selection. In the thymus, developing T cells undergo a two-step selection process to check, firstly that they have a functioning TCR, i.e., they can bind antigen in the form of short peptides loaded onto MHCs on the surface of other cells (positive selection), and secondly that they do not bind strongly to self antigens (negative selection). T cells which fail either of these tests die, either through neglect or through induced cell death.

Some self-reactive T cells may escape negative selection in the thymus, whilst others may recognise non-self but otherwise harmless antigens, such as those derived from food. In either case these cells must be prevented from launching an unnecessary immune response. One of the ways in which T cells are regulated once they have left the thymus and are spread throughout the body is through co-stimulation and co-inhibition. Or, together, co-signalling. These are signals which T cells receive in addition to the one transmitted via the TCR and which provide some additional context to the antigen recognition event.

Co-signalling receptors & their ligands

When professional APCs present T cells with antigen they do so in conjunction with co-signalling molecules. These co-signalling molecules provide T cells with the ability to sense and respond to environmental conditions. The canonical example of co-signalling molecules are the ligands to CD28: CD80 and CD86. Naïve T cells, those which have not yet encountered their cognate antigen in the periphery, were previously shown to require both the antigen recognition signal (signal I) via the TCR, as well as a second signal (signal II) through the co-signalling receptor CD28 in order to become fully activated [50]. Co-signalling also affects a T cell's effector response throughout its lifetime. Either enhancing it, or in the case of co-inhibitory molecules, acting to limit it after the infection or malignancy has been cleared. As well as regulating effector function, co-signalling molecules also regulate activation, differentiation and survival.

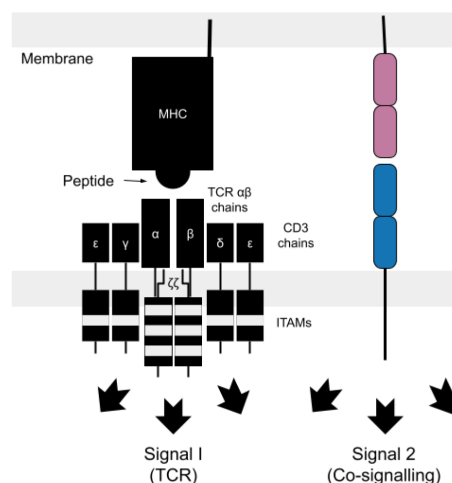


Figure 4: **T cells require signalling through both the TCR and co-signalling molecules to become fully activated**. T cells which only receive a signal through the TCR (signal I) become anergic, whilst T cells which receive a signal through both the TCR and a co-signalling molecule such as CD28 become fully activated.

There is great diversity in the structure, function, and expression of the various co-signalling molecules. The downstream signalling pathways of both the TCR-CD3 complex and co-signalling receptors often overlap significantly, so it is hard to identify the signalling events that lead to the unique function of the various co-signalling receptors.

Prominent T cell co-signalling receptors include CD28, 41BB (CD137), CD27, CD2 and LFA-1 (Figure 5). These are the ones which we will consider in this thesis.

CD28: CD28 is a member of a family of co-signalling molecules, the 'immunoglobulin superfamily' (IgSF). All of whom are characterised by the presence of an immunoglobulin-like extracellular domain. It is expressed on only 50% of $CD8^+$ T cells, and the proportion of $CD28^+$ T cells declines with age [51]. CD28 has two ligands, CD80 and CD86. The latter is expressed constitutively on APCs whereas the former is induced at later timepoints by stimulation of the antigen presenting cell [52]. CD28 competes with another receptor on the T cell surface, CTLA4, for its ligands. CTLA4 has a higher affinity to both CD80 and CD86 than does CD28; allowing it to out-compete CD28 for these ligands and suppress the T cell response. A competitive mechanism of action is not uncommon amongst co-signalling receptors. Indeed a number of co-signalling receptors interact with more than one ligand, giving several functions from a single molecule (e.g. HVEM [53])

The cytoplasmic tail of CD28 contains conserved motifs, one of which can become a docking site for SH2 domain containing proteins. CD28 can interact with a number of kinases involved in T cell signalling — like *PI3K* [54] and *Lck* [55]. The proline rich motif (PYAP) in the tail of CD28 associates with *Lck* and *GRB2* to increase the production of the cytokine IL2 by promoting the localisation of NFAT to the nucleus [56]. Signalling via CD28 both amplifies TCR signalling as well as triggers CD28 unique events [56]. In this way CD28 has a role in regulating everything from proliferation and survival to T cell differentiation.

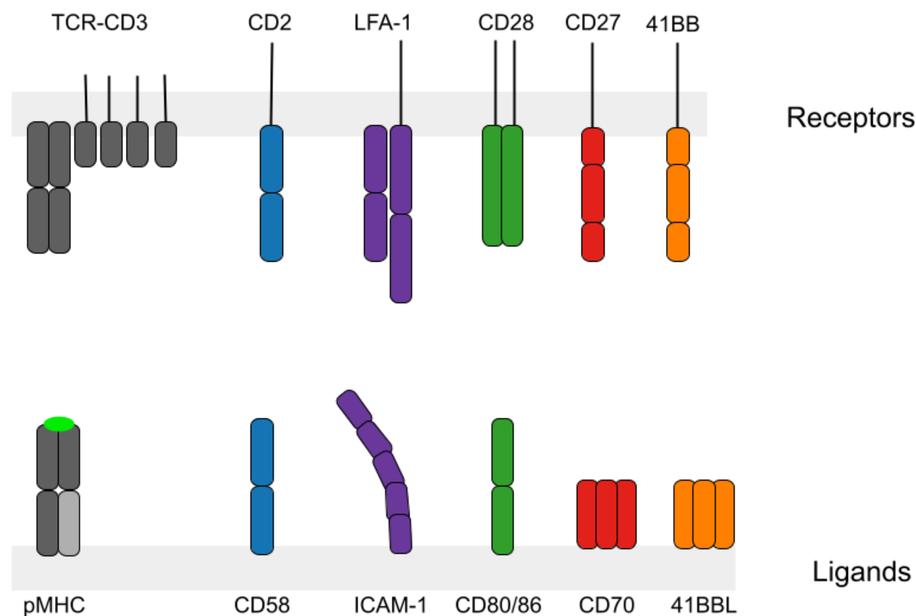


Figure 5: **APCs present T cells with antigen in conjunction with co-signalling molecules.** Schematic of prominent T cell accessory receptors and their ligands.

CD2: CD2 is also member of the immunoglobulin superfamily (IgSF). The IgSF family contains both stimulatory and inhibitory receptors and displays a great deal of heterogeneity both in terms of structure and function. CD2 is constitutively expressed on the surface of T cells and NK cells. The ligand for CD2, CD58, is widely expressed — including on other white blood cells and endothelial cells and has two different isoforms: one with a transmembrane region and cytoplasmic tail, and one which is lipid-anchored.

CD2 has a low affinity for CD58, though the overall avidity of the CD2–CD58 interaction is increased upon T cell activation as a result of an increase in both expression level and 2D affinity [57, 58]. The overall size of the CD2–CD58 interaction is similar to that of TCR–pMHC and CD28–CD86 and so it is able to co-localise with with these receptor-ligand pairs in nanometre scale close contact regions between the T cell and APC. Larger molecules such as LFA-1 appear to surround these smaller molecules [59]. Along with other adhesion molecules, CD2 can can facilitate TCR binding and exclude large phosphatases from the area around the TCR [33].

CD2 is a potent co-stimulator of T cell activation [60]. CD2 can activate T cells via ZAP70 and can induce activation in the absence of either TCR–CD3 signalling or CD28 signalling [61, 62]. It has also been shown to compensate for the role of CD28 in $CD28^-$ cells [63] and is able to reduce the antigen dose required to induce T cell proliferation and cytokine production by up to 100-fold [60, 64, 65]. This may be why some viruses, such as CMV, have evolved an ability to downregulate the CD2 ligand — CD58 — as a means of evading the immune response [66].

Previous studies have correlated immunotherapy response rate with CD58 expression; where cancers with low CD58 expression have correspondingly poor response rates [67]. This has also been shown experimentally, with CD58 knockout cancer cells resulting in reduced lysis and IFNγ production by T cells, suggesting that CD58 expression by cancer cells can modulate T cell sensitivity [67].

41BB: 41BB is a member of the tumour necrosis factor receptor superfamily (TNFRSF), a structurally diverse family of co-signalling receptors. TNFRSF receptors contain a number of extracellular cysteine rich domains, whilst their ligands contain TNF homology domains. Like other receptors in this family, 41BB after binding to its ligand 41BBL, promotes cell cycle progression, cytokine secretion and T cell survival [68]. In particular 41BB preferentially enhances the expansion of $CD8^+$ T cells [68]. On becoming activated T cells upregulate expression of 41BB and it can therefore be used as a maker of T cell activation. After being upregulated, 41BB monomers interact with trimers of 41BBL on antigen presenting cells. Causing it to also trimerise, or form higher order clusters of trimers. This clustering is believed to be the triggering mechanism that leads to the recruitment of TNF receptor-associated factor (TRAF) proteins and downstream signalling. Whilst different TNFRSF members associate with different TRAF proteins this alone is insufficient to explain the variation in their function.

CD27: CD27 is a member of the TNF receptor superfamily. Unlike 41BB however it is constitutively expressed on the T cell surface. The ligand for CD27, CD70, like that of 41BB is found on professional APCs and is expected to act early in the T cell response, that is, during the priming of naïve cells. CD27 promotes the survival of both $CD4^+$ and $CD8^+$ T cells by suppressing programmed cell death and triggering proliferation [69, 70]. Although CD27 appears to rely on CD28 in order to trigger cell cycling [70].

LFA-1: LFA-1 is an integrin and, like other integrins, is a dimeric protein. LFA-1 in particular is a dimer of CD11a and CD18. Although integrins are expressed on a wide variety of cells, LFA-1 is unique to white blood cells. On these cells it is primarily involved in T cell–APC contact

formation, and the migration of T cells from the blood stream into tissue. LFA-1 has multiple binding partners: ICAMs 1-5 and JAM-A. ICAM-1 is considered to be the most important of these and is initially expressed at low levels on epithelial and other immune cells but then later increased upon stimulation by IL-1 and TNFα.

LFA-1 has three different conformations, and each of these has a different ligand affinity. They are termed closed, closed-bent, and open; with low, intermediate and high affinity respectively. The conformation of LFA-1 can be altered by signalling (so-called inside-out signalling). Although signalling via the TCR can cause a shift in the conformation of LFA-1 from low to intermediate it cannot lead to the high affinity conformation; which is instead dependent on ligand binding [71]. Provided that the ICAM-1 is immobilised, LFA-1/ICAM-1 forms a catch bond whose strength increases under force [72, 73].

LFA-1 is larger than the TCR, CD2 and CD28 and is consequently segregated away from these molecules in areas of close contact between the T cell and antigen presenting cell [74]. LFA-1 has been shown to be a potent co-stimulator of T cell activation, decreasing, the required dose of antigen by 10-100 fold [75, 76]. LFA-1 has also been shown to synergise with CD2, producing an additive effect in lowering the required antigen dose [60].

The primary means by which the co-signalling receptors are regulated is through their cell surface expression. Both in time, as many co-signalling receptors are induced following T cell activation, but also in the differential expression between pairs of receptors and their ligands. Upon engagement it is possible for both the co-signalling receptor and the ligand to induce signalling, that is, co-signalling ligands can also induce signalling in the antigen presenting cell [77].

As regulators of the immune response co-signalling molecules have found an important role in immunotherapy. Blocking inhibitory co-signalling molecules such as CTLA4 and PDL1 has been found to increase the efficacy of tumour clearance by T cells (reviewed in [78]) whilst engaging stimulatory molecules, such as 41BB, is also being explored as a means to 'boost' the immune response [79, 80, 81].

The intracellular signalling domains of co-signalling molecules are also of use in another therapy: chimeric antigen receptor (CAR) T cell therapy. Here T cells are genetically engineered with a chimeric receptor that allows them to be redirected against novel antigens. These engineered receptors often contain domains from co-signalling molecules to modulate signalling through the chimeric receptor.

Chimeric antigen receptors

Adoptive cell therapy is a form of cancer therapy where T cells are infused into patients so that they can recognise and eliminate tumour cells (Figure 6). Abnormal cancer cells can be recognised by T cells because they abnormally express high levels of self antigens and/or express neoantigens, which are mutated self antigens that can be recognised as non-self by T cells [82]. Historically this form of therapy used tumour infiltrating T cells taken from the patient, which were expanded *ex vivo* before being put back into the patient. This approach however could not be applied for patients who lack tumour infiltrating T cells.

An alternative would be to generate tumour specific T cell clones by stimulating patient or donor T cells with the desired antigen *in vitro*. Better still, T cells to target any tumour antigen can be produced by engineering the T cells to express a specific T cell receptor [83]. However, although these TCRs would target tumour antigens, they are still HLA-restricted. HLA or 'human leukocyte antigen' is the MHC system in humans. HLA-restriction means that each

TCR is specific to a peptide and MHC combination (whilst maintaining some cross-reactivity), requiring careful evaluation of specificity for each antigen and HLA allele.

Chimeric antigen receptors allow the HLA-restriction to be removed so that any desired antigen may be targeted. These receptors were initially constructed by grafting the variable domains from an antibody into the variable regions of the TCR [84], sometimes called a 'T-Body'. Although this construction is now experiencing something of a comeback as 'STAR/HITs' [85, 86], it wasn't until the two chimeric TCR chains were replaced with a single chain construct that CAR T cell therapy really took off. These singe-chain constructs [87] consisted of single-chain variable fragments (scFvs) of antibodies (as described in [88]) fused with the TCR ζ or Fcγ chain via a 'hinge' and transmembrane domain taken from another T cell surface protein. With the antibody part conferring antigen specificity and the signalling domain provoking a T cell response.

These CARs, which are now known as '1st generation' CARs, performed poorly in mouse models of cancer owing to their inability to trigger proliferation or to produce high levels of cytokine [89]. We know that when triggered via the TCR, T cells require an additional signal, via a co-signalling molecule, to become fully activated; so CARs were subsequently engineered that contained the domains from one of these receptors. Such CARs are referred to as '2nd generation'. Both CD28 and 41BB co-signalling domains have been found to greatly improve CAR efficacy [90, 91, 92].

Current CAR therapies

Worldwide, there are currently approved CAR T cell therapies for several haematological malignancies: acute lymphoblastic leukemia (ALL), B-cell lymphoma, follicular lymphoma, mantle cell lymphoma and multiple myeloma. The CARs for multiple myeloma target B-cell maturation antigen (BCMA) whilst the others target the B-cell antigen CD19.

To provide some additional context for the way in which CAR-T therapy is used we briefly discuss here their use and outcomes for the treatment of acute lymphoblastic leukemia (ALL) and

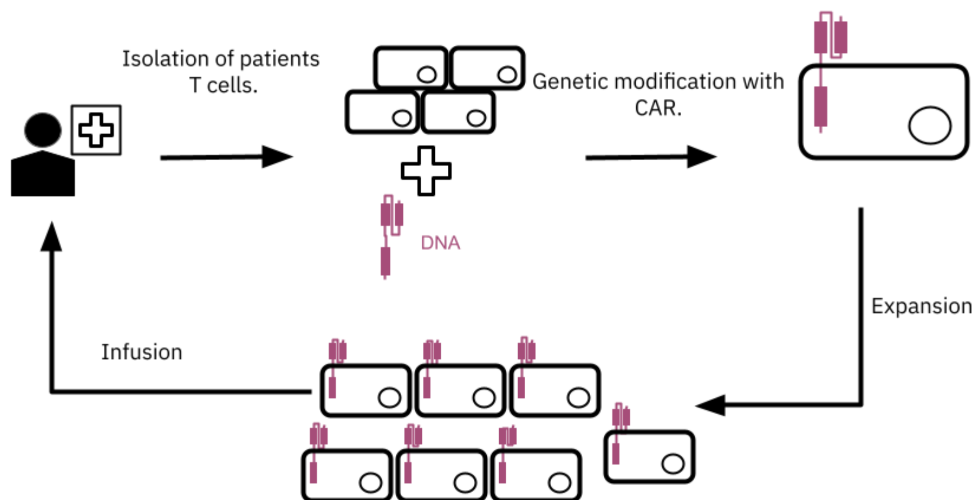


Figure 6: **Adoptive cell therapy is used in the treatment of some cancers**. A patients T cells are harvested and engineered to express a chimeric receptor before being grown *in vitro* and re-infused back into the patient where it is hoped that they will kill the target cells.

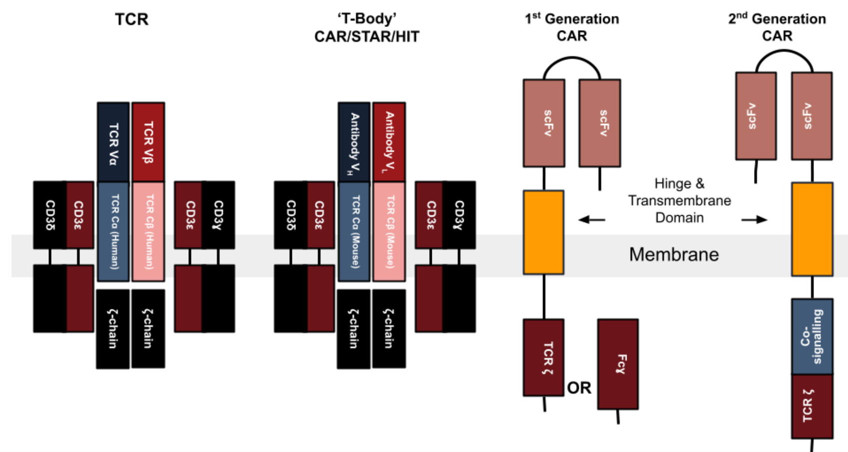


Figure 7: **The development of chimeric antigen receptors (CARs)**. CARs were originally produced by grafting the variable domains of an antibody into the variable regions of the TCR ('T-Body/STAR/HIT') before they were changed to single chain constructs consisting of a single chain variable fragment from an antibody fused to a signalling domain ('1st generation CAR') and subsequently developed by the addition of a co-signalling domain ('2nd generation CAR'), often from CD28 or 41BB.

B-cell lymphoma. About 30-40% of people with (diffuse large) B cell lymphoma will experience relapse and 10% of patients will not respond to the first line treatment, which is chemotherapy [93]. Of those who do not respond to first line treatment and are given the second line treatment (salvage chemotherapy followed by a stem-cell transplant) around 50% will relapse further [93]. It is this group of patients who receive CAR-T therapy. Median survival for this group, prior to CAR-T therapy is 6-12 months [93]. Relapse is also the the most common cause of treatment failure in child patients with ALL and results in a poor prognosis: a 22% survival rate at 1 year and 7% at 5 years post-relapse. CARs therefore primarily represent a 'last-resort' treatment option for patients who have highly treatment resistant disease.

A meta-analysis [93] of B-cell lymphoma treatment using CAR-T cells reports overall survival at 12 months of between 48-59%. In the phase II efficacy studies of tisagenlecleucel for ALL the overall response rate was 34% of all enrolled patients. In all of the studies considered by the meta-analysis [93] 99-100% of patients experienced an adverse event, and 68-98% experienced serious adverse events. A common adverse effect of CAR-T therapy is cytokine release syndrome (discussed below) which occurred in 42-100% of patients. The key challenges from a clinical perspective are therefore addressing complex disease cases, reducing toxicity and reducing risk.

Limitations of CAR T cells

CAR T cell therapies have been remarkably successful in treating haematological tumours, but are not perfect and there are still a number of factors that limit the more widespread use of CAR-T cell therapy. These include high toxicity, induced T cell exhaustion, poor efficacy against solid tumours, poor infiltration and persistence as well as reduced antigen sensitivity.

Toxicity: One of the main side effects of CAR-T cell therapy is cytokine release syndrome (CRS). This occurs when the engineered T cells become overactivated, leading to widespread and uncontrolled production of pro-inflammatory cytokines. These cytokines subsequently

activate other cells of the immune system which causes a feedback loop leading to further uncontrolled inflammation. The severity of CRS is variable, it can result in anything from a high fever to multiple organ failure [94], but in most cases it can be managed with the administration of anti-IL6 therapy [94]. Despite the ready availability of treatment for CRS, the high frequency of this side effect increases the risks of CAR-T therapy. Recognition of the CAR construct itself by the immune system can also contribute to cytokine-related toxicity [95].

Exhaustion: The efficacy of CAR T cell therapy depends on the ability to CAR-T cells to repeatedly kill target cells, yet persistent exposure of T cells to antigen causes their differentiation into a dysfunctional state where they display poor effector function and low proliferation [96]. This state is referred to as the 'exhausted' state. T cell exhaustion is associated with poor responses in CAR-T therapy [97]. Exhaustion in CAR T cells has been suggested to be the result of tonic signalling through the CAR [98]. Tonic signals are thought to be a consequence of the architecture of the extracellular part of the CAR and, in particular, it is suggested that tonic signalling is induced by CAR aggregation as a result of interactions between the scFvs (indicated on Figure 7) [98]. This means that the effect of tonic signalling varies from CAR-to-CAR and is dependent on the target antigen.

Difficulties posed by solid tumours: Although CAR-T therapy has been relatively successful for haematological malignancies, less success has been found targeting the CARs against solid tumours [99]. The ability of CAR T cells to infiltrate into solid tumours is limited as a result of the immunosupressive tumour microenvironment. Many cells that work to suppress the immune response, such as regulatory T cells are able to reach inside of tumours, where they can produce tumour facilitating cytokines [100]. The professional APCs found within the tumour can also have defects, for example displaying a poor ability to cross present tumour antigens [101]. Tumour cells can also upregulate 'classical' immune checkpoint inhibitors such as PD-L1 [100].

Successful CAR-T cell therapy is also correlated with long-term persistence [102], which tends not to occur for solid tumours [103]. Poor persistence may be due to the phenotype of the T cells which are infused — an analysis of leukemia patients treated with CAR-T therapy identified that complete remission was correlated with an increase in the number of cells displaying 'effector memory' markers, these are the cells which are mostly responsible for cytotoxic action [104].

A number of tumour antigens are also expressed on normal tissue, though usually at a lower level. This means the activity of CARs has to be carefully tuned in order to limit their toxicity towards healthy tissues (called 'on-target off-tumour' toxicity). That is, CARs have to respond to high antigen levels whilst ignoring low antigen levels.

Poor sensitivity: A current issue is the high rate of relapse following CAR-T therapy as a result of developed tumour resistance [105]. In one phase II CAR study 25% of patients who initially completely responded to treatment later relapsed with cancers which had an undetectable level of the target antigen, CD19 [105].

This 'antigen escape' is thought to be the result of CAR-T cells requiring high doses of antigen in order to become responsive. That is to say, CAR-T cells show poor antigen sensitivity, and this leads to the survival of antigen-low tumour cells whose out-growth can later cause relapse. The sensitivity defect in CAR-T cells has been observed *in vitro*, where CARs required between 100-1000 times as much antigen to become as activated as a TCR for the same target [106, 107].

Causes of the sensitivity defect in CAR-T cells

In the development of CARs their architecture has diverged significantly from that of the native TCR. Some of these structural differences may be the origin of their reduced sensitivity.

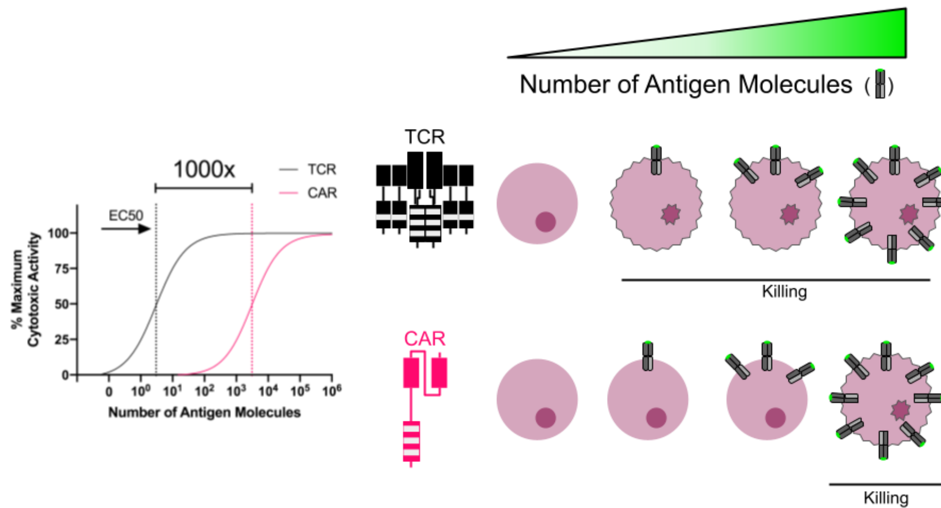


Figure 8: **CARs require higher levels of antigen to become activated and kill target cells.** CAR-T cells are less sensitive than TCR-T cells, requiring higher levels of antigen expression on target cells before they can kill them. This allows antigen low cells to survive and can potentially cause relapse.

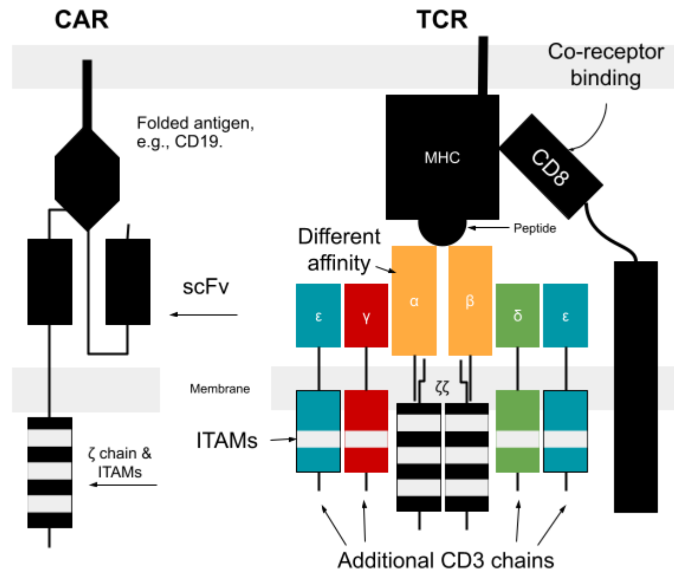


Figure 9: **A number of structural differences between CARs and the TCR could explain the reduced sensitivity of CARs.** The TCR complex contains more signalling motifs from the additional CD3 chains, benefits from co-receptor binding to stabilise its interactions, has a different affinity compared to CARs and imposes certain spatial constraints on other T cell surface molecules when it binds to peptide-MHC antigen.

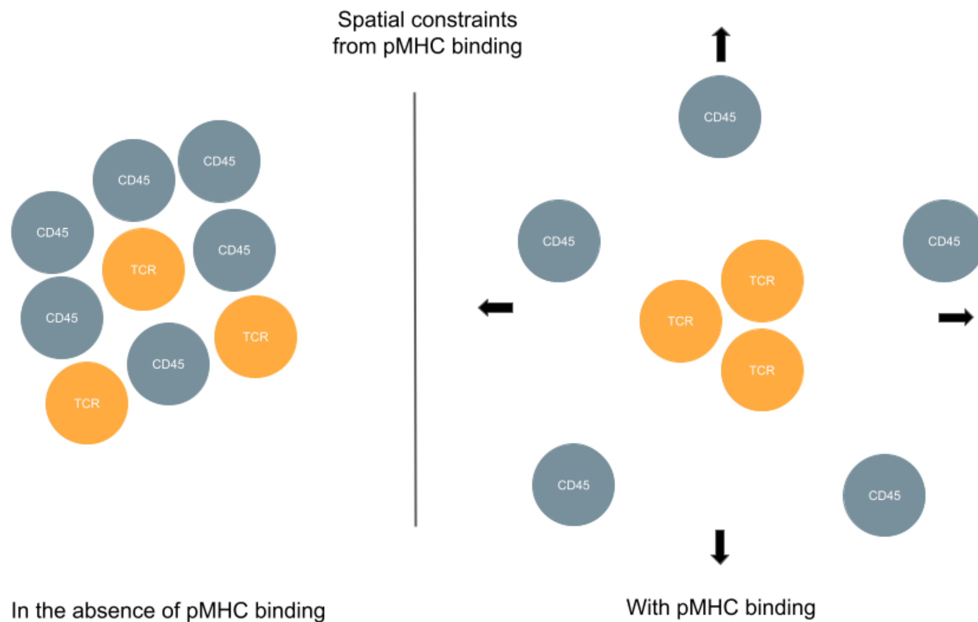


Figure 10: **TCR–pMHC binding imposes spatial constraints on other T cell surface molecules.** A top-down view of the T cell surface with and without pMHC binding. The binding of TCR–pMHC creates a close contact region between the T cell and APC which excludes the bulky extracellular domains of large phosphatases like CD45 from the contact area.

The CD3 chains: Perhaps the most glaring difference between CARs and the TCR is the absence of the various CD3 chains, which, together with the TCR α and β chains, form the TCR complex. Each of the CD3 chains present in the TCR contains an immunoreceptor tyrosine-based activation motif (ITAM); these are key signal transduction motifs for immune receptors. Because CARs lack these additional ITAMs their signalling capacity is naturally reduced relative to that of the TCR. Not only do the CD3 chains contain these ITAMs but they also contain diverse other signalling regions whose importance is only just being recognised. This quantitative and qualitative difference in signalling between CARs and the TCR may be one source of the sensitivity defect.

Co-receptors: The CD4 and CD8 co-receptors, like the TCR, also bind pMHC. The co-receptors have been proposed to increase the sensitivity of T cells by both stabilising the TCR-pMHC interaction as well as recruiting the associated kinase *Lck*, to the site of the TCR-pMHC interaction. CARs on the other hand typically target non-MHC antigens so the co-receptors cannot increase the antigen sensitivity of CARs.

Affinity: Being derived from antibodies, which typically have high affinity for their targets, CARs too have typically higher affinity for the same antigen than does the TCR. There is evidence that high affinity TCRs show reduced antigen sensitivity [108], and this may be the case for high affinity CARs as well [109, 110].

Spatial constraints: Molecules on the T cell surface function under the constrains imposed by the size of the TCR/pMHC interaction. We mentioned previously that two co-signalling interactions: CD2/CD58 and CD28/CD86 share a size similar to that of the TCR/pMHC interaction and that this leads to them co-localising in the close contact area between the T cell and the APC [111, 112]. This size constraint also segregates large phosphatases away from the TCR. The true size of many CARs is unknown, so it is not clear if they can also segregate away

from inhibitory phosphatases or co-localise with co-stimulatory molecules, either of which may reduce their sensitivity.

Co-signalling domains: The choice of co-signalling domain included within the signalling portion of the CAR can have dramatic impact on its function. For instance, constitutive signalling through the CAR, which can lead to ineffective exhausted T cells can be alleviated by the addition of the 41BB co-signalling domain. Various other signalling domains have been used and even smaller units, signalling motifs, have been used to modulate CAR-T performance [113, 114].

The effects of the different co-signalling domains within CARs on sensitivity is largely unknown. Some co-signalling domains, such as those derived from CD28, have been examined and found not to have an impact on antigen sensitivity [115]. There is however evidence that the intrinsic co-stimulation delivered by signalling domains included within the CAR may differ from that provided by intact co-signalling molecules [116]. In general little is known about how co-signalling molecules interact with CAR-T cells; something which this thesis will try to rectify.

Proposed methods to improve CAR-T cell therapies

One of the issues with CAR-T therapy is excessive cytokine release, which is not usually observed in TCR mediated T cell responses. Acute toxicity can be managed by engineering CAR-T cells to express proteins that make the amenable to antibody depletion, to engineer 'kill switches' into the cells which allow them to be depleted by small molecules [117] or to swap the affinity of the scFv or block its binding [118]. Alternatively, lowering the number of transfused CAR-T cells would also reduce the toxicity. This approach is not preferred because it might concurrently lower the efficacy of the therapy.

The toxicity of the CAR can also be reduced directly, for example by inserting inhibitory domains into the CAR. In particular, an inhibitory domain from CD3ε has been shown to reduce cytokine production by CAR-T cells [119]. Others have altered the co-stimulatory domain. The co-stimulatory domains usually found in CARs are those taken from CD28 and 41BB. But some researchers have opted for those taken from non-traditional co-stimulatory receptors, for example ICOS [120] and MyD88 [121].

As well as inserting co-stimulatory domains, CARs can also be engineered to express specific cytokines. CARs engineered to express IL-15, for example, have shown improved persistence over longer periods of time [122]. Similar improvements in CAR functionality have been observed with IL-12 and IL-18 [123]. The success of engineering CAR-T cells to secrete cytokines has also lead to the development of CARs which directly signal through the JAK/STAT pathway, as many cytokines do. These CARs have been found to display improved *in vivo* persistence and antitumour activity [113]. Although studies with engineered cytokine secretion improved persistence, antigen escape variants of the targeted tumour were still observed [122].

To address the problem of antigen escape, others have looked at ways in which the potency of CARs could be improved in order to reduce the likelihood of antigen bearing cells surviving. One approach for this is to utilise the entirety of the TCR associated signalling machinery, namely the CD3 chains, in the CAR context where they would usually otherwise be absent. This can be done in a number of ways. One way is to link the single chain variable fragment (scFv) from a CAR to the TCR-CD3 complex using a short linker. This is called a TRuC [124]. TRuCs have been found to kill tumour cells as potently as CARs containing the CD28 co-stimulatory domain, whilst also causing lower levels of cytokine release [124].

A related approach is to directly replace the TCR αβ variable regions with those of a CAR.

This is called a STAR or HIT [85, 86]. Like the TRuC, these constructs have been shown to perform better than traditional '2nd generation CARs', displaying better control of several different mouse tumour models. They have also been shown to have higher sensitivity which may reduce issues of antigen escape. Both the TRuC and STAR approaches are discussed in more detail in chapter 2.

Increasing the potency of CAR-T cells may also aid in reducing the likelihood of cytokine release syndrome and other adverse events since with higher potency the same efficacy could be achieved with fewer CAR-T cells, thus reducing the number of CAR-T cells producing excessive amounts of cytokines and subsequent toxicity.

An alternative approach to reduce the likelihood of antigen escape is the use of CAR-T cells which have multiple targets. It has been shown that CAR-T cells targeting CD19 and HER2 simultaneously resulted in increased *in vivo* antitumour activity [125] and that CAR-T cells which dual target CD19 and either CD20, CD22, or CD133 can potentially reduce the risk of relapse with $CD19^-$ tumour cells [126, 127, 128]. Dual targeting can be done by engineering the T cells to express two different CARs, or to express a CAR with dual scFvs [129]. The effect of the latter CAR format on antigen escape has not yet been shown. Trials of a dual specific CAR have shown however that different target antigens result in different selective pressures, in a dual CAR against CD22 and CD19, CD22 was not lost as strongly from the target cells as CD19 [129] and as such antigen escape can still occur even with these CARs.

Having multiple targets can also be used to reduce toxicity by increasing the specificity of CARs such that they require more than one antigen to be present to become activated. This can allow them to be targeted more precisely to tumour tissue rather than healthy tissue. For example, creating 'AND gates' that lead to maximal CAR activation when two target antigens are present rather than only a single antigen. It is also possible to decompose the CARs signal I and signal II, that is, ζ-chain signalling from that of the CAR intrinsic co-signalling (e.g. from CD28 or 41BB). In this way the optimal effect is produced when both the ζ chain CAR is engaged *and* the CD28/41BB co-signalling CAR is engaged [118].

The above described methods focus on manipulating the CAR construct itself, but there is recent evidence that suggests that the location and regulation of the CAR transgene is also important. Ordinarily, adoptive cell therapy uses retroviruses to carry the transgene into the genome of the cell and this results in the random insertion of the CAR into the genome. This has fortuitously revealed that disruption of some genes can alter the performance of CAR-T cells. For example, it has been found that disruption of the *TET2* locus alters the differentiation state and proliferative capacity of the T cell in a manner which confers a therapeutic advantage [130]. The role of TET2 in DNA methylation suggests that targeting the epigenome may improve the efficacy of CAR T cells.

Whilst the TET2 disruption occurred by chance, advances in genome engineering mean that the CAR can now be inserted by design into any location of the genome. Researchers have used this tool to insert the CAR into the T cell receptor α constant (TRAC) locus, which was found to increase T cell potency and reduce tonic signalling [131].

Amongst all the proposed CAR improvements, it is the prevention of antigen escape which is most key for increasing the clinical potential of CAR-T cells. As described, several strategies have been proposed to address this shortcoming, however it is difficult to compare the effectiveness of these approaches as they have been developed and evaluated in different contexts; in this thesis will compare these strategies directly.

This thesis

Here, we take a systematic approach to identify the defect in CAR-T cell antigen sensitivity and use this knowledge to develop novel CAR-T cells with improved antigen sensitivity. Chapter 1 of this thesis explores the sensitivity defect between a CAR and TCR targeting the same antigen, and then examines the role of several prominent co-signalling receptors on the sensitivity of the same CAR and TCR pair. Finding that CARs are unable to exploit the CD2 and LFA-1 mediated co-signalling receptor interactions.

Chapter 2 then investigates the sensitivity of some alternative chimeric receptors which have been proposed to have increased sensitivity compared to the traditional CAR format. Finding that the sensitivity of the these CARs can be linked to their ability to exploit co-stimulation, and suggesting a means to recapitulate TCR sensitivity.

In chapter 3 we explore *why* CARs are unable to capitalise on co-signalling via the CD2/CD58 interaction and propose a means to improve CAR sensitivity by engineering a chimeric CD2 receptor.

References

[1] Carol SK Leung. “Endogenous antigen presentation of MHC class II epitopes through non-autophagic pathways”. In: *Frontiers in immunology* 6 (2015), p. 464.

[2] Louis N Schoettle, Morgan E Poindexter, and Joseph N Blattman. “T-cell Receptors”. In: *eLS* (2015), pp. 1–12.

[3] Hongbing Yang et al. “Elimination of latently HIV-infected cells from antiretroviral therapy-suppressed subjects by engineered immune-mobilizing T-cell receptors”. In: *Molecular Therapy* 24.11 (2016), pp. 1913–1925.

[4] Niels AW Lemmermann et al. “Immune evasion proteins of murine cytomegalovirus preferentially affect cell surface display of recently generated peptide presentation complexes”. In: *Journal of virology* 84.3 (2010), pp. 1221–1236.

[5] Lauren M Sompayrac. *How the immune system works.* John Wiley & Sons, 2019.

[6] Kenneth Murphy and Casey Weaver. *Janeway’s immunobiology.* Garland science, 2016.

[7] Yunmin Jung et al. “Three-dimensional localization of T-cell receptors in relation to microvilli using a combination of superresolution microscopies”. In: *Proceedings of the National Academy of Sciences* 113.40 (2016), E5916–E5924.

[8] Gina López-Cantillo et al. “CAR-T Cell Performance: How to Improve Their Persistence?” In: *Frontiers in immunology* (2022), p. 1757.

[9] Federica Sallusto, Jens Geginat, and Antonio Lanzavecchia. “Central memory and effector memory T cell subsets: function, generation, and maintenance”. In: *Annual review of immunology* 22 (2004), p. 745.

[10] Jinfang Zhu and William E Paul. “Heterogeneity and plasticity of T helper cells”. In: *Cell research* 20.1 (2010), pp. 4–12.

[11] Dario AA Vignali, Lauren W Collison, and Creg J Workman. “How regulatory T cells work”. In: *Nature reviews immunology* 8.7 (2008), pp. 523–532.

[12] Don Mason. “A very high level of crossreactivity is an essential feature of the T-cell receptor”. In: *Immunology today* 19.9 (1998), pp. 395–404.

[13] Linda Wooldridge et al. “Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface”. In: *Journal of Biological Chemistry* 280.30 (2005), pp. 27491–27501.

[14] Arrate Mallabiabarrena, Manuel Fresno, and Balbino Alarcón. “An endoplasmic reticulum retention signal in the CD3e chain of the T-cell receptor”. In: *Nature* 357.6379 (1992), pp. 593–596.

[15] De Dong et al. “Structural basis of assembly of the human T cell receptor–CD3 complex”. In: *Nature* 573.7775 (2019), pp. 546–552.

[16] Dennis X Beringer et al. “T cell receptor reversed polarity recognition of a self-antigen major histocompatibility complex”. In: *Nature immunology* 16.11 (2015), pp. 1153–1161.

[17] Veronica T Chang et al. “Initiation of T cell signaling by CD45 segregation at’close contacts’”. In: *Nature immunology* 17.5 (2016), pp. 574–582.
[18] Jennifer D Stone, Adam S Chervin, and David M Kranz. “T-cell receptor binding affinities and kinetics: impact on T-cell activity and specificity”. In: *Immunology* 126.2 (2009), pp. 165–176.
[19] Darienne R Myers, Julie Zikherman, and Jeroen P Roose. “Tonic signals: why do lymphocytes bother?” In: *Trends in immunology* 38.11 (2017), pp. 844–857.
[20] Yi Li et al. “Directed evolution of human T-cell receptors with picomolar affinities by phage display”. In: *Nature biotechnology* 23.3 (2005), pp. 349–354.
[21] Minh Ngoc Duong et al. “Chronic TCR-MHC (self)-interactions limit the functional potential of TCR affinity-increased CD8 T lymphocytes”. In: *Journal for immunotherapy of cancer* 7.1 (2019), pp. 1–17.
[22] Evan M Beckman et al. “Recognition of a lipid antigen by CD1-restricted $\alpha\beta$+ T cells”. In: *Nature* 372.6507 (1994), pp. 691–694.
[23] Alessandro Vacchini et al. “MR1-restricted T cells are unprecedented cancer fighters”. In: *Frontiers in immunology* 11 (2020), p. 751.
[24] Keenan T Bashour et al. “CD28 and CD3 have complementary roles in T-cell traction forces”. In: *Proceedings of the National Academy of Sciences* 111.6 (2014), pp. 2241–2246.
[25] Dibyendu Kumar Das et al. “Force-dependent transition in the T-cell receptor β-subunit allosterically regulates peptide discrimination and pMHC bond lifetime”. In: *Proceedings of the National Academy of Sciences* 112.5 (2015), pp. 1517–1522.
[26] K Christopher Garcia et al. “An $\alpha\beta$ T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex”. In: *Science* 274.5285 (1996), pp. 209–219.
[27] Stephen C Bunnell et al. “T cell receptor ligation induces the formation of dynamically regulated signaling assemblies”. In: *The Journal of cell biology* 158.7 (2002), pp. 1263–1275.
[28] Gabriele Campi, Rajat Varma, and Michael L Dustin. “Actin and agonist MHC–peptide complex–dependent T cell receptor microclusters as scaffolds for signaling”. In: *The Journal of experimental medicine* 202.8 (2005), pp. 1031–1036.
[29] Enfu Hui and Ronald D Vale. “In vitro membrane reconstitution of the T-cell receptor proximal signaling network”. In: *Nature structural & molecular biology* 21.2 (2014), pp. 133–142.
[30] Jun Huang et al. “A single peptide-major histocompatibility complex ligand triggers digital cytokine secretion in CD4+ T cells”. In: *Immunity* 39.5 (2013), pp. 846–857.
[31] Mario Brameshuber et al. “Monomeric TCRs drive T cell antigen recognition”. In: *Nature immunology* 19.5 (2018), pp. 487–496.
[32] P Anton van der Merwe et al. “Topology of the CD2–CD48 cell-adhesion molecule complex: implications for antigen recognition by T cells”. In: *Current Biology* 5.1 (1995), pp. 74–84.
[33] P Anton van der Merwe. “A subtle role for CD2 in T cell antigen recognition”. In: *The Journal of experimental medicine* 190.10 (1999), pp. 1371–1374.
[34] Olivier Leupin et al. “Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes”. In: *Current Biology* 10.5 (2000), pp. 277–280.
[35] Kaushik Choudhuri et al. “T-cell receptor triggering is critically dependent on the dimensions of its peptide-MHC ligand”. In: *Nature* 436.7050 (2005), pp. 578–582.
[36] Jennifer E Smith-Garvin, Gary A Koretzky, and Martha S Jordan. “T cell activation”. In: *Annual review of immunology* 27 (2009), p. 591.
[37] Elizabeth K Barber et al. “The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex.” In: *Proceedings of the National Academy of Sciences* 86.9 (1989), pp. 3277–3281.
[38] Alexander M Mørch et al. “Coreceptors and TCR signaling–the strong and the weak of it”. In: *Frontiers in cell and developmental biology* 8 (2020), p. 597627.
[39] Makio Iwashima et al. “Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases”. In: *Science* 263.5150 (1994), pp. 1136–1139.
[40] Weiguo Zhang et al. “LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation”. In: *Cell* 92.1 (1998), pp. 83–92.
[41] Kaushik Choudhuri et al. “Peptide-major histocompatibility complex dimensions control proximal kinase-phosphatase balance during T cell activation”. In: *Journal of Biological Chemistry* 284.38 (2009), pp. 26096–26105.

[42] Hong-Sheng Lim et al. "Costimulation of IL-2 production through CD28 is dependent on the size of its ligand". In: *The Journal of Immunology* 195.11 (2015), pp. 5432–5439.

[43] Tadashi Yokosuka et al. "Spatiotemporal basis of CTLA-4 costimulatory molecule-mediated negative regulation of T cell activation". In: *Immunity* 33.3 (2010), pp. 326–339.

[44] Tadashi Yokosuka et al. "Programmed cell death 1 forms negative costimulatory microclusters that directly inhibit T cell receptor signaling by recruiting phosphatase SHP2". In: *Journal of Experimental Medicine* 209.6 (2012), pp. 1201–1217.

[45] Matthew H Bakalar et al. "Size-dependent segregation controls macrophage phagocytosis of antibody-opsonized targets". In: *Cell* 174.1 (2018), pp. 131–142.

[46] Karsten Köhler et al. "Matched sizes of activating and inhibitory receptor/ligand pairs are required for optimal signal integration by human natural killer cells". In: *PloS one* 5.11 (2010), e15374.

[47] Ryan D Guest et al. "The role of extracellular spacer regions in the optimal design of chimeric immune receptors: evaluation of four different scFvs and antigens". In: *Journal of immunotherapy* 28.3 (2005), pp. 203–211.

[48] Waleed Haso et al. "Anti-CD22–chimeric antigen receptors targeting B-cell precursor acute lymphoblastic leukemia". In: *Blood, The Journal of the American Society of Hematology* 121.7 (2013), pp. 1165–1174.

[49] Veronika I Zarnitsyna et al. "Estimating the diversity, completeness, and cross-reactivity of the T cell repertoire". In: *Frontiers in immunology* 4 (2013), p. 485.

[50] Carl H June et al. "T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression". In: *Molecular and cellular biology* 7.12 (1987), pp. 4472–4481.

[51] Nan-ping Weng, Arne N Akbar, and Jorg Goronzy. "CD28- T cells: their role in the age-associated decline of immune function". In: *Trends in immunology* 30.7 (2009), pp. 306–312.

[52] Deborah J Lenschow et al. "Differential up-regulation of the B7-1 and B7-2 costimulatory molecules after Ig receptor engagement by antigen." In: *The Journal of Immunology* 153.5 (1994), pp. 1990–1997.

[53] ML Del Rio et al. "HVEM/LIGHT/BTLA/CD160 cosignaling pathways as targets for immune regulation". In: *Journal of leukocyte biology* 87.2 (2010), pp. 223–235.

[54] Fabien Garçon et al. "CD28 provides T-cell costimulation and enhances PI3K activity at the immune synapse independently of its capacity to interact with the p85/p110 heterodimer". In: *Blood, The Journal of the American Society of Hematology* 111.3 (2008), pp. 1464–1471.

[55] Amy D Holdorf et al. "Proline residues in CD28 and the Src homology (SH) 3 domain of Lck are required for T cell costimulation". In: *The Journal of experimental medicine* 190.3 (1999), pp. 375–384.

[56] Jonathan S Boomer and Jonathan M Green. "An enigmatic tail of CD28 signaling". In: *Cold Spring Harbor perspectives in biology* 2.8 (2010), a002436.

[57] WC Hahn, SJ Burakoff, and BE Bierer. "Signal transduction pathways involved in T cell receptor-induced regulation of CD2 avidity for CD58." In: *The Journal of Immunology* 150.7 (1993), pp. 2607–2619.

[58] De-Min Zhu et al. "Mechanisms of cellular avidity regulation in CD2–CD58-mediated T cell adhesion". In: *ACS chemical biology* 1.10 (2006), pp. 649–658.

[59] Akiko Hashimoto-Tane et al. "Micro–adhesion rings surrounding TCR microclusters are essential for T cell activation". In: *Journal of Experimental Medicine* 213.8 (2016), pp. 1609–1625.

[60] Martin F Bachmann, Marijke Barner, and Manfred Kopf. "CD2 sets quantitative thresholds in T cell activation". In: *The Journal of experimental medicine* 190.10 (1999), pp. 1383–1392.

[61] Edgar Meinl et al. "Differential requirement of ZAP-70 for CD2-mediated activation pathways of mature human T cells". In: *The Journal of Immunology* 165.7 (2000), pp. 3578–3583.

[62] Jonathan M Green et al. "Coordinate regulation of T cell activation by CD2 and CD28". In: *The Journal of Immunology* 164.7 (2000), pp. 3591–3595.

[63] Judith Leitner et al. "CD58/CD2 is the primary costimulatory pathway in human CD28- CD8+ T cells". In: *The Journal of Immunology* 195.2 (2015), pp. 477–487.

[64] Enas Abu-Shah et al. "Human CD8+ T cells exhibit a shared antigen threshold for different effector responses". In: *The Journal of Immunology* 205.6 (2020), pp. 1503–1512.

[65] Tetsuro Sasada and Ellis L Reinherz. "A critical role for CD2 in both thymic selection events and mature T cell function". In: *The Journal of Immunology* 166.4 (2001), pp. 2394–2403.

[66] Eddie CY Wang et al. "Suppression of costimulation by human cytomegalovirus promotes evasion of cellular immune defenses". In: *Proceedings of the National Academy of Sciences* 115.19 (2018), pp. 4998–5003.

[67] Patricia Ho et al. "The CD58: CD2 axis is co-regulated with PD-L1 via CMTM6 and governs anti-tumor immunity". In: *bioRxiv* (2022).

[68] Michael Croft. “The role of TNF superfamily members in T-cell function and diseases”. In: *Nature Reviews Immunology* 9.4 (2009), pp. 271–285.
[69] Kazushi Izawa et al. “Inherited CD70 deficiency in humans reveals a critical role for the CD70–CD27 pathway in immunity to Epstein-Barr virus infection”. In: *Journal of Experimental Medicine* 214.1 (2017), pp. 73–89.
[70] Julie Denoeud and Muriel Moser. “Role of CD27/CD70 pathway of activation in immunity and tolerance”. In: *Journal of leukocyte biology* 89.2 (2011), pp. 195–203.
[71] Nancy Hogg, Irene Patzak, and Frances Willenbrock. “The insider’s guide to leukocyte integrin signalling and function”. In: *Nature Reviews Immunology* 11.6 (2011), pp. 416–426.
[72] William A Comrie et al. “The dendritic cell cytoskeleton promotes T cell adhesion and activation by constraining ICAM-1 mobility”. In: *Journal of Cell Biology* 208.4 (2015), pp. 457–473.
[73] Wei Chen, Jizhong Lou, and Cheng Zhu. “Forcing switch from short-to intermediate-and long-lived states of the αA domain generates LFA-1/ICAM-1 catch bonds”. In: *Journal of Biological Chemistry* 285.46 (2010), pp. 35967–35978.
[74] Colin RF Monks et al. “Three-dimensional segregation of supramolecular activation clusters in T cells”. In: *Nature* 395.6697 (1998), pp. 82–86.
[75] Martin F Bachmann et al. “Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation”. In: *Immunity* 7.4 (1997), pp. 549–557.
[76] Yinan Wang et al. “LFA-1 decreases the antigen dose for T cell activation in vivo”. In: *International immunology* 20.9 (2008), pp. 1119–1127.
[77] Günther Eissner, Walter Kolch, and Peter Scheurich. “Ligands working as receptors: reverse signaling by members of the TNF superfamily enhance the plasticity of the immune system”. In: *Cytokine & growth factor reviews* 15.5 (2004), pp. 353–366.
[78] Caroline Robert. “A decade of immune-checkpoint inhibitors in cancer therapy”. In: *Nature Communications* 11.1 (2020), pp. 1–3.
[79] Ignacio Melero et al. “Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors”. In: *Nature medicine* 3.6 (1997), pp. 682–685.
[80] Rajesh K Sharma et al. “Costimulation as a platform for the development of vaccines: a peptide-based vaccine containing a novel form of 4-1BB ligand eradicates established tumors”. In: *Cancer research* 69.10 (2009), pp. 4319–4326.
[81] Rajesh K Sharma, Esma S Yolcu, and Haval Shirwan. “SA-4-1BBL as a novel adjuvant for the development of therapeutic cancer vaccines”. In: *Expert review of vaccines* 13.3 (2014), pp. 387–398.
[82] Drew Pardoll. “Does the immune system see tumors as foreign or self?” In: *Annual review of immunology* 21 (2003), p. 807.
[83] Michael H Kershaw, Jennifer A Westwood, and Phillip K Darcy. “Gene-engineered T cells for cancer therapy”. In: *Nature Reviews Cancer* 13.8 (2013), pp. 525–541.
[84] Yoshihisa Kuwana et al. “Expression of chimeric receptor composed of immunoglobulin-derived V resions and T-cell receptor-derived C regions”. In: *Biochemical and biophysical research communications* 149.3 (1987), pp. 960–968.
[85] Yue Liu et al. “Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors”. In: *Science Translational Medicine* 13.586 (2021), eabb5191.
[86] Jorge Mansilla-Soto et al. “HLA-independent T cell receptors for targeting tumors with low antigen density”. In: *Nature Medicine* 28.2 (2022), pp. 345–352.
[87] Zelig Eshhar et al. “Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors.” In: *Proceedings of the National Academy of Sciences* 90.2 (1993), pp. 720–724.
[88] Robert E Bird et al. “Single-chain antigen-binding proteins”. In: *Science* 242.4877 (1988), pp. 423–426.
[89] Thomas Brocker. “Chimeric Fv-ζ or Fv-ε receptors are not sufficient to induce activation or cytokine production in peripheral T cells”. In: *Blood, The Journal of the American Society of Hematology* 96.5 (2000), pp. 1999–2001.
[90] Xiao-Song Zhong et al. “Chimeric antigen receptors combining 4-1BB and CD28 signaling domains augment PI3kinase/AKT/Bcl-XL activation and CD8+ T cell–mediated tumor eradication”. In: *Molecular therapy* 18.2 (2010), pp. 413–420.
[91] De-Gang Song et al. “In Vivo Persistence, Tumor Localization, and Antitumor Activity of CAR-Engineered T Cells Is Enhanced by Costimulatory Signaling through CD137 (4-1BB) Costimulated FRα-Specific CAR Therapy of Cancer”. In: *Cancer research* 71.13 (2011), pp. 4617–4627.

[92] Syam Tammana et al. “4-1BB and CD28 signaling plays a synergistic role in redirecting umbilical cord blood T cells against B-cell malignancies”. In: *Human gene therapy* 21.1 (2010), pp. 75–86.

[93] M. Ernst et al. “Chimeric antigen receptor (CAR) T-cell therapy for people with relapsed or refractory diffuse large B-cell lymphoma”. In: *Cochrane Database of Systematic Reviews* 9 (2021). issn: 1465-1858. doi: 10.1002/14651858.CD013365.pub2. url: https://doi.org//10.1002/14651858.CD013365.pub2.

[94] Sattva S Neelapu et al. “Chimeric antigen receptor T-cell therapy—assessment and management of toxicities”. In: *Nature reviews Clinical oncology* 15.1 (2018), pp. 47–62.

[95] Daniel Sommermeyer et al. “Fully human CD19-specific chimeric antigen receptors for T-cell therapy”. In: *Leukemia* 31.10 (2017), pp. 2191–2199.

[96] Christine M Bucks et al. “Chronic antigen stimulation alone is sufficient to drive CD8+ T cell exhaustion”. In: *The Journal of Immunology* 182.11 (2009), pp. 6697–6708.

[97] Zhi-Zhang Yang et al. “TIGIT Expression Is Associated with T-cell Suppression and Exhaustion and Predicts Clinical Outcome and Anti–PD-1 Response in Follicular LymphomaTIGIT in Follicular Lymphoma”. In: *Clinical Cancer Research* 26.19 (2020), pp. 5217–5231.

[98] Taku Kouro, Hidetomo Himuro, and Tetsuro Sasada. “Exhaustion of CAR T cells: potential causes and solutions”. In: *Journal of Translational Medicine* 20.1 (2022), pp. 1–10.

[99] Öykü Umut et al. “CAR T cell therapy in solid tumors: A short review”. In: *memo-Magazine of European Medical Oncology* 14.2 (2021), pp. 143–149.

[100] Ming Yi et al. “Regulation of PD-L1 expression in the tumor microenvironment”. In: *Journal of Hematology & Oncology* 14.1 (2021), pp. 1–13.

[101] David H Munn and Vincenzo Bronte. “Immune suppressive mechanisms in the tumor microenvironment”. In: *Current opinion in immunology* 39 (2016), pp. 1–6.

[102] David L Porter et al. “Chimeric antigen receptor T cells persist and induce sustained remissions in relapsed refractory chronic lymphocytic leukemia”. In: *Science translational medicine* 7.303 (2015), 303ra139–303ra139.

[103] Jiaqiao Fan et al. “Development of CAR-T cell persistence in adoptive immunotherapy of solid tumors”. In: *Frontiers in oncology* 10 (2021), p. 574860.

[104] Donna L Farber, Naomi A Yudanin, and Nicholas P Restifo. “Human memory T cells: generation, compartmentalization and homeostasis”. In: *Nature Reviews Immunology* 14.1 (2014), pp. 24–35.

[105] Robbie G Majzner and Crystal L Mackall. “Tumor antigen escape from CAR T-cell therapy”. In: *Cancer discovery* 8.10 (2018), pp. 1219–1226.

[106] Venugopal Gudipati et al. “Inefficient CAR-proximal signaling blunts antigen sensitivity”. In: *Nature Immunology* 21.8 (2020), pp. 848–856.

[107] Daniel T Harris et al. “Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains”. In: *The Journal of Immunology* 200.3 (2018), pp. 1088–1100.

[108] Sharyn Thomas et al. “Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen”. In: *Blood, The Journal of the American Society of Hematology* 118.2 (2011), pp. 319–329.

[109] Michael L Olson et al. “Low-affinity CAR T cells exhibit reduced trogocytosis, preventing rapid antigen loss, and increasing CAR T cell expansion”. In: *Leukemia* (2022), pp. 1–4.

[110] Sara Ghorashian et al. “Enhanced CAR T cell expansion and prolonged persistence in pediatric patients with ALL treated with a low-affinity CD19 CAR”. In: *Nature medicine* 25.9 (2019), pp. 1408–1414.

[111] Shannon K Bromley et al. “The immunological synapse and CD28-CD80 interactions”. In: *Nature immunology* 2.12 (2001), pp. 1159–1166.

[112] Simon J Davis and P Anton Van Der Merwe. “The structure and ligand interactions of CD2: implications for T-cell function”. In: *Immunology today* 17.4 (1996), pp. 177–187.

[113] Yuki Kagoya et al. “A novel chimeric antigen receptor containing a JAK–STAT signaling domain mediates superior antitumor effects”. In: *Nature medicine* 24.3 (2018), pp. 352–359.

[114] Johannes Breuning, Brian Philip, and Marion H Brown. “Addition of the C-terminus of CD6 to a chimeric antigen receptor enhances cytotoxicity and does not compromise expression”. In: *Immunology* 156.2 (2019), pp. 130–135.

[115] M Chmielewski, AA Hombach, and H Abken. “CD28 cosignalling does not affect the activation threshold in a chimeric antigen receptor-redirected T-cell attack”. In: *Gene therapy* 18.1 (2011), pp. 62–72.

[116] Robbie G Majzner et al. “CD58 aberrations limit durable responses to CD19 CAR in large B cell lymphoma patients treated with axicabtagene ciloleucel but can be overcome through novel CAR engineering”. In: *Blood* 136 (2020), pp. 53–54.

[117] Sarwish Rafiq, Christopher S Hackett, and Renier J Brentjens. “Engineering strategies to overcome the current roadblocks in CAR T cell therapy”. In: *Nature reviews Clinical oncology* 17.3 (2020), pp. 147–167.

[118] Jang Hwan Cho, James J Collins, and Wilson W Wong. “Universal chimeric antigen receptors for multiplexed and logical control of T cell responses”. In: *Cell* 173.6 (2018), pp. 1426–1438.

[119] Wei Wu et al. “Multiple signaling roles of CD3ε and its application in CAR-T cell therapy”. In: *Cell* 182.4 (2020), pp. 855–871.

[120] Sonia Guedan et al. “Enhancing CAR T cell persistence through ICOS and 4-1BB costimulation”. In: *JCI insight* 3.1 (2018).

[121] Melinda Mata et al. “Inducible Activation of MyD88 and CD40 in CAR T Cells Results in Controllable and Potent Antitumor Activity in Preclinical Solid Tumor ModelsInducible MyD88 and CD40 in CAR T Cells”. In: *Cancer discovery* 7.11 (2017), pp. 1306–1319.

[122] Giedre Krenciute et al. “Transgenic expression of IL15 improves antiglioma activity of IL13Rα2-CAR T cells but results in antigen loss variants”. In: *Cancer immunology research* 5.7 (2017), pp. 571–581.

[123] Elizabeth R Hawkins, Reena R D’Souza, and Astero Klampatsa. “Armored CAR T-cells: The next chapter in T-cell cancer immunotherapy”. In: *Biologics: targets & therapy* 15 (2021), p. 95.

[124] Patrick A Baeuerle et al. “Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response”. In: *Nature communications* 10.1 (2019), pp. 1–12.

[125] Zakaria Grada et al. “TanCAR: a novel bispecific chimeric antigen receptor for cancer immunotherapy”. In: *Molecular Therapy-Nucleic Acids* 2 (2013), e105.

[126] Alexandra Martyniszyn et al. “CD20-CD19 bispecific CAR T cells for the treatment of B-cell malignancies”. In: *Human gene therapy* 28.12 (2017), pp. 1147–1157.

[127] Hejin Jia et al. “Haploidentical CD19/CD22 bispecific CAR-T cells induced MRD-negative remission in a patient with relapsed and refractory adult B-ALL after haploidentical hematopoietic stem cell transplantation”. In: *Journal of hematology & oncology* 12.1 (2019), pp. 1–9.

[128] Dan Li et al. “TanCAR T cells targeting CD19 and CD133 efficiently eliminate MLL leukemic cells”. In: *Leukemia* 32.9 (2018), pp. 2012–2016.

[129] Jay Y Spiegel et al. “CAR T cells with dual targeting of CD19 and CD22 in adult patients with recurrent or refractory B cell malignancies: a phase 1 trial”. In: *Nature medicine* 27.8 (2021), pp. 1419–1431.

[130] Joseph A Fraietta et al. “Disruption of TET2 promotes the therapeutic efficacy of CD19-targeted T cells”. In: *Nature* 558.7709 (2018), pp. 307–312.

[131] Justin Eyquem et al. “Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection”. In: *Nature* 543.7643 (2017), pp. 113–117.

Chapter 1

A comparison between T cell receptor and chimeric antigen receptor ability to exploit accessory receptors

Introduction

Antigen sensitivity of the T cell receptor

One of the remarkable features of the T cell receptor is its exquisite sensitivity. There is evidence that a T cell can respond to a single foreign peptide-MHC antigen (pMHC) [1]. This sensitivity is required for two reasons. First, that the copy number of individual pMHCs is likely to be low. In *Yang et al.* [2], after *in vitro* infection with HIV, cells were observed to have between 8 and 46 viral pMHCs. Second, many viruses are known to downregulate MHC as an escape mechanism. The production of viral proteins known as immunoevasins which interfere with MHC, for example by decreasing peptide transport into the ER, can aid them in avoiding detection by the immune system. *In vitro* infection with human cytomegalovirus (HCMV) has been shown to reduce the number of MHC molecules by 10-fold, and the number of viral specific pMHCs from approximately 10^4 to fewer than 100 [3].

The T cell obtains its sensitivity through a diverse range of mechanisms. These include the use of the CD8 and CD4 co-receptors, having a large number of ITAMs in the TCR-CD3 complex (10 in total), amplification of downstream signalling by co-stimulatory molecules such as 41BB, and so on [4, 5]. By and large these mechanisms act to either promote TCR-pMHC binding, induce rapid phosphorylation of the TCR ITAMs and ZAP70 or increase signalling downstream of the TCR. These features are likely to prove useful in the design of highly sensitive engineered receptors. The prime example of engineered receptors today being chimeric antigen receptors, or CARs, as used in immunotherapy.

Chimeric antigen receptors & immunotherapy

T cells are able to recognise a variety of cells, including cancer cells [6]. Though this is a particular challenge for the immune system owing to the similarity of cancer antigens to self antigens, their small number [7], and the weaker affinity of TCR to these antigens [8].

Notably, of course, the immune system is unable to recognise all cancers and so there has been much interest in developing tools that would enable the immune system to be redirected against novel targets. The primary approach for this is the use of chimeric antigen receptors (CARs). These are engineered receptors which fuse antibody derived single chain variable fragments (scFvs) to a hinge and transmembrane domain (often derived from CD28 or CD8α) followed by intracellular signalling domains from the T cell receptor complex. When transduced into T cells these CARs can selectively induce T cell responses towards cells expressing the target antigen.

These CARs have been shown to function effectively against liquid tumours [9, 10] but, despite their high efficacy, a significant proportion of patients later relapse with tumours arising from cells that have low or no detectable expression of the original target antigen [11]. This is known as antigen escape and is thought to arise because CARs have a reduced sensitivity to antigen — requiring between 100 and 1000 times more antigen to become activated compared to the TCR [12, 13].

Mechanisms underlying the defect in antigen sensitivity of CARs

Understanding why CARs require larger quantities of antigen compared to the TCR is vital if we are to engineer new CARs capable of preventing antigen escape. However, the sensitivity of CAR T cells is often not assessed. The reason for this is that it is often not possible to easily titrate the concentration of folded antigens on target cells. In studies where an antigen titration was performed the CARs that were used either targeted a peptide-MHC antigen [13] allowing for titration of peptide on APCs or purified folded antigen was titrated on planar lipid bilayers [12].

Collectively, these and other studies have shown that inclusion of signalling domains taken from various co-signalling receptors in the cytoplasmic tail of CARs can alter sensitivity [14, 15], as can altering the number of ITAMs [16], and the identity of the hinge region [17]. However, increasing the number of ITAMs in a CAR did not lead to an increase in antigen sensitivity [13] and there is no clear hypothesis for how changes in the hinge could impact antigen sensitivity. Therefore, although the hinge and signalling domains of CARs can impact antigen sensitivity, this information has not translated into novel CARs that can recapitulate the antigen sensitivity of the TCR.

An aspect which has not previously been addressed is the role of co-signalling receptors on the antigen sensitivity of CAR-T cells. These receptors, which are expressed on the surface of T cells and augment T cell signalling events, can increase the sensitivity of T cells to antigen [5, 18, 19, 20]. It has previously been shown that some CARs also benefit from these interactions [21], but whether they increase CAR antigen sensitivity is unclear.

We hypothesised that CAR-T cells are unable to capitalise on these co-signalling interactions in the same way as TCR-T cells and that this is the cause of their reduced sensitivity when compared to TCR-T cells. To study the effect of co-signalling molecules we utilise a solid-phase system consisting of immobilised antigen on streptavidin coated plates. Remarkably when presented with only antigen in this manner both CAR and TCR-T cells exhibit the same sensitivity. By systematically adding purified ligands to different co-signalling receptors we

identify that the CD2 and LFA-1 co-signalling receptors dramatically improve the sensitivity of TCR, but not CAR-T cells. Further controls identify that this is the result of an intrinsic CAR defect.

Results

A CAR against pMHC antigen displays reduced sensitivity compared to a TCR against the same pMHC antigen

To date, only a single study [13] has systematically compared antigen sensitivity between a TCR and CAR. We first wanted to confirm this observation, and to do so we use both a TCR (1G4) and CAR (D52N) that target the same antigen; the NY-ESO-1 peptide on HLA-A*02:01 (Figure 11, A). Since we are using a peptide antigen it is possible to titrate peptide on target cells, enabling sensitivity to be measured. Throughout this study we use a variant of the NY-ESO-1 peptide which is mutated such that there is a valine at position 9 that improves HLA-A*02:01 binding affinity and solution stability [22], hence we refer to it as the 9V peptide or pMHC.

In this study we used $CD8^+$ T cells isolated from human blood donors, lentivirally transduced with either the TCR or CAR and expanded using IL-2 and CD3/CD28 bead stimulation for 6 days followed by expansion with IL-2 alone until the cells were discarded at 14 days post isolation. This is a protocol which is similar to that used to produce the cells required for adoptive cell therapy. Transduced and blasted T cells were then co-cultured for 24 hours with T2 target cells pulsed with differing concentrations of peptide antigen (Figure 11, B). At the end of the co-culture period the fraction of target cells killed (% cytotoxicity) and fraction of activated T cells (those expressing the activation marker CD69) are assessed for each antigen dose.

We find that D52N CARs show a reduced sensitivity compared to the 1G4 TCR when antigen is presented on APCs (Figure 11, C). This remains true even when we systematically alter the CAR architecture. We tested CAR variants with two hinge regions taken from clinically approved CARs, which are taken from regions of other T cell surface proteins: CD8α (D52N-CD8α-z) and CD28 (D52N-CD28-z), as well as a hinge region which we estimated to be larger than either of these which was derived from IgG1 (D52N-IgG1-z) (Figure 12). All of these CARs were much less sensitive than the TCR; 300, 590 and 1900-fold respectively (Figure 11, C-D).

We also systematically altered the CAR signalling domain, testing both '1st generation' and '2nd generation CARs' (Figure 12). The latter having an additional signalling domain taken from CD28 in addition to that derived from the TCR ζ-chain. We found no significant difference in the sensitivity of 1st and 2nd generation constructs with the same hinge region (Figure 11, C-D).

These findings were consistent across both sensitivity read-outs — cytotoxicity (Figure 11, C) or CD69 expression (Figure 11, D). Indeed, both of these read-outs are correlated ($R^2 = 0.93$, slope = 1.13, see Figure 13) and therefore CD69 can be used as a proxy metric for cytotoxicity when assessing sensitivity. As the T cells we use here are already 'antigen experienced' (having been expanded with CD3/CD28 stimulation), the have pre-formed lytic granules which can be released without further transcription. Since we observe a poor antigen sensitivity when measuring cytotoxicity this is consistent with the defect being more proximal to receptor triggering.

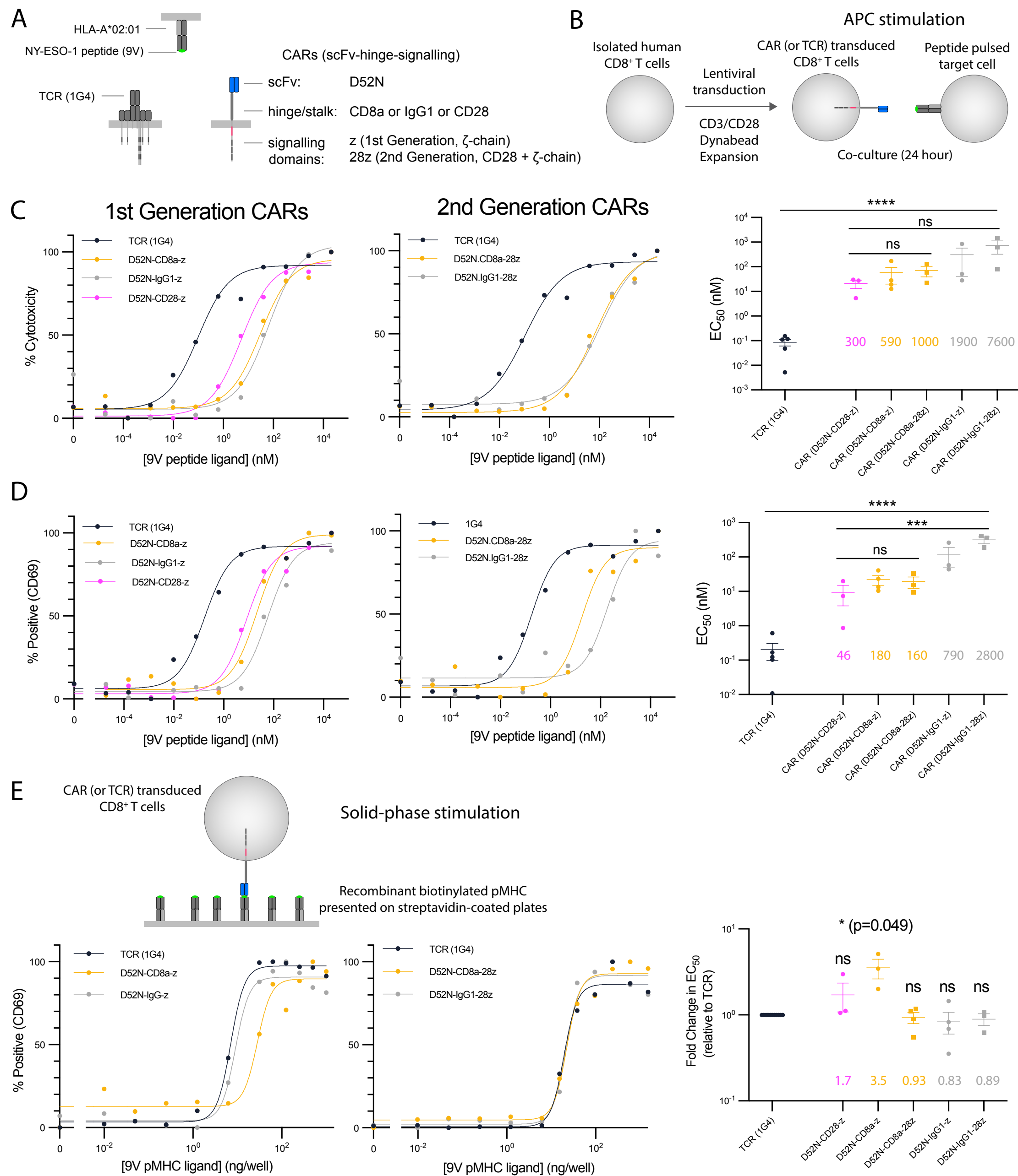


Figure 11: **CARs show reduced sensitivity compared to the TCR when antigen is presented on APCs but not when presented as purified protein.** **(A)** Schematic of antigen receptors. The 1G4 TCR and the D52N scFv both recognise the 9V NY-ESO-1 peptide antigen (having a valine at position 9 of the original NY-ESO-1 peptide) presented on HLA-A*02:01. CARs using the CD8a hinge contain the CD8a transmembrane domain whereas CARs using the IgG1 or CD28 hinges contain the CD28 transmembrane domain. **(B)** Assay schematic. **(C-D)** Representative dose-response showing **(C)** cytotoxicity by LDH release and **(D)** surface expression of CD69 for the TCR and the indicated CARs along with EC_{50} values from at least 3 independent experiments determined by fitting a Hill function to each dose-response curve. **(E)** Representative dose-response when purified biotinylated 9V pMHC ligand is presented on streptavidin-coated plates (left two plots) and EC_{50} values from at least 3 independent experiments (right). The EC_{50} values are compared using (C, D) one-way ANOVA or (E) one-sample t-test for a hypothetical mean of 1.0 on log-transformed values. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

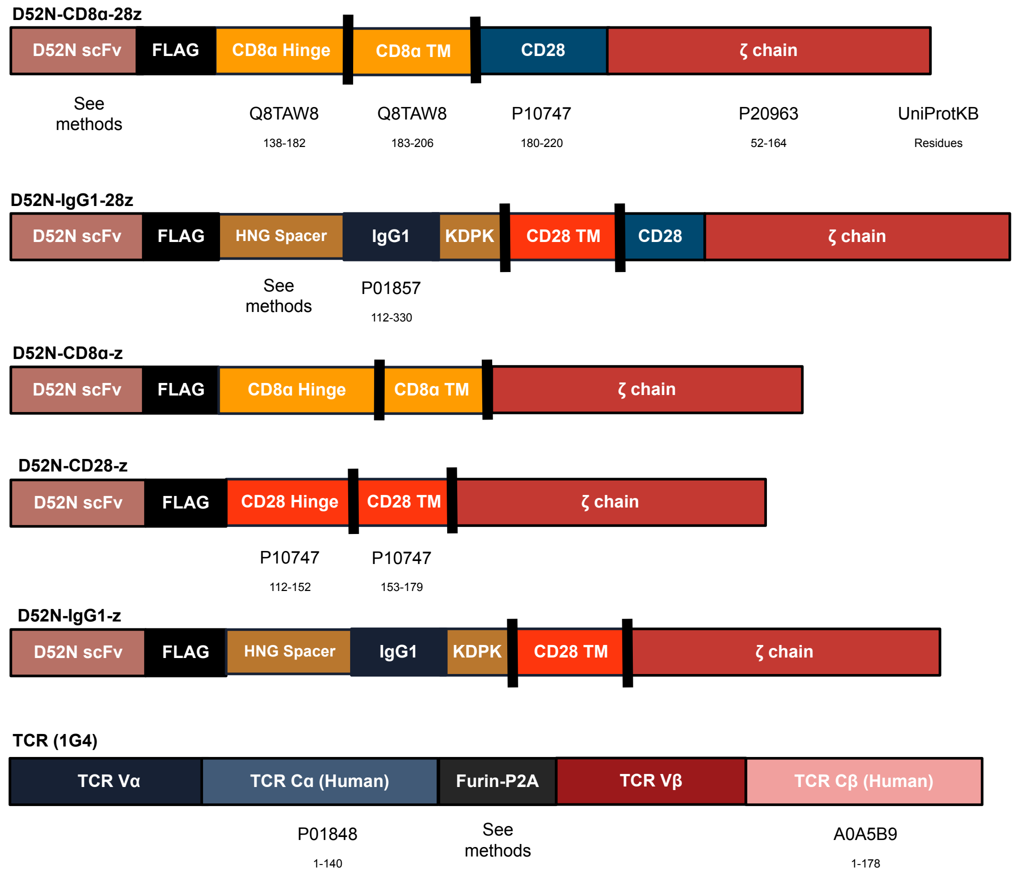


Figure 12: **We tested a variety of chimeric receptors with different 'hinge' and signalling domains.** CARs consisting of a D52N antibody derived single chain variable fragment coupled to a FLAG-tag and a hinge region derived from CD8, IgG1, or CD28 and transmembrane domain from either CD28 or CD8 and a signalling region consisting of TCRζ ('1st generation') or TCRζ and CD28 ('2nd generation') were lentivirally transduced into human CD8+ T cells.

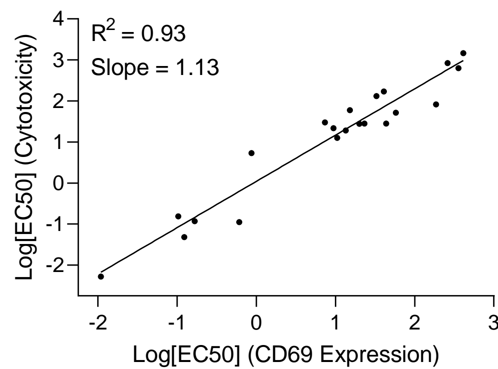


Figure 13: **Sensitivity measured by CD69 expression and cytotoxicity are correlated.** Data from Figure 11, C & D are plotted against each other and the log-transformed EC_{50} values fitted by linear regression giving the coefficient of determination (R^2) and slope indicated.

When antigen is presented as purified protein TCR and CARs display similar antigen sensitivity

One of the mechanisms by which the T cell is thought to achieve its remarkable sensitivity (reviewed in [4, 23]) is through co-signalling receptors expressed on the surface of the T cell which are able to modulate T cell signalling. This includes those adhesion receptors which modulate signalling by increasing the effective on-rate of the TCR or by improving the stability of the TCR–pMHC interaction.

To asses whether these varied receptors have a differential impact on CAR compared to TCR-T cells we use a reductionist solid-phase system whereby antigen is presented as recombinant biotinylated pMHC on streptavidin coated plates (Figure 11, E). In contrast to the cellular assay where peptide is titrated on APCs, in this plate-based assay the re-folded purified pMHC is titrated directly on plates and then T cells are added and their response at each concentration of antigen assessed.

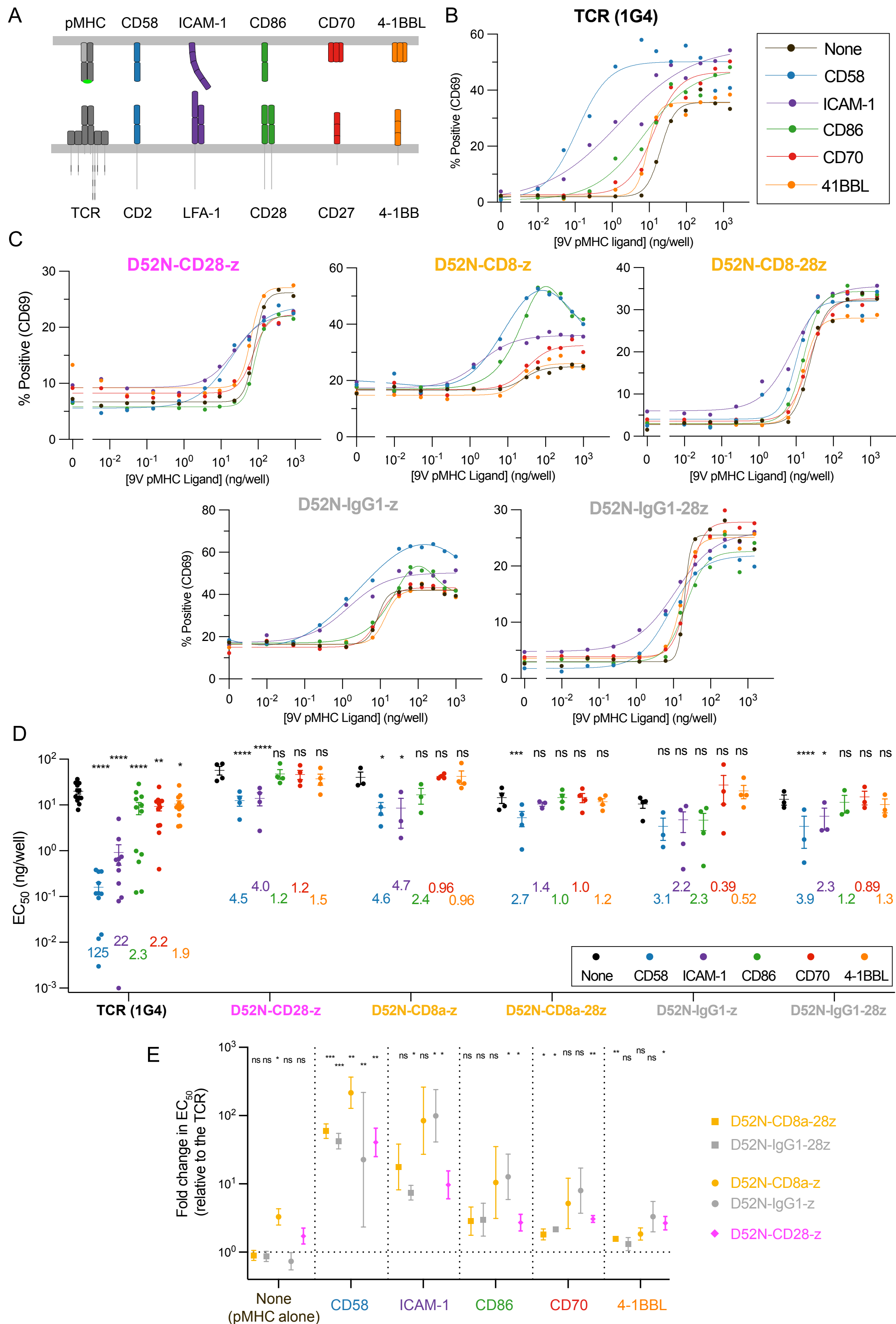
We found that when presented as purified protein the difference in sensitivity between CARs and the TCR became less than 4-fold (compared to >46 fold in the cellular assay, c.f. Figure 11 D & E). With only a significant difference between TCR and CAR sensitivity for the D52N-CD8a-z CAR (p=0.049). Taken together, this suggests that other factors not present in the plate-based stimulation, such as ligands to co-signalling receptors, may be important in increasing the antigen sensitivity of the TCR relative to CARs.

Co-signalling adhesion receptors increase TCR, but not CAR, antigen sensitivity

We hypothesised that a reason for the CAR sensitivity defect observed in our cellular assay was that CAR-T cells are unable to capitalise on co-signalling molecules to the same extent as TCR-T cells. To test this hypothesis we used the same solid-phase system, only this time in addition to antigen we also immobilised a fixed concentration of a purified co-signalling ligand.

We opted to screen a range of prominent co-signalling receptors: the adhesion ligands CD58 and ICAM-1, whose receptors are respectively CD2 and LFA-1; CD86, the ligand to CD28; and TNF family members CD70 and 4-1BBL (CD137L), the ligands to CD27 and 41BB (CD137) respectively. In doing so, we found that CD58 and ICAM-1 dramatically increase TCR sensitivity (by 100- and 20-fold respectively) whilst the TNF family ligands showed only a small impact on sensitivity (Figure 14, B & D). This is consistent with previous results [5]. We also found only a small impact on sensitivity from the CD28-CD86 interaction (Figure 14, D); again consistent with previous reports [20].

CD58 and ICAM-1 have a much less dramatic effect on CAR-T cell sensitivity (<5-fold). This was true for a whole range of CARs; we saw no difference in the effect of CD58 and ICAM-1 when changing either the hinge region of the CAR or its signalling domain (Figure 14, C-D). As with the TCR, we observed that both CD86 and the TNF family ligands also had a small impact on CAR-T sensitivity.



A
pMHC
CD58
ICAM-1
CD86
CD70
4-1BBL
TCR
CD2
LFA-1
CD28
CD27
4-1BB
B
TCR (1G4)
% Positive (CD69)
[9V pMHC ligand] (ng/well)
None
CD58
ICAM-1
CD86
CD70
41BBL
C
D52N-CD28-z
D52N-CD8-z
D52N-CD8-28z
D52N-IgG1-z
D52N-IgG1-28z
% Positive (CD69)
[9V pMHC Ligand] (ng/well)
D
EC50 (ng/well)
None
CD58
ICAM-1
CD86
CD70
4-1BBL
TCR (1G4)
D52N-CD28-z
D52N-CD8a-z
D52N-CD8a-28z
D52N-IgG1-z
D52N-IgG1-28z
E
Fold change in EC50 (relative to the TCR)
D52N-CD8a-28z
D52N-IgG1-28z
D52N-CD8a-z
D52N-IgG1-z
D52N-CD28-z
None (pMHC alone)
CD58
ICAM-1
CD86
CD70
4-1BBL

Figure 14: **Systematic engagement of accessory receptors identifies that CARs are inefficient at exploiting the adhesion receptors CD2 and LFA-1 relative to the TCR. (A)** Schematic of accessory receptors and their ligands. **(B-C)** Representative dose-response curves showing T cell activation by upregulation of surface CD69 measured by flow cytometry after 24 hours using the solid-phase stimulation assay. T cells were presented with purified pMHC alone ('None') or with a fixed concentration of 250 ng/well of the indicated accessory receptor ligand (colours) for the (B) TCR and (C) the indicated CARs. **(D)** The EC_{50} values for the indicated antigen receptor and purified ligand condition were obtained by fitting a Hill function to each dose-response curve. Individual EC_{50} values for each antigen receptor are from an independent experiment (N≥3). The numbers indicate the fold-change in EC_{50} induced by the accessory receptor ligand relative to pMHC alone ('None') and statistical significance is determined by a paired t-test on log-transformed data. **(E)** The data in (D) is presented in a different format showing the fold-change in EC_{50} between the TCR and the indicated CAR for pMHC alone or the indicated accessory receptor ligand. The fold-change is compared using a one-sample t-test to a hypothetical value of 0 on log-transformed data. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001

We confirmed that, in addition to to failing to increase sensitivity when measuring CD69 expression, CAR-T cells were also inefficient at exploiting adhesion receptors when measuring IFNγ production (Figure 15). Though the sensitivity impact of ICAM-1 and CD58 for TCR-T cells was less when measured this way than with CD69 expression (52- and 8.6-fold for CD58 and ICAM-1 respectively) this was still dramatically higher than for the CARs (<6.4- and <3.2-fold for CD58 and ICAM-1 respectively). The magnitude of the effect of CD86, CD70 and 41BBL was similar when measuring cytokine production as when measuring CD69 expression (cf. Figure 15, B & 11, B).

Together these results suggest that CAR-T cells fail to capitalise on adhesion interactions mediated by CD58 and ICAM-1 to the same extent as TCR-T cells.

CAR-T cells fail to efficiently exploit ICAM-1 and CD58 in a cellular system

To test whether our findings in the solid-phase system could explain the sensitivity defect observed in the cellular system we tested the effect of abrogating either, or both, of the CD58 and ICAM-1 interactions in a cellular assay. To do so we performed a similar co-culture assay as previously, this time using U87 cells rather than T2 cells as the target. As before, we titrated the peptide antigen on target cells with different concentrations of antigen before incubating them with T cells and then measuring the expression of T cell activation markers. This time, we also added blocking antibodies to CD58, ICAM-1 or both at a fixed concentration to the media before the incubation period (Figure 16, A).

We saw that blocking CD58 and ICAM-1 had a greater effect on TCR T cells than CAR-T cells. The loss of sensitivity when blocking the CD58 interaction was significantly higher for T cells than for CAR-T cells (Figure 16, C-D (right)), as was also the case when blocking both the ICAM-1 and CD58 interactions together. The fold change between the TCR and CAR was also significantly reduced in these two conditions (Figure 16, C-D (left)). We did not observe any significant difference in the sensitivity reduction between CAR and TCR-T cells when blocking only the ICAM-1 interaction.

In addition to using blocking antibodies we also used CRISPR (Figure 16, E) to knock-out the endogenous ICAM-1, CD58, or both, from U87 cells and performed the same assay. In this system we were able to reproduce the same result as with blocking antibodies — the loss of

sensitivity from knocking out either CD58 or both ICAM-1 was significantly higher for TCR than CAR-T cells and there was no significant difference when knocking out ICAM alone (Figure 16, F-H).

Given that the expression of CD58 and ICAM-1 can vary across different target cells, it was important to reproduce our findings using a different target cell. We therefore repeated this experiment using NALM6 cells as the target cell line. The NALM6 cell line expressed lower

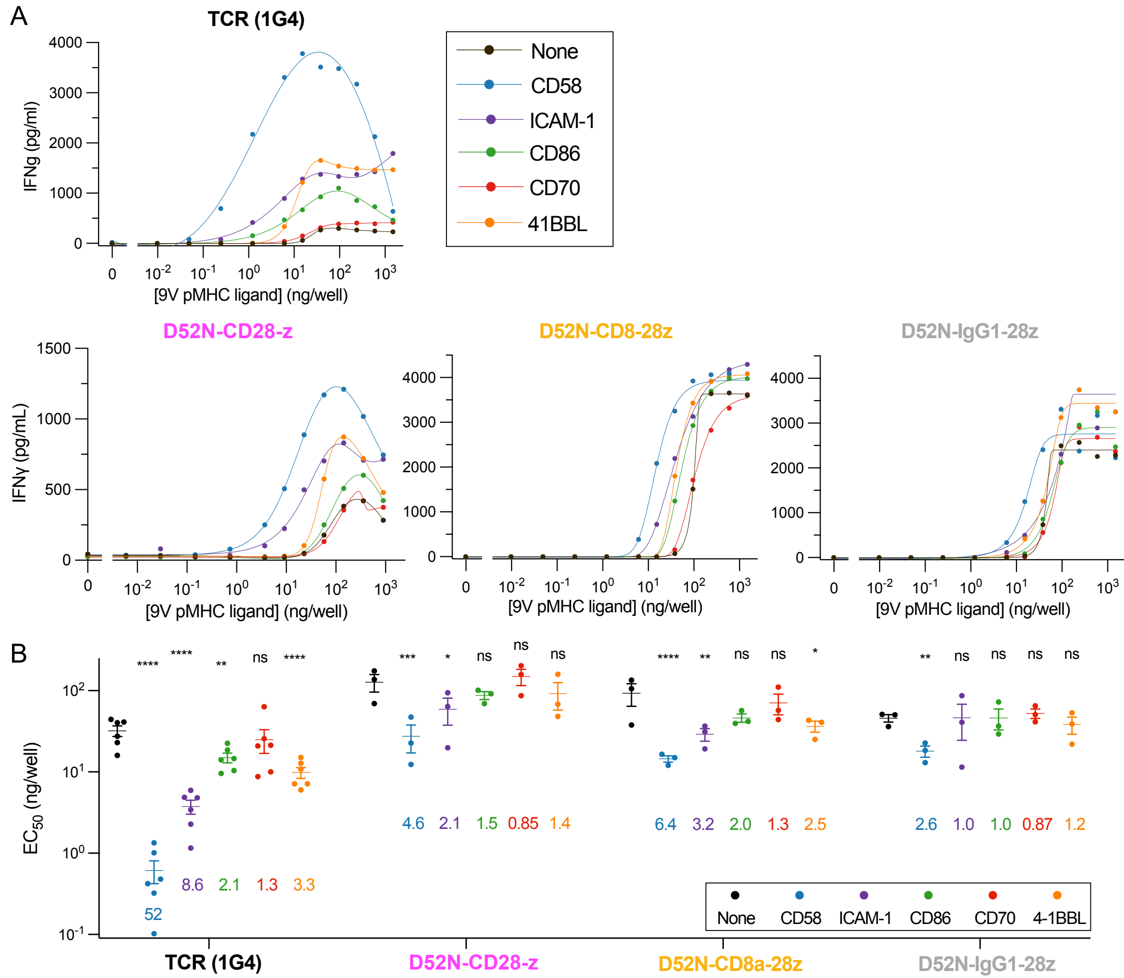


Figure 15: **CARs are inefficient at exploiting adhesion receptors when measuring IFNγ production.** **(A)** Representative dose-responses using T cells expressing the indicated antigen receptor stimulated by a titration of purified pMHC alone ('None') or in combination with a fixed concentration of the indicated purified accessory receptor ligand (colours). The supernatant concentration of IFNγ was determined after 24 hours by ELISA. Each dose-response curve was fitted to obtain the EC_{50} and E_{max} values. **(B)** The EC_{50} values for the indicated antigen receptor and purified ligand. The coloured numbers indicate the fold-change in EC_{50} induced by the addition of the indicated accessory receptor ligand relative to pMHC alone ('None'). The EC_{50} values for each ligand are compared to the 'None' condition using a paired t-test on log-transformed data. **(C)** The fold-change in E_{max} relative to pMHC alone ('None') for each antigen receptor and purified ligand. The fold-change is compared using a one-sample t-test to a hypothetical value of 0 on log-transformed data. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

levels of CD58 but higher levels of ICAM-1 when compared to the U87 cell line and both showed lower expression of both ligands relative to the T2 target cells (Figure 17, E).

As with U87 target cells we found that blocking CD58, or both CD58 and ICAM-1 had a larger effect on reducing TCR compared to CAR sensitivity (Figure 17, C-D). We also observed that blocking ICAM-1 alone had a small but significantly higher effect on TCR sensitivity than CAR sensitivity, which we did not observe with the U87 target cells. This could be explained by the slightly higher levels of ICAM-1 we observed on NALM6 compared to U87 cells (Figure 17, E).

Surface expression of CARs does not explain a sensitivity defect

A straightforward explanation for the reduced sensitivity of CAR-T cells would be that that the CARs were expressed at lower levels than the TCR. The transduction efficiency, that is the percentage of cells which were positive for a given receptor construct, varies between repeats and is never 100% as each experimental repeat was performed with T cells isolated from an individual donor and then subsequently transduced with a fresh lentiviral preparation. What we are interested in however, is the surface expression of each receptor. This is measured as the gMFI of the receptor positive cells by flow cytometry.

Though they had, on average, poorer transduction efficiency than the TCR (Figure 18, C) we found that the surface expression of the chimeric receptors was similar to or higher than the TCR (Figure 18, D). This rules out lower CAR expression levels as an explanation for the sensitivity defect in our experimental system.

CAR induced tonic signalling cannot explain a sensitivity defect

It has previously been shown that tonic signalling induced by CARs can lead to T cell exhaustion [24]. The exhausted T cell phenotype includes a loss of T cell function and an increase in the expression of inhibitory receptors [25]. Both of which could lead to a reduction in sensitivity. It could therefore be suggested that the sensitivity defect observed in CAR-T cells is a result of tonic signalling induced exhaustion.

One way in which exhaustion might alter the behaviour of CAR T cells is through modulating the expression of the various co-signalling receptors. Tonic signalling induced downregulation of these receptors may lead to the CAR-T cell failing to exploit these signals to increase sensitivity. However, we found that this was not the case as we observed no significant difference in the expression of co-signalling receptors in CAR compared to TCR-T cells (Figure 19, A).

We then looked to see if our T cells were becoming exhausted. To do so we measured the expression of several surface markers for T cell exhaustion. These were LAG-3, TIM-3 and PD-1. We found no significant difference in the surface expression of LAG-3 and PD-1 in CAR-T cells compared with TCR-T cells. We did however observe a small ($\leq$ 2-fold) increase in TIM-3 expression in CAR-T cells relative to TCR-T cells (Figure 19, B).

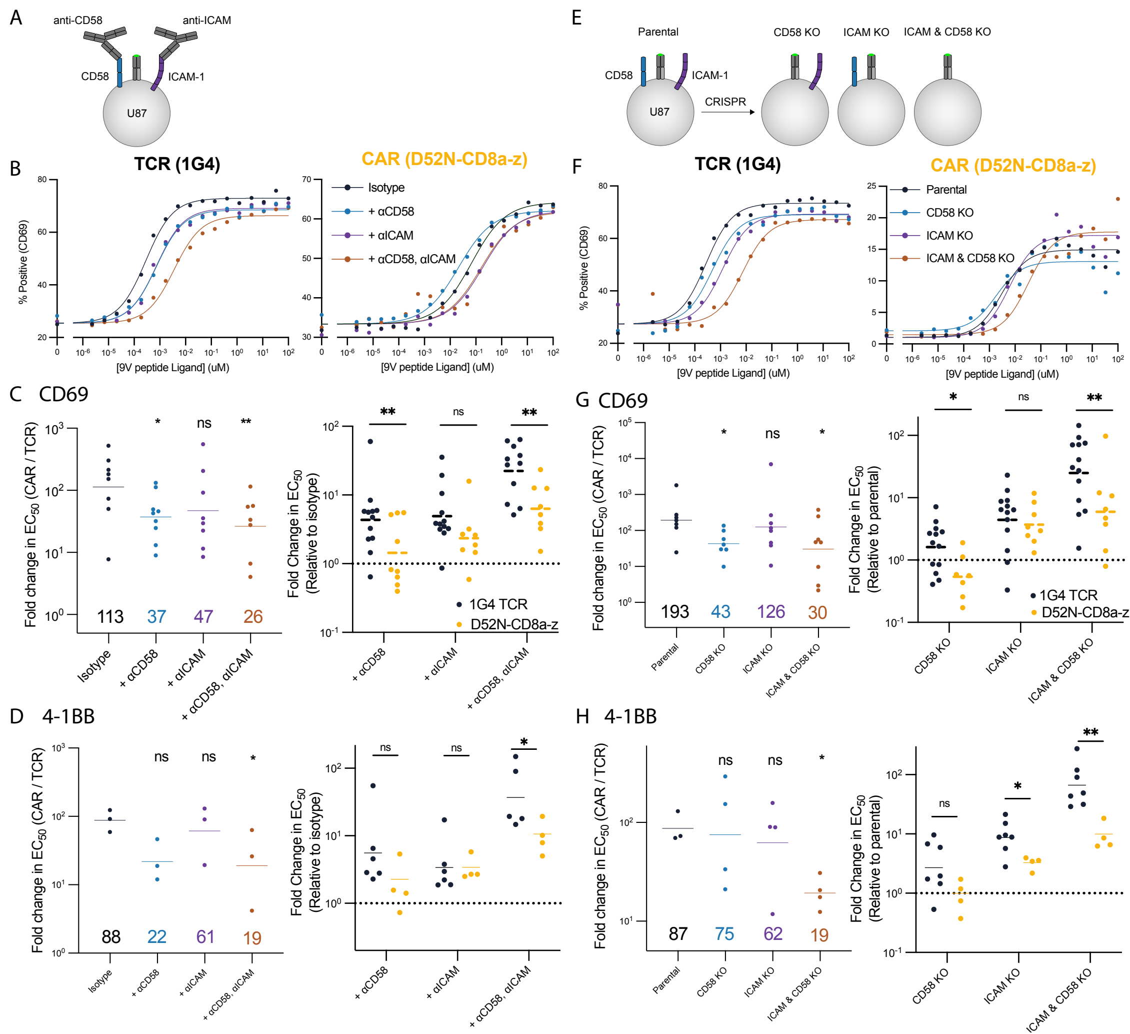


Figure 16: **Abrogating the CD2 and LFA-1 adhesion interactions disproportionately impacts the antigen sensitivity of the TCR compared to a CAR. (A)** Schematic of CD58 and ICAM-1 blocking experiment on the HLA-A2+ glioblastoma U87 target cell line. **(B)** Representative dose-response curves for the indicated blocking conditions for the TCR (left) and CAR (right). **(C-D)** Fold-change in EC_{50} between the CAR and TCR (left) or relative to the isotype (right) for (C) CD69 and (D) 4-1BB upregulation. **(E)** Schematic of CD58 and ICAM-1 knockout experiments. **(F)** Representative dose-response curves for the indicated target cell lines for the TCR (left) and CAR (right). **(G-H)** Fold-change in EC_{50} between the CAR and TCR (left) or relative to the isotype (right) for (G) CD69 and (H) 4-1BB. Individual EC_{50} values for CD69 or 4-1BB are determined by a fit to the dose-response curve from at least 3 independent experiments (each data point in C, D, G, H is from an independent experiment). The fold-change between the TCR and CAR is compared using a two-sample t-test to the isotype or parental line condition (left panel in C, D, G, H) or directly between the TCR and CAR (right panels in C, D, G, H) on log-transformed values. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

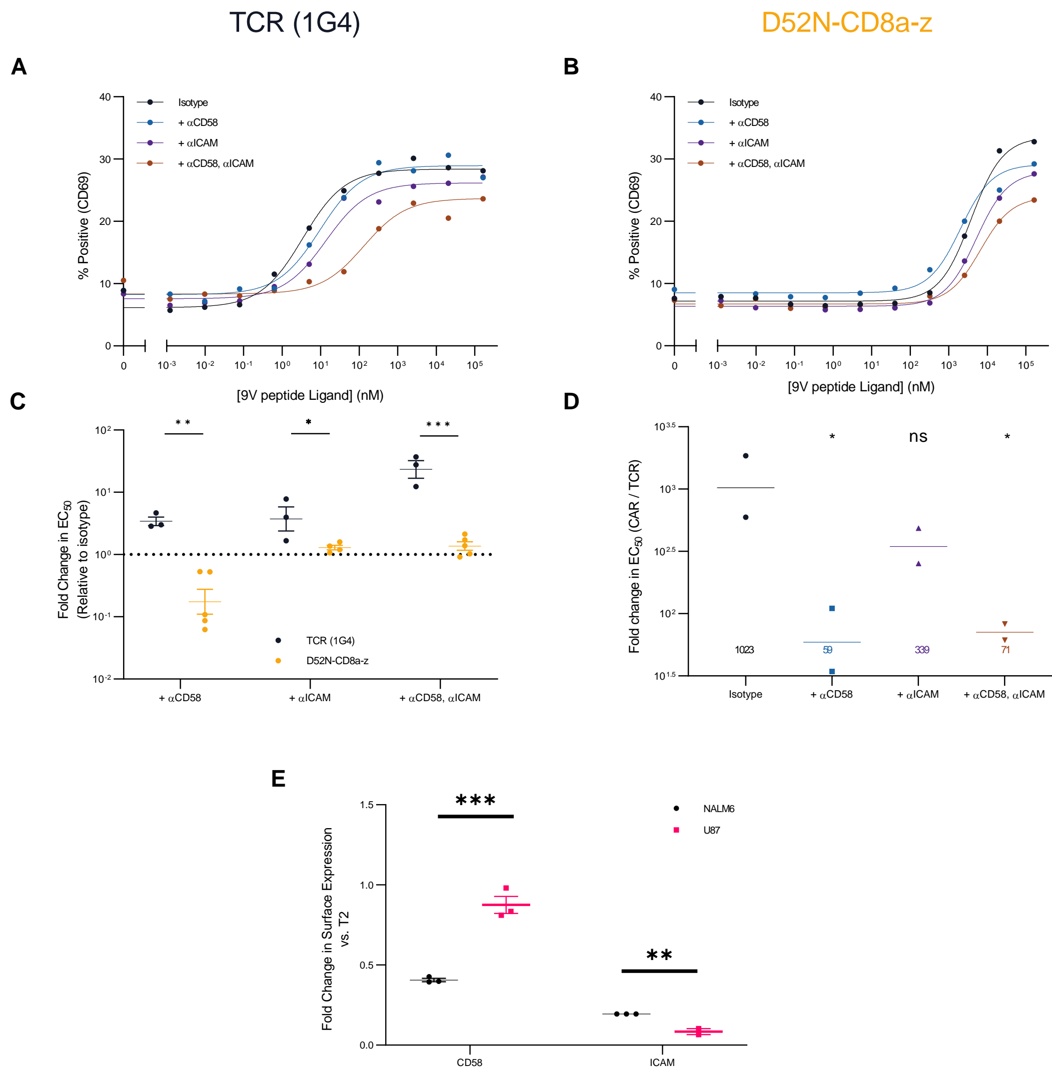
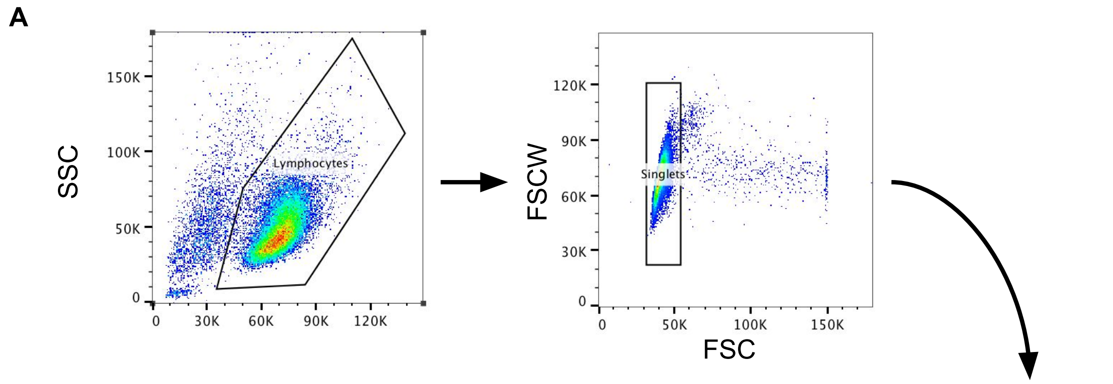
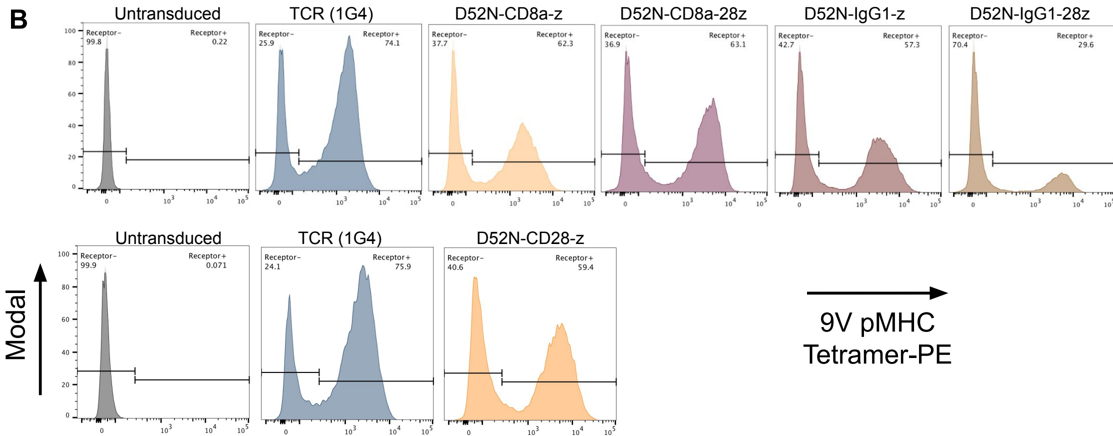
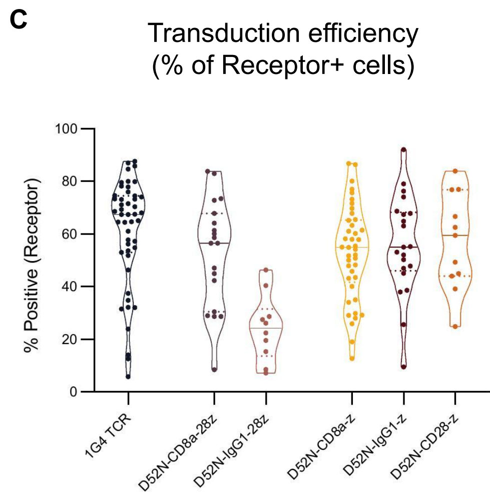


Figure 17: **The disproportionate impact of abrogating CD58 and ICAM-1 interactions on CAR sensitivity was also observed in the NALM6 cell line.** NALM6 cells are a B cell precursor leukemia cell line. **(A)** Representative dose-response curves for the indicated blocking conditions for the TCR and **(B)** CAR. **(C)** Fold-change in EC_{50} relative to the isotype condition or **(D)** between the CAR and TCR. **(E)** Surface expression levels of CD58 and ICAM-1 on NALM6 compared to U87 cells by normalisation against a reference cell line (T2s). U87 data collected by Ashna Patel. The fold-change between the TCR and CAR is compared using a two-sample t-test to the isotype condition or directly between the TCR and CAR on log-transformed values. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.


A
SSC
FSCW
FSC
Lymphocytes
Singlets


B
Untransduced
TCR (1G4)
D52N-CD8a-z
D52N-CD8a-28z
D52N-IgG1-z
D52N-IgG1-28z
D52N-CD28-z
Modal
9V pMHC
Tetramer-PE


C
Transduction efficiency
(% of Receptor+ cells)
% Positive (Receptor)
1G4 TCR
D52N-CD8a-28z
D52N-IgG1-28z
D52N-CD8a-z
D52N-IgG1-z
D52N-CD28-z

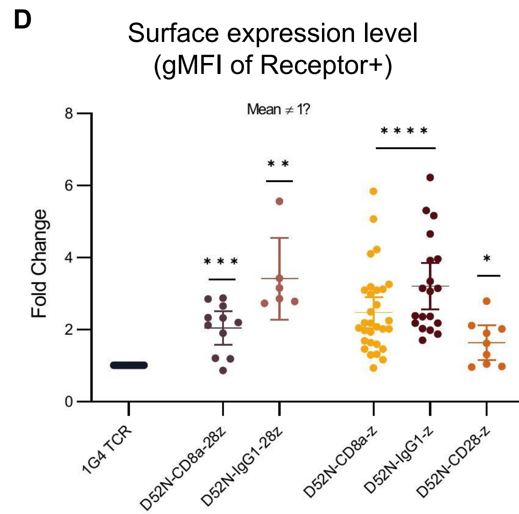

D
Surface expression level
(gMFI of Receptor+)
Mean ≠ 1?
Fold Change
1G4 TCR
D52N-CD8a-28z
D52N-IgG1-28z
D52N-CD8a-z
D52N-IgG1-z
D52N-CD28-z

Figure 18: **Surface expression of chimeric receptors was similar or higher compared to the TCR. (A)** Gating strategy to identify single lymphocytes. **(B)** Representative flow cytometry histograms showing surface expression of the indicated surface receptor using fluorescent 9V pMHC tetramers. Untransduced T cells are used to determine the negative gate. Each row is an independent experiment and each column is the indicated antigen receptor with the TCR being included in all experiments performed in the study. **(C)** The percent of T cells expressing the indicated receptor (i.e., within receptor positive gate). **(D)** The fold-change in the surface expression of each chimeric antigen receptor relative to the TCR determined by the gMFI of T cells in the receptor positive gate. The surface expression of each antigen receptor was determined for every experiment carried out in the study and is shown in aggregate in panel C and D. Individual data points for each antigen receptor represent an independent experiment (N is shown below the labels in panel C), which is generated by producing lentivirus and transducing a new sample of primary human T cells (see chapter 5). A one-sample t-test is used to compare each chimeric receptor to the expression of the TCR (1.0) on log-transformed values. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

Despite this being only a small increase in TIM-3 expression we wanted to confirm that exhaustion did not lead to a functional difference in ability to exploit co-stimulation. To do this we took CAR transduced and untransduced T cells and electroporated an orthogonal TCR (Figure 19, C). In this case, the 868 TCR that is specific to the HIV SL9 peptide [26]. This enabled us to stimulate the T cells independently of the CAR. We saw no significant difference in sensitivity between the cells which co-expressed the CAR with the SL9 TCR and those with the TCR alone (Figure 19, C). We also saw no difference in the ability of either T cells to capitalise on the CD2-CD58 interaction (Figure 19, C).

Taken together, we found no evidence that the transduction of the CAR impaired the ability of T cells to display high antigen sensitivity by capitalising on the CD2-CD58 interaction.

Supraphysiological affinity of CARs cannot explain a sensitivity defect

It has previously been shown that high affinity to antigen can reduce the sensitivity of the TCR [27] and the D52N CAR tested here has a higher affinity for the 9V pMHC than does the 1G4 TCR. The 9V pMHC was shown in surface plasmon resonance experiments to result in an approximately 50-fold higher affinity for the CAR than for the TCR at 37 °C (Figure 20, A). Since the CAR has a supraphysiological affinity (K_D=0.15 μM) this may explain the corresponding reduction in sensitivity we observe relative to the TCR.

To control for this possibility we identified an 9V peptide mutant to which the CAR showed a similar affinity as the TCR does for the 9V NY-ESO-1 variant (7 μM). This mutation was an alanine at position 4, referred to as '4A'. When using this lower affinity peptide we observe that the sensitivity of the CAR is reduced (Figure 20, B). The difference between the TCR and the CAR is therefore bigger with matched affinity peptides than when both target the 9V pMHC. We also find that TCR-T cells have a 10-fold better sensitivity than CAR-T cells when their affinities are matched (Figure 20, C).

The lower affinity CAR-T cells still remained unable to capitalise on the CD2-CD58 interaction as TCR-T cells were 1000-fold more sensitive than their matched affinity CAR counterparts in the presence of CD58 (Figure 20, B-C). The low affinity CAR-T cells were only able to achieve a 10-fold improvement in sensitivity (Figure 20, D), similar to that seen with the higher affinity peptide. We therefore conclude that the higher affinity of the CAR to the 9V pMHC cannot

explain the difference in antigen sensitivity in the presence or absence of CD58. In all of these experiments the different pMHCs were presented at matched levels (Figure 20, E).

The CAR antigen sensitivity defect is independent of the CD8 co-receptor

$CD8^+$ T cells carry the CD8 co-receptor that binds MHC at a site that is independent of the TCR. This allows the CD8 co-receptor to increase the sensitivity of T cells by both stabilising the TCR-pMHC interaction [28] as well as recruiting the associated kinase *Lck* (which phosphorylates the TCR) to the site of the TCR-pMHC interaction [29].

Given the low affinity of CD8 to MHC (10 μM to 200 μM [30]) it is thought that cooperativity with the TCR is required for stable CD8-pMHC-TCR complexes to form [31]. It is not known if CD8 is able to associate with CARs or to bind MHC in the presence of our pMHC targeting D52N CAR. It could be suggested that the TCR may benefit from an increased sensitivity as a result of the the CD8 co-receptor, but that the CAR is unable to do so. Given that the majority of CARs target non-MHC surface proteins, it was important to determine whether CD8 was impacting the relative differences in antigen sensitivity we were observing between the TCR and CARs.

To test if the sensitivity defect in CAR-T cells could be explained by a failure to exploit the CD8 co-receptor, we used an HLA-A*02:01 mutant (DT227/8KA) [32] to abrogate CD8 binding and then tested the effect on TCR and CAR sensitivity (Figure 21, A). The effects of CD8 are known to be affinity dependent, with CD8 playing a greater role in low affinity interactions [32] so we tested the effects of abrogating CD8 binding under different affinity regimes.

For the TCR we found that at K_D=7 μM affinity (i.e., for the 9V NY-ESO-1 peptide) abrogating CD8 binding had no effect on sensitivity (Figure 21, C). This was to be expected as this is a within the higher affinity regime where CD8 is predicted to have a limited effect [32]. We did however observe that abrogating CD8 binding had a small but significant effect on the ability of TCR-T cells to capitalise on CD58 (Figure 21, C). Consistent with previous findings [33], when we reduced the affinity of the TCR-pMHC interaction by using the NYESO 4A peptide (K_D=299 μM) we abolished the T cell response at all concentrations of antigen tested when using the CD8 binding mutant. This was independent of whether CD58 was included or not.

These results would suggest that the CAR, which has a higher affinity than the TCR for the 9V (K_D=0.15 μM) pMHC should also see no effect on sensitivity from the possible loss of CD8. Indeed, when we use the DT227/8KA mutant we see no difference in sensitivity compared to wild-type HLA-A*02:01 (Figure 21, E). Unlike with the TCR we see no difference in the ability of CAR-T cells to capitalise on CD58 when CD8 binding is abrogated.

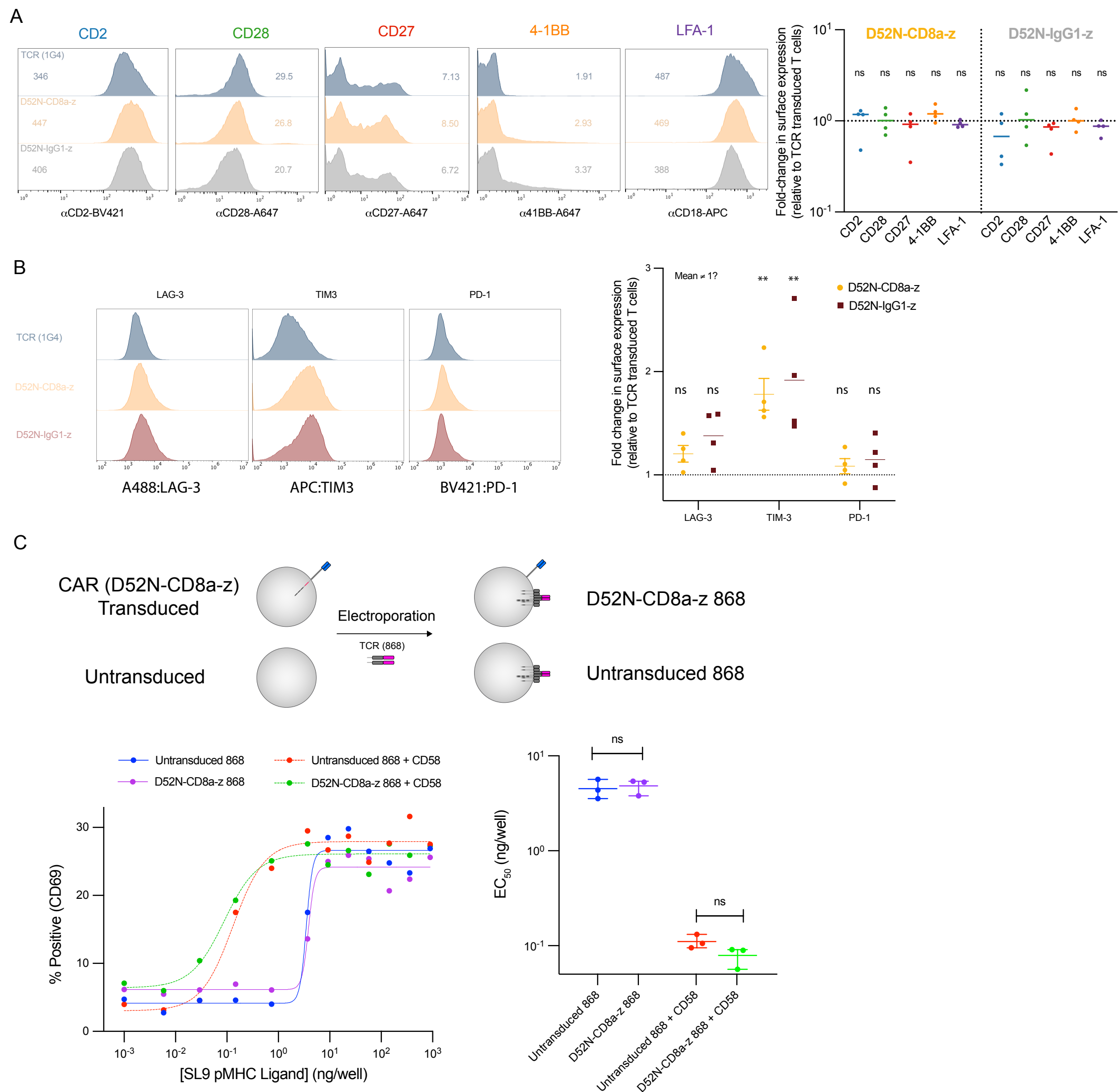
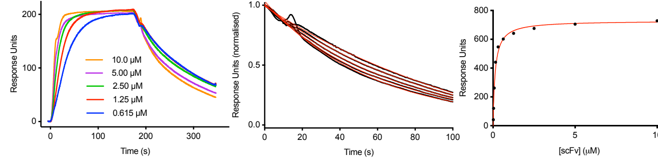


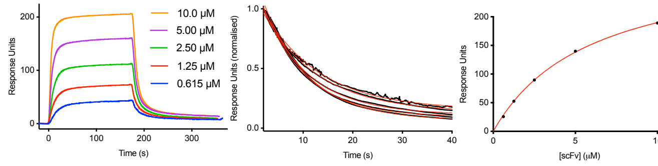
Figure 19: **The CAR antigen sensitivity defect is not a result of exhaustion induced by tonic signalling**. **(A)** Surface expression of the indicated co-stimulatory receptors on T cells transduced with the TCR or the indicated CAR. Representative flow cytometry histograms (top) and fold-change across independent experiments (N=4, bottom). **(B)** As (A) for the indicated co-inhibitory receptors. **(C)** CAR transduced or untransduced T cells are electroporated with the 868 TCR before being stimulated by purified SL9 pMHC with or without CD58. Representative dose-response (bottom left) and EC_{50} values across independent experiments (N=3, bottom right). A one-sample t-test is used to obtain a p-value for the null hypothesis that the indicated surface receptor expression differs from 1.0 on log-transformed values (panel A and panel B, right) and a two-sample t-test is used to compare log-transformed EC_{50} values (panel C, bottom right). Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

A

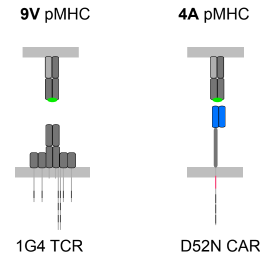
Recombinant D52N scFv injected over 9V pMHC



Recombinant D52N scFv injected over 4A pMHC

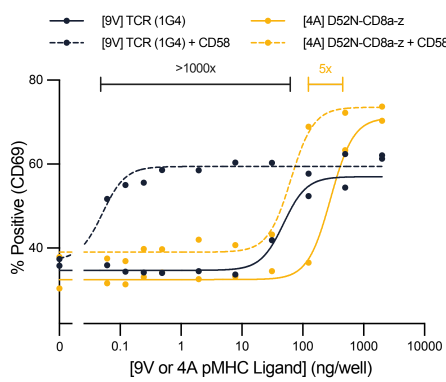


Matched Affinity (K_D ~ 7 μM)

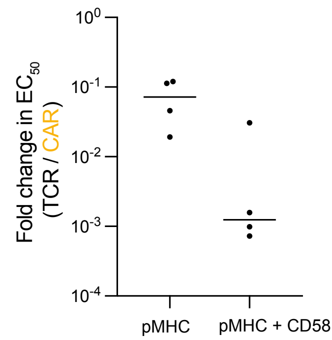


	D52N scFv				1G4 TCR
pMHC	k_{on} ($\mu M^{-1}s^{-1}$)	k_{off} (s^{-1})	K_D (μM)	n	K_D (μM)
9V (SLLMWITQV)	0.087	0.013±0.001	0.15±0.03	3	**7.2±0.5**
4A (SLLAWITQV)	0.021	0.15±0.07	**7.0±1.4**	2	299±49

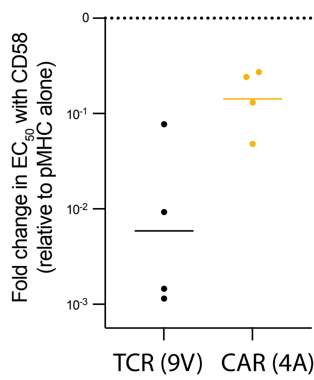
B



C



D



E

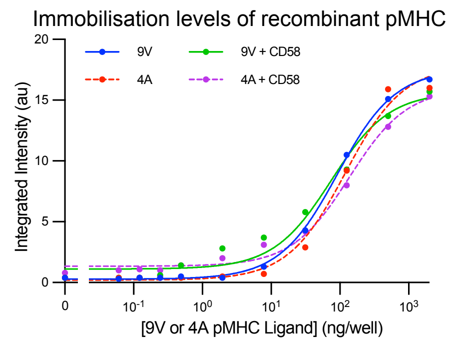


Figure 20: **Matching the antigen affinity of the TCR and CAR increases the antigen sensitivity defect of CARs**. **(A)** Binding between purified D52N scFv and the 9V (top) or 4A (bottom) pMHCs measured by surface plasmon resonance (SPR) showing the full sensogram (left), dissociation phase (middle) and steady state binding response (right). The kinetic k_{off} and equilibrium K_D are obtained by fitting the dissociation phase and steady-state binding response, respectively (red line is model fit). The kinetic k_{on} is derived from K_D and k_{off}. The K_D values for the TCR are obtained from previous work [18]. **(B)** Representative dose-response for the TCR recognising the 9V pMHC and for the CAR recognising the 4A pMHC with (dashed line) or without (solid line) purified CD58 (250 ng/well). **(C-D)** Fold-change in EC_{50} between (C) the TCR recognising 9V and the CAR recognising 4A and (D) induced by the addition of CD58 for the indicated pMHC and antigen receptor across independent experiments (N=4). **(E)** Levels of presented pMHC for each condition as detected by the conformationally sensitive W6/32 antibody. The SPR data and kinetic rates shown in panel A were produced by Benjamin Salzer.

Reducing the affinity of the CAR using the 4A peptide, so that it is matched with the TCR to 9V, we see that abrogating CD8 binding has a small but significant effect on CAR-T sensitivity. This suggests that CD8 may be altering CAR sensitivity (Figure 21, E). As with 9V, at this affinity we find no difference in ability to capitalise on co-stimulation with and without CD8 binding (Figure 21, E).

Together, these results show that CD8 has no impact on the antigen sensitivity of CARs when recognising the 9V pMHC and a modest impact on TCR antigen sensitivity when recognising the 9V pMHC with CD58, and therefore, we can conclude that CD8 binding cannot account for the large different in antigen sensitivity we have observed between the TCR and CARs.

Dimerisation of the CAR has no impact on ability to exploit co-stimulation

We next explored whether the different stoichiometry of CARs compared to the TCR may contribute to their differences in antigen sensitivity. Unlike the monovalent and monomeric TCR [34], CARs rely on hinges from proteins that are natively dimeric (CD8α, CD28, and IgG1) and therefore, they are dimeric and divalent receptors. To address whether the stoichiometry impacts antigen sensitivity, we introduced mutations to the two cysteins in the CD8a hinge that were previously reported to mediate dimer formation (D52N-CD8a(Ser)-z, see [35] for details).

We then assessed the sensitivity of this monovalent CAR compared to the dimeric CAR and the monovalent TCR. All the constructs had a similar transduction efficiency and expression level (Figure 22, A-C). Abrogating dimerisation did not alter the impact of CD58 on the CAR. Both the original and mutant CARs failed to capitalise on CD58 relative to the TCR (Figure 22, D-E). Taken together, this shows that both standard dimeric and divalent CARs and monomeric and monovalent CARs are inefficient at exploiting CD58 compared to the TCR.

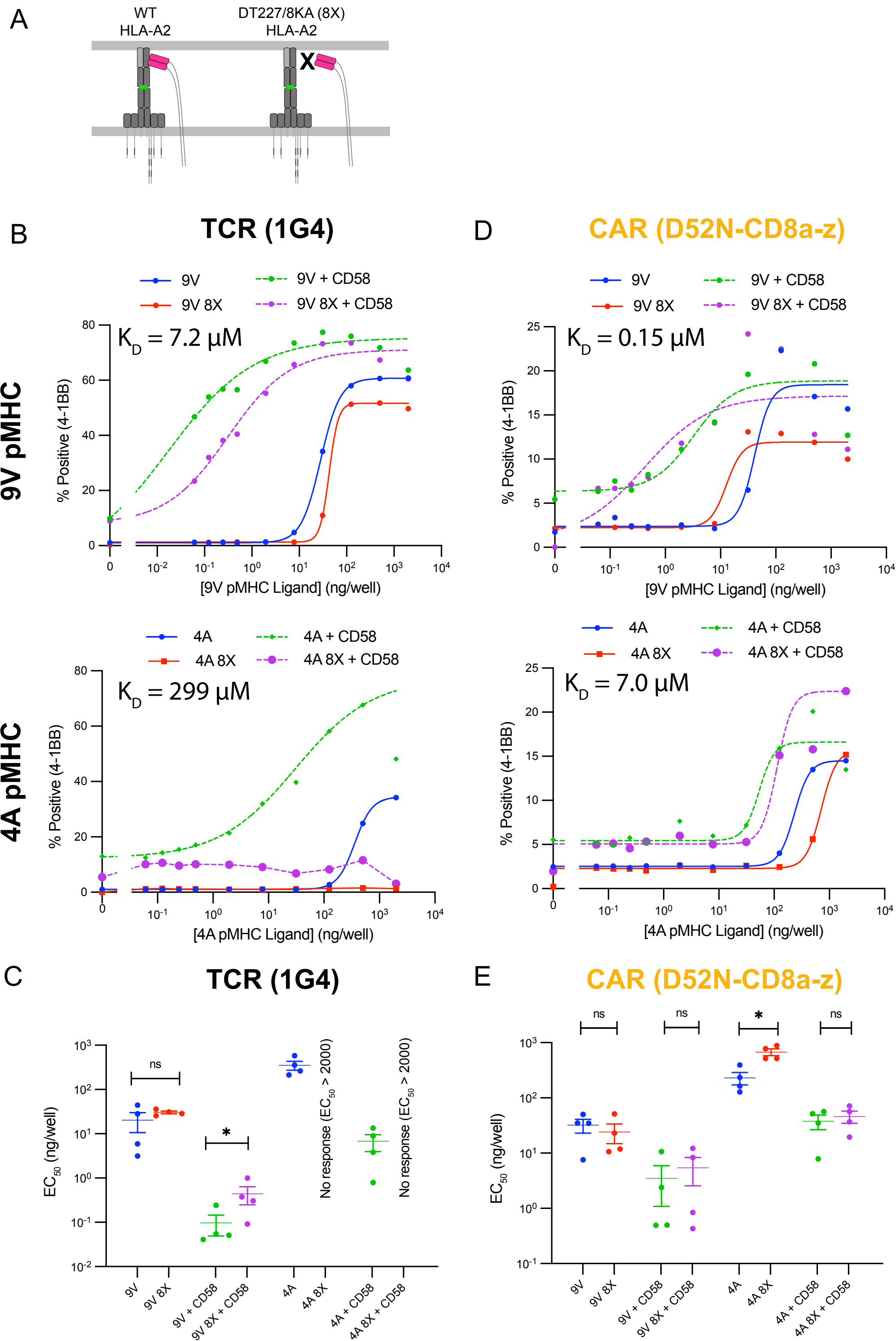

A
WT
HLA-A2
DT227/8KA (8X)
HLA-A2
B
TCR (1G4)
9V pMHC
9V
9V 8X
9V + CD58
9V 8X + CD58
K$_D$ = 7.2 µM
% Positive (4-1BB)
[9V pMHC Ligand] (ng/well)
4A pMHC
4A
4A 8X
4A + CD58
4A 8X + CD58
K$_D$ = 299 µM
[4A pMHC Ligand] (ng/well)
D
CAR (D52N-CD8a-z)
K$_D$ = 0.15 µM
K$_D$ = 7.0 µM
C
TCR (1G4)
EC$_{50}$ (ng/well)
ns
*
No response (EC$_{50}$ > 2000)
E
CAR (D52N-CD8a-z)
ns
ns
*
ns

Figure 21: **The CAR antigen sensitivity defect is independent of the CD8 co-receptor.** **(A)** The DT227/8KA mutations in the HLA-A2 heavy-chain prevent binding by the CD8 co-receptor (referred to as 8X). **(B-E)** Representative dose-response curves (B,D) and summary measures across independent experiments (N=4) (C,E) for the 9V and 4A pMHC variants with or without CD58 for the TCR (B,C) and the CAR (D,E). A t-test is used to compare the EC_{50} values on log-transformed data. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

Discussion

Using systematic antigen titrations, we have confirmed previous reports [12, 13] that CARs require 100-1000-fold more antigen compared to the TCR in order to activate T cells. However, using purified antigen in isolation we found that CAR and TCR antigen sensitivity are similar (<4-fold across all tested CARs, see Figure 11, E). This allowed us to test whether the lower antigen sensitivity of CARs can be attributed, at least in part, to co-signalling receptor/ligand interactions. Indeed, we found that the CD2/CD58 and LFA-1/ICAM-1 mediated adhesion interactions dramatically increased the the sensitivity of the TCR (>125-fold; Figure 11, D) but not that of CARs (<5-fold across all tested CARs; Figure 11, D). We controlled for a number of other explanations for the CAR sensitivity defect, including, receptor expression (Figure 18), exhaustion (Figure 19), supraphysiological affinity (Figure 20), and co-receptor binding (Figure 21). We found that none of these provided an adequate explanation.

There have been two previous studies in which CAR and TCR sensitivity were directly compared. In *Harris et al.* [13] the TCR is compared with a CAR whose antigen binding region is formed from a single chain of the TCR Vβ and Vα. Consistent with our results they show a 10-100 fold defect in sensitivity when measuring cytokine production, a similar magnitude to what we observe when measuring CD69 expression (46-2800x, Figure 11D) . They also saw no difference in sensitivity between a 1st and 2nd generation CAR for the same antigen, again consistent with our result (see Figure 11, C & D). The TCR and CAR used by *Harris et al.* is engineered for high affinity, which is in the nanomolar range, as is the affinity of our CAR (Figure 20, A) further supporting our conclusion that high affinity is not the cause of the CAR sensitivity defect. *Harris et al.* also show a sensitivity defect in CD4 T cells similar to our finding with the HLA*02:01 mutant having abrogated CD8 binding (Figure 21).

As they performed titrations of peptide on APCs we note that their results include the effect of co-stimulation. However *Harris et al.* [13] propose that the CAR sensitivity defect can be explained by either the presence of fewer ITAMs in the CAR or by inefficient downstream signalling (e.g. reduced LAT phosphorylation by the CAR). Our finding that co-signalling molecules disproportionately impact TCR but not CAR sensitivity is consistent with these proposals. The behaviour of co-signalling molecules simply provides a biological mechanism for each of these. For example, co-signalling molecules which acted to 'amplify' signalling, would show an increased impact on the TCR with its inherently greater signalling capacity[1]. Likewise co-signalling molecules such as CD2 have been shown to effectively phosphorylate downstream signalling molecules such as LAT, and to do so in a way that is enhanced by simultaneous CD3 stimulation [36]. Consequently, inefficient downstream signalling can be explained by a failure to exploit co-signalling molecules.

[1] We do show however, in chapter 2, that signalling capacity cannot completely explain the failure of CARs to capitalise on co-stimulation.

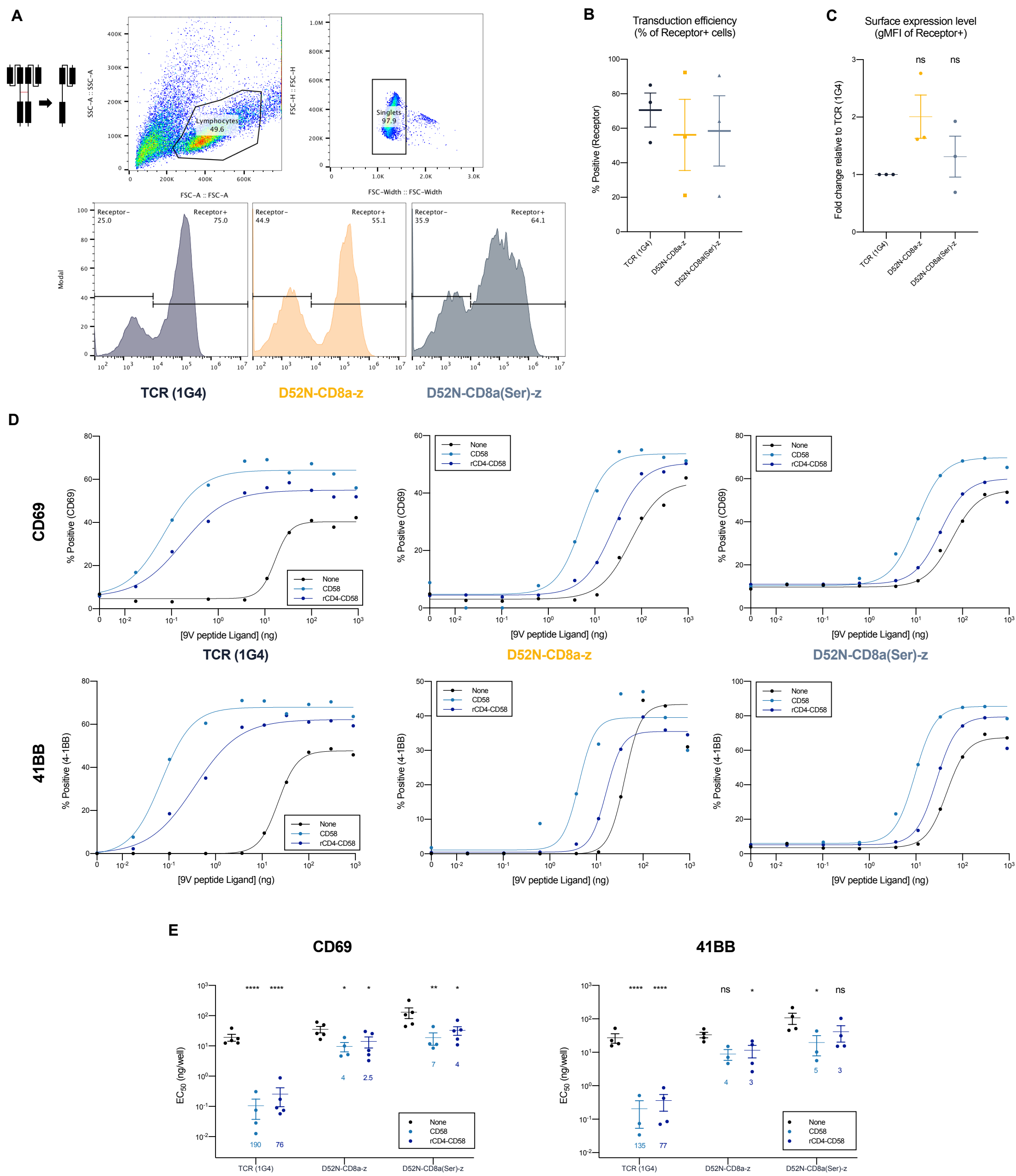


Figure 22: **Dimerisation state of CAR has no impact on ability to exploit co-stimulation. (A-C)** Surface expression of CD8α hinge CARs with a C→S mutation (D52N-CD8α(Ser)-z). **(A)** Shown by representative histograms, **(B)** transduction efficiency and **(C)** surface expression level for $n = 3$ independent transductions of primary $CD8^+$ human T cells. **(D)** Representative curves showing dose-response of TCR (left), D52N-CD8α-z CAR (middle) and D52N-CD8α(Ser)-z mutant (right). **(E)** Extracted EC_{50} values for each construct by % CD69 positive (left) and % 41BB positive (right) cells. Comparison to TCR by paired t-test. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001

The second study to compare a TCR and CAR, *Gudipati et al.* [12], use a CAR and TCR which target different antigens. The folded antigen ROR1, and a pMHC respectively. Rather then using antigen presenting cells, both antigens are titrated on lipid bilayers in the presence of the co-signalling ligands CD80 and ICAM-1 (engaging CD28 and LFA-1 respectively). Consistent with our findings and with those of *Harris et al.*, *Gudipati et al.* show that the CAR required 1000x more antigen than the TCR to become activated, and that there was no impact on sensitivity from adding or changing the co-signalling domain of the CAR. *Gudipati et al.* also find that TCR sensitivity was reduced by abrogating CD8 binding but that this did not explain the entire sensitivity defect. That they were able to see a difference in sensitivity that was not complete abrogation of the T cell response (which is what we observe, see Figure 21) when abrogating CD8 binding is likely due to a difference in the affinity of their receptor and range of antigen used.

Gudipati et al. find poor ITAM and ZAP-70 phosphorylation by CARs compared to the TCR, consistent with the modelling performed by *Harris et al.*. This remains consistent with a failure to capitalise on co-signalling molecules due to the important role of ZAP-70 in mediating the eventual activation of LFA-1 via SLP76 [37] and the presence of ICAM-1 in this study.

Various aspects of CAR structure have been examined previously for their effect on therapeutic outcomes. Most notably the finding that the presence of a 4-1BB signalling domain increases *in vivo* persistence compared to the CD28 signalling domain [24]. We see a very limited effect from co-stimulation by either the CD28 or 4-1BB ligands in our acute short-term cytokine production assays. However, this is consistent with improved outcomes *in vivo* where long term effects such as improved proliferation and cytokine production would have a larger effect. Other *in vitro* studies also find a small effect from the 41BB and CD28 co-signalling domains embedded in the CAR [16, 38] consistent with our work. It has also been reported that the effect of co-signalling may vary depending on whether the co-signalling receptor is the native protein or the domain embedded within a CAR [39].

It has previously been shown that downregulation of CD58 is correlated with resistance to CAR-T therapy, and reduces progression free survival [39, 40], suggesting that CAR-T cells do capitalise on co-signalling via CD2/CD58. Our results are consistent with this as we do find that CARs are able to capitalise on CD58 — there is a statistically significant increase in CAR antigen sensitivity (Figures 11D & 15B). However this effect size is very small, especially when compared to the TCR, and so this capitalisation is inefficient. Together these results raise the prospect that antigen escape may still arise due to loss of either CD58 and ICAM-1 in addition to low levels of antigen.

Our findings show that CARs with differing hinge regions have different antigen sensitivity. We found that a CAR with a larger predicted hinge size, the IgG1 derived hinge, exhibited worse antigen sensitivity than CD28 and CD8α hinge CARs (Figure 11, C). Some studies have found that switching the CD8α hinge for CD28 improves sensitivity [16], whilst in other studies the inverse improves sensitivity [41]. However, the CARs in these studies target different antigens and so this conflict is consistent with reports that the optimal CAR hinge region depends on the antigen binding epitope [17].

We observed that CARs exhibit a sensitivity defect compared to the TCR when measuring both surface activation markers as well as cytokine production. Though the effect of co-signalling molecules on sensitivity was diminished when measuring cytokine production. This may be due to a difference in the sensitivity of the two assays or due to a difference in the dynamics of cytokine production as compared to surface marker expression. This could be addressed by measuring single cell cytokine expression by flow cytometry or allowing more cytokine to

accumulate over time before performing the ELISA.

We show that CAR T cells fail to capitalise on co-stimulation in both a solid-phase as well as a cellular system. However, the magnitude of the sensitivity improvement conferred by co-signalling ligands was much larger in the solid phase system than in the cellular system. This could suggest that in the cellular system there are other co-signalling ligands which are able to compensate for the loss of CD58 and ICAM-1; underlining the importance of the reductionist solid-phase system to identify these interactions. Differences in the mobility of the co-signalling ligands when presented in the solid phase system may also contribute to the magnitude of their effect in the cellular vs. solid-phase stimulation systems. It will be important to repeat these experiments using a reductionist system where the purified ligands are mobile.

The reduced sensitivity observed in CAR-T cells could be due to a defect in either antigen engagement, signalling or both. Moreover, we have not ruled out that other mechanisms, beyond that of a failure to capitalise on CD2 and LFA-1 could also contribute to the sensitivity defect. We found that an approximately 50-fold difference in CAR affinity (K_D) could alter CAR sensitivity relative to the TCR by approximately 10-fold (c.f. Figure 20, C & 11, E). Other mechanisms such as a deficiency in the number of ITAMs may also play a part in reducing CAR sensitivity. Though we have tested 3 and 6 ITAM CARs (by altering how they dimerise), and others have shown that increasing ITAM count does not improve CAR sensitivity [12], we cannot rule out that a larger number of ITAMs might restore CAR sensitivity. Our data does suggest however, that a failure to capitalise on co-signalling molecules is the predominant mechanism causing the CAR sensitivity defect. Though we recognise that the interplay between different mechanisms that modulate T cell sensitivity would benefit from being explored.

We have established that CARs are inefficient at exploiting the co-signalling receptors LFA-1 and CD2, compared to the TCR. However, the mechanism underlying this inefficiently is unclear. CARs differ from the TCR both in terms of their signalling capacity and in their overall structure, and this may provide an explanation.

In terms of signalling, CARs contain a total of 6 ITAMs in two ζ-chains whereas the TCR contains 10 ITAMs across two ζ-chains, two CD3ε chains, one CD3γ chain, and one CD3δ chain. Previous reports have suggested that the nature of the signalling chain may be important; identifying important roles for components of the ε-chain [42, 43]. In terms of structure, differences in the size and signalling machinery between the CAR and TCR could fail to allow for signalling between the CAR and the co-signalling receptors, cause the CARs to fail to properly engage antigen or to be segregated away from other important co-signalling molecules in the close contact region. Both these aspects are explored in chapters 2 and 3.

References

[1] Jun Huang et al. "A single peptide-major histocompatibility complex ligand triggers digital cytokine secretion in CD4+ T cells". In: *Immunity* 39.5 (2013), pp. 846–857.

[2] Hongbing Yang et al. "Elimination of latently HIV-infected cells from antiretroviral therapy-suppressed subjects by engineered immune-mobilizing T-cell receptors". In: *Molecular Therapy* 24.11 (2016), pp. 1913–1925.

[3] Niels AW Lemmermann et al. "Immune evasion proteins of murine cytomegalovirus preferentially affect cell surface display of recently generated peptide presentation complexes". In: *Journal of virology* 84.3 (2010), pp. 1221–1236.

[4] Jesús A Siller-Farfán and Omer Dushek. "Molecular mechanisms of T cell sensitivity to antigen". In: *Immunological reviews* 285.1 (2018), pp. 194–205.

[5] John Nguyen et al. “Quantitative contributions of TNF receptor superfamily members to CD8+ T-cell responses”. In: *Molecular systems biology* 17.11 (2021), e10560.

[6] Malcolm JW Sim and Peter D Sun. “T Cell Recognition of Tumor Neoantigens and Insights Into T Cell Immunotherapy”. In: *Frontiers in Immunology* 13 (2022).

[7] Marco A Purbhoo et al. “Quantifying and imaging NY-ESO-1/LAGE-1-derived epitopes on tumor cells using high affinity T cell receptors”. In: *The Journal of Immunology* 176.12 (2006), pp. 7308–7316.

[8] Milos Aleksic et al. “Different affinity windows for virus and cancer-specific T-cell receptors: implications for therapeutic strategies”. In: *European journal of immunology* 42.12 (2012), pp. 3174–3179.

[9] Renier J Brentjens et al. “CD19-targeted T cells rapidly induce molecular remissions in adults with chemotherapy-refractory acute lymphoblastic leukemia”. In: *Science translational medicine* 5.177 (2013), 177ra38–177ra38.

[10] Nikhil C Munshi et al. “Idecabtagene vicleucel in relapsed and refractory multiple myeloma”. In: *New England Journal of Medicine* 384.8 (2021), pp. 705–716.

[11] Robbie G Majzner and Crystal L Mackall. “Tumor antigen escape from CAR T-cell therapy”. In: *Cancer discovery* 8.10 (2018), pp. 1219–1226.

[12] Venugopal Gudipati et al. “Inefficient CAR-proximal signaling blunts antigen sensitivity”. In: *Nature Immunology* 21.8 (2020), pp. 848–856.

[13] Daniel T Harris et al. “Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains”. In: *The Journal of Immunology* 200.3 (2018), pp. 1088–1100.

[14] Helene M Finney, Arne N Akbar, and Alastair DG Lawson. “Activation of resting human primary T cells with chimeric receptors: costimulation from CD28, inducible costimulator, CD134, and CD137 in series with signals from the TCRζ chain”. In: *The Journal of Immunology* 172.1 (2004), pp. 104–113.

[15] CMIHARA Imai et al. “Chimeric receptors with 4-1BB signaling capacity provoke potent cytotoxicity against acute lymphoblastic leukemia”. In: *Leukemia* 18.4 (2004), pp. 676–684.

[16] Robbie G Majzner et al. “Tuning the Antigen Density Requirement for CAR T-cell ActivityTuning the Antigen Density Requirement for CAR T Cells”. In: *Cancer discovery* 10.5 (2020), pp. 702–723.

[17] Michael Hudecek et al. “The nonsignaling extracellular spacer domain of chimeric antigen receptors is decisive for in vivo antitumor activity”. In: *Cancer immunology research* 3.2 (2015), pp. 125–135.

[18] Johannes Pettmann et al. “The discriminatory power of the T cell receptor”. In: *Elife* 10 (2021), e67092.

[19] Martin F Bachmann, Marijke Barner, and Manfred Kopf. “CD2 sets quantitative thresholds in T cell activation”. In: *The Journal of experimental medicine* 190.10 (1999), pp. 1383–1392.

[20] Martin F Bachmann et al. “Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation”. In: *Immunity* 7.4 (1997), pp. 549–557.

[21] EJ Cheadle et al. “Ligation of the CD2 co-stimulatory receptor enhances IL-2 production from first-generation chimeric antigen receptor T cells”. In: *Gene therapy* 19.11 (2012), pp. 1114–1120.

[22] Shannon Bownds et al. “Induction of tumor-reactive cytotoxic T-lymphocytes using a peptide from NY-ESO-1 modified at the carboxy-terminus to enhance HLA-A2. 1 binding affinity and stability in solution”. In: *Journal of Immunotherapy* 24.1 (2001), pp. 1–9.

[23] Lieping Chen and Dallas B Flies. “Molecular mechanisms of T cell co-stimulation and co-inhibition”. In: *Nature reviews immunology* 13.4 (2013), pp. 227–242.

[24] Adrienne H Long et al. “4-1BB costimulation ameliorates T cell exhaustion induced by tonic signaling of chimeric antigen receptors”. In: *Nature medicine* 21.6 (2015), pp. 581–590.

[25] John S Yi, Maureen A Cox, and Allan J Zajac. “T-cell exhaustion: characteristics, causes and conversion”. In: *Immunology* 129.4 (2010), pp. 474–481.

[26] Angel Varela-Rohena et al. “Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor”. In: *Nature medicine* 14.12 (2008), pp. 1390–1395.

[27] Sharyn Thomas et al. “Human T cells expressing affinity-matured TCR display accelerated responses but fail to recognize low density of MHC-peptide antigen”. In: *Blood, The Journal of the American Society of Hematology* 118.2 (2011), pp. 319–329.

[28] Linda Wooldridge et al. “Interaction between the CD8 coreceptor and major histocompatibility complex class I stabilizes T cell receptor-antigen complexes at the cell surface”. In: *Journal of Biological Chemistry* 280.30 (2005), pp. 27491–27501.

[29] Phillip D Holler and David M Kranz. “Quantitative analysis of the contribution of TCR/pepMHC affinity and CD8 to T cell activation”. In: *Immunity* 18.2 (2003), pp. 255–264.

[30] Jennifer D Stone and David M Kranz. “Role of T cell receptor affinity in the efficacy and specificity of adoptive T cell therapies”. In: *Frontiers in immunology* 4 (2013), p. 244.

[31] Ning Jiang et al. “Two-stage cooperative T cell receptor-peptide major histocompatibility complex-CD8 trimolecular interactions amplify antigen discrimination”. In: *Immunity* 34.1 (2011), pp. 13–23.

[32] Sarah L Hutchinson et al. “The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities”. In: *Journal of Biological Chemistry* 278.27 (2003), pp. 24285–24293.

[33] Bruno Laugel et al. “Different T cell receptor affinity thresholds and CD8 coreceptor dependence govern cytotoxic T lymphocyte activation and tetramer binding properties”. In: *Journal of Biological Chemistry* 282.33 (2007), pp. 23799–23810.

[34] Mario Brameshuber et al. “Monomeric TCRs drive T cell antigen recognition”. In: *Nature immunology* 19.5 (2018), pp. 487–496.

[35] Benjamin Salzer et al. “Engineering AvidCARs for combinatorial antigen recognition and reversible control of CAR function”. In: *Nature communications* 11.1 (2020), pp. 1–16.

[36] Maria Paola Martelli et al. “Signaling via LAT (linker for T-cell activation) and Syk/ZAP70 is required for ERK activation and NFAT transcriptional activation following CD2 stimulation”. In: *Blood, The Journal of the American Society of Hematology* 96.6 (2000), pp. 2181–2190.

[37] Jessica Horn et al. “Src homology 2-domain containing leukocyte-specific phosphoprotein of 76 kDa is mandatory for TCR-mediated inside-out signaling, but dispensable for CXCR4-mediated LFA-1 activation, adhesion, and migration of T cells”. In: *The Journal of Immunology* 183.9 (2009), pp. 5756–5767.

[38] Xueyin Wang et al. “Extensive functional comparisons between chimeric antigen receptors and T cell receptors highlight fundamental similarities”. In: *Molecular Immunology* 138 (2021), pp. 137–149.

[39] Robbie G Majzner et al. “CD58 aberrations limit durable responses to CD19 CAR in large B cell lymphoma patients treated with axicabtagene ciloleucel but can be overcome through novel CAR engineering”. In: *Blood* 136 (2020), pp. 53–54.

[40] Livnat Jerby-Arnon et al. “A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade”. In: *Cell* 175.4 (2018), pp. 984–997.

[41] Xiang Chen et al. “The CD8α hinge is intrinsically disordered with a dynamic exchange that includes proline cis-trans isomerization”. In: *Journal of Magnetic Resonance* (2022), p. 107234.

[42] Wei Wu et al. “Multiple signaling roles of CD3ε and its application in CAR-T cell therapy”. In: *Cell* 182.4 (2020), pp. 855–871.

[43] Frederike A Hartl et al. “Noncanonical binding of Lck to CD3ε promotes TCR signaling and CAR function”. In: *Nature immunology* 21.8 (2020), pp. 902–913.

Chapter 2

The sensitivity of novel chimeric antigen receptors

Introduction

The role of TCR structure in signalling

The TCR-CD3 complex and CARs have different architectures. Whilst the CAR is a single chain protein, the TCR-CD3 complex is composed of 4 different subunits: the αβ chains (which form the antigen binding region) that assemble with the associated ζζ, CD3εγ and CD3εδ dimers. Each of these has a number of immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic tails. 3 in each of the ζ chains and 1 in each of the CD3 chains. Therefore the TCR-CD3 complex contains a total of 10 ITAMs.

In addition to the ITAMs, the CD3 chains contain a diverse range of other protein binding motifs whose role in TCR signalling is a topic of active research. For example, CD3ε has been

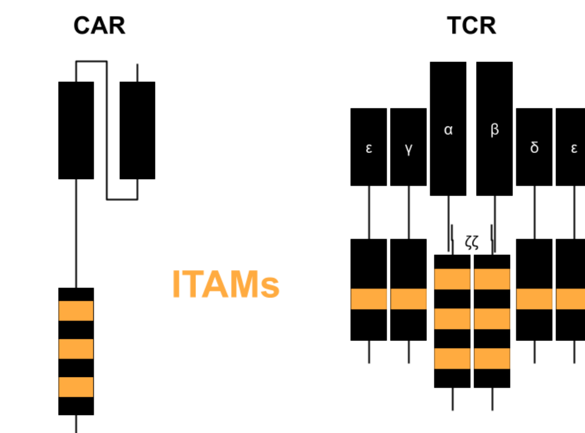


Figure 23: **CARs and the TCR differ in their number of ITAMs.** The CAR ζ chain contains 3 ITAMs whilst the TCR is composed of 4 subunits, the two ζ chains each contain 3 ITAMs whilst the remaining CD3 chains (ε, γ, δ) each contain 1 ITAM.

found to interact with proteins regulating TCR downregulation [1]. It has been suggested that the diverse CD3 signals are important for T cell functionality [2]. CARs can interact with endogenous CD3ζ [3] and can be engineered to interact with the TCR-CD3 complex [4] but it is not clear if they can capitalise on CD3 signalling in the same way as the TCR. We suggest that reduced CAR sensitivity could be a result of having quantitatively and qualitatively different signals than the TCR.

The extracellular size of TCRαβ has been suggested as being important, particularly for the exclusion of large phosphatases [5, 6, 7] from the vicinity of the receptor. There is now some evidence that, depending on the size of the CAR, CD45 is not as efficiently excluded from the close contact region [8] and this may alter CAR signalling.

This notion that the additional signalling components of the TCR-CD3 complex may be important, as with the TCR size, has lead to the development of several novel CAR architectures. These have been proposed to have increased antigen sensitivity when compared with the TCR.

Recent CARs have been proposed to have increased antigen sensitivity

There has been some debate as to whether the different CD3 ITAMs are unique or redundant. A recent study found that the CD3ε chain is able to recruit the inhibitory molecule *Csk* leading to reduced cytokine production by the T cell [9]. When CD3ε is used in addition to the ζ-chain in standard CARs the resulting CAR was found to have increased persistence, leading to a corresponding improvement in *in vivo* tumour control.

This is not the only way by which the ε chain might improve CAR performance. Another recent study has shown that CD3ε additionally contains a, binding domain for the kinase *Lck* which phosphorylates the TCR signalling machinery in the early stages of T cell signalling [10]. *Lck* also appears to phosphorylate CARs [11, 12], so it was hypothesised that including this domain within the CAR would lead to increased CAR phosphorylation and more efficient signalling. Indeed the authors found that CAR-T cells endowed with a fragment of CD3ε showed enhanced tumour cell killing.

Given the apparent importance of the CD3ε chain, it stands to reason that the remaining CD3 chains are also beneficial for T cell signalling. To engage the entirety of the TCR signalling machinery, whilst still maintaining the specificity and HLA-independence of CARs, others have grafted the CAR scFv onto each of the TCR chains (an εTRuC is shown in Figure 24). These receptors are known as TRuCs [13]. In *Baeuerle et al.* [13], the authors find that such receptors lead to lower cytokine release compared to 2nd generation CARs, but with similar cytotoxicity. They also find that an εTRuC (the scFv being fused to CD3ε) was found to have better anti-tumour activity than CARs and increased antigen sensitivity *in vitro*.

The same study found that α or β TRuCs performed worse than several of the other TRuC constructs and hypothesised this to be the result of the increased extracellular reach of such a receptor failing to exclude large phosphatases from the T cell–APC contact region. A way to avoid increasing the size of the receptor, whilst still engaging the entire T cell signalling machinery is to replace the variable regions of the TCR α and β chains with the variable heavy and light chains from the scFv of a CAR. These receptors thus recapitulate exactly the same size as the TCR.

Such a receptor has been termed a STAR or HIT [14, 15] (a STAR constructed from the D52N antibody is shown in Figure 24). In order to express properly these receptors are engineered with mouse TCR constant regions and additional cysteine mutations to promote α-β chain

pairing. These receptors have also been found to exhibit higher sensitivity than traditional CARs [14].

CARs, TRuCs and STARs are typically used to target folded antigens like CD19. This makes it difficult to accurately titrate the antigen in order to study sensitivity. Using a variety of cell lines it has been shown that in some models TRuCs outperform the traditional CAR format in terms of tumour control [13]. In particular the εTRuC was shown to result in better tumour control than a '2nd generation' CAR containing the 4-1BB signalling domain. *Hartl et al.* [10] also compare a CD3ε containing CAR against a CAR containing the same 41BB domain and find that it showed better tumour control at lower CAR doses. However the antigen sensitivity of either of these synthetic receptors was not assessed. *Liu et al.* [14] did assess the sensitivity of a STAR using a cell line expressing a range of antigen doses and found that it was more sensitive than a similar 41BB-CAR.

About this study

A quantitative comparison between these receptors, traditional CARs, and the TCR has not been performed. We wanted to determine how each of these novel receptors compared with the TCR and CARs and to determine, if they showed improved sensitivity, by what mechanism they are able to achieve sensitivity beyond that of the traditional CAR construct.

Here we generate versions of these novel receptors which use the D52N scFv (Figure 25, A), and therefore all target the NY-ESO-1 peptide on HLA-A*02:01. This allows them to be compared directly against one another. We then explore a possible mechanism through which these novel receptors achieve improved sensitivity.

Results

Some 'TCR-like' chimeric receptors display improved sensitivity and can recapitulate TCR sensitivity

We began by comparing the surface expression levels of 'TCR-like' synthetic receptors (Figure 24). Although we found variability in the transduction efficiency between independent experiments (using T cells from different donors), the surface expression of each antigen receptor was similar to that of the TCR as detected using pMHC tetramers (Figure 25, B-C). We next confirmed that the STAR but not CARs assembled into the full TCR-CD3 complex by transducing each receptor into TCRαβ$^-$ Jurkat cells and measuring CD3 expression (Figure 26).

As in chapter 1, we assessed the relative sensitivity of each of these receptors compared to the TCR by presenting them with T2 target cells that had been pulsed with varying concentrations of peptide antigen. We found that exchanging the ζ chain for CD3ε in the tail of the CAR (Figure 24, bottom right) had either no effect (when measuring cytotoxicity) or a small effect in reducing sensitivity (when measuring CD69 expression) (Figure 28, C-E) when compared to a reference CAR (the D52N-CD8α-z).

We found the εTRuC to have an intermediate sensitivity between that of the TCR and our previously studied D52N-CD8α-z CAR — 130 fold less sensitive than the TCR by cytotoxicity (Figure 28, C) and 66 fold less sensitive by CD69 expression (Figure 28, E). Lastly, we saw that a STAR had a TCR like sensitivity to antigen; observing no significant difference between these two receptors (Figure 28, C & E).

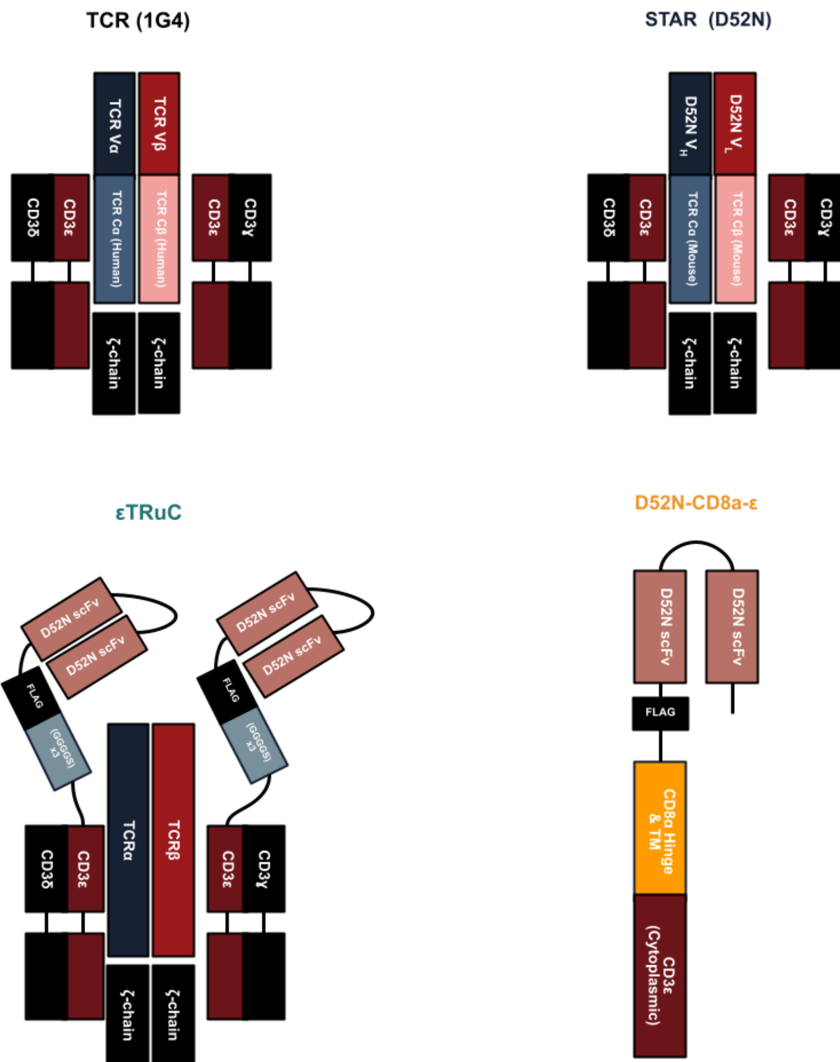
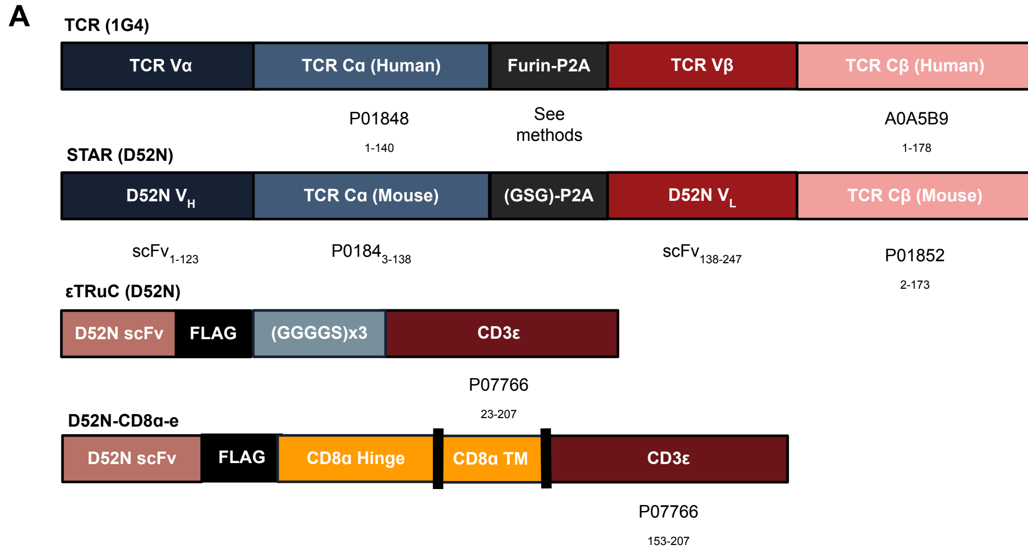


Figure 24: **A variety of TCR-like receptors**. Schematic diagram showing a TCR (top left), a STAR (top right), ε-TRuC (bottom left), and traditional CAR with the ζ chain swapped for the CD3ε chain (bottom right). All the synthetic constructs show a region of the D52N (anti-NY-ESO-1) antibody as used in this study.


A
TCR (1G4)
TCR Vα
TCR Cα (Human)
Furin-P2A
TCR Vβ
TCR Cβ (Human)
P01848
1-140
See methods
A0A5B9
1-178
STAR (D52N)
D52N V_H
TCR Cα (Mouse)
(GSG)-P2A
D52N V_L
TCR Cβ (Mouse)
scFv 1-123
P0184 3-138
scFv 138-247
P01852
2-173
εTRuC (D52N)
D52N scFv
FLAG
(GGGGS)x3
CD3ε
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D52N scFv
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CD3ε
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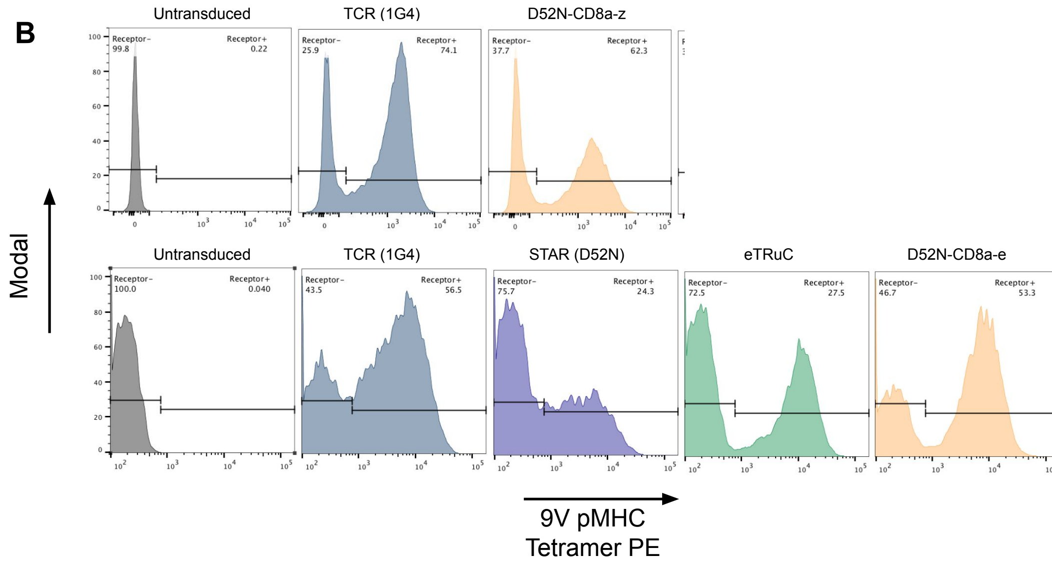
B
Untransduced
TCR (1G4)
D52N-CD8a-z
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TCR (1G4)
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eTRuC
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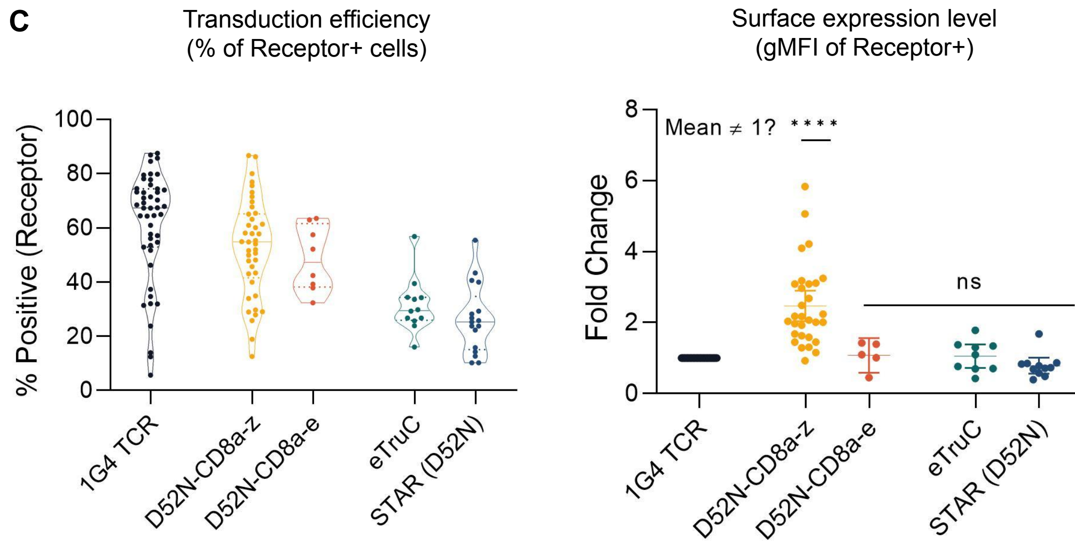
C
Transduction efficiency
(% of Receptor+ cells)
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1G4 TCR
D52N-CD8a-z
D52N-CD8a-e
eTruC
STAR (D52N)
Surface expression level
(gMFI of Receptor+)
Fold Change
Mean ≠ 1?
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ns

Figure 25: **TCR-like chimeric receptors express at similar levels to the TCR. (A)** The construction of TCR-like chimeric receptors by engrafting the D52N variable regions into the TCR or fusion of the scFv with portions of CD3ε **(B)** Histograms showing typical expression profiles of the indicated chimeric receptors, each row represents a single experiment. **(C)** Transduction efficiency (left) and surface expression levels (right) of various TCR-like receptors. The mean surface expression level is compared to a hypothetical mean of 1 (equal to the TCR) by one-sample t-test. **** = p-value≤0.0001.

The ability of TCR-like chimeric antigen receptors to recapitulate TCR sensitivity depends on their ability to exploit the CD2-CD58 interaction

Since we had previously shown (chapter 1) that the sensitivity defect in CARs is an apparent failure to exploit co-signalling receptors, we wanted to examine if the εTRuC and STAR receptors were able to more efficiency exploit the CD2 and LFA-1 accessory receptors.

We investigated the ability of each novel receptor to capitalise on co-signalling receptors. First by assessing their sensitivity in the absence of co-signalling. This was done using the solid-phase system described in chapter 1. Briefly, cells are presented with titrations of purified biotinylated pMHC antigen immobilised on streptavidin coated plates. We observed that the antigen sensitivity of all the receptors became similar (Figure 28, F, G & I).

In contrast, when we presented antigen in the same manner along with a fixed concentration of purified biotinylated CD58, we were able to recapitulate the same sensitivity hierarchy as we observed in the cellular assay (Figure 28, G). That is, there was no significant difference in sensitivity between the TCR and STAR, whilst the εTRuC construct had an intermediate sensitivity between that of the TCR and the D52N-CD8α-z CAR, with the ε-chain CAR having a worse sensitivity than the ζ chain CAR. We observed the same hierarchy when measuring sensitivity by IFNγ expression (Figure 28, I).

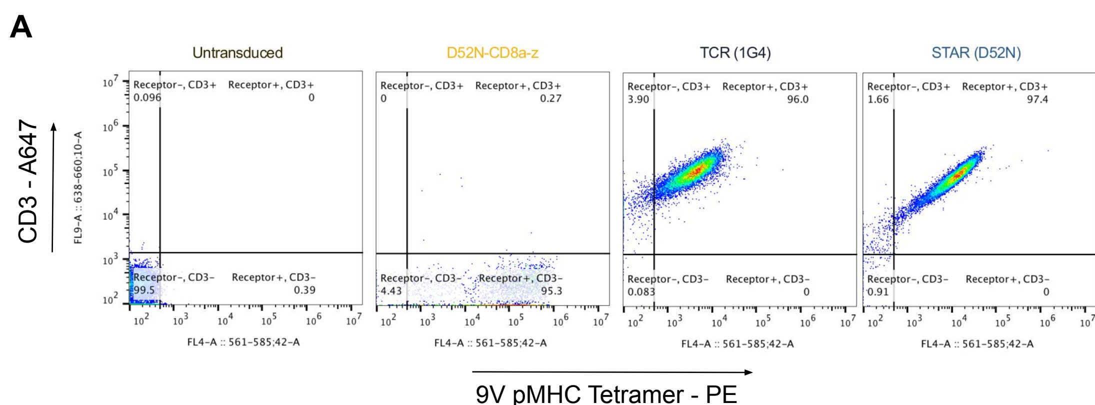


Figure 26: **The D52N-CD8α-z CAR does not assemble with CD3 (A)** Jurkat T cells which are αβTCR⁻ are transduced with the indicated chimeric receptors and then stained for the presence of both the receptor and CD3.

These results suggest that the performance of each receptor in the cellular system is dependent on its ability to exploit the CD2-CD58 interaction, and indeed we found a significant correlation ($r^2 = 0.96$) between sensitivity in the cellular system and the improvement in sensitivity conferred by CD58 on plates (Figure 28, J). The ability of chimeric antigen receptors to recapitulate the sensitivity of the TCR is therefore dependent on their ability to exploit the CD2-CD58 interaction.

The ability of TCR-like chimeric antigen receptors to recapitulate TCR sensitivity depends on their ability to exploit the LFA-1/ICAM-1 interaction

The other co-signalling interaction which we had previously found disproportionately increased the sensitivity of the TCR compared to the CAR was the LFA-1/ICAM-1 interaction. We wanted to examine if the TCR-like chimeric antigen receptors were also able to capitalise on this interaction, and so we additionally measured their sensitivity in the presence of a fixed concentration of purified biotinylated ICAM-1.

Consistent with our previous findings, we found that ICAM-1 increased the antigen sensitivity of the TCR by a large amount compared to the CAR (Figure 27, A). Moreover, we saw that the presence of ICAM-1 had a smaller improvement on sensitivity compared to CD58 (c.f. Figure 27, B and 28, G). We also observed that the presence of ICAM-1 produces the same sensitivity hierarchy as does CD58. Again, ability to capitalise on the LFA-1/ICAM-1 interaction is strongly ($r^2 = 0.96$) correlated with sensitivity in the cellular assay (Figure 27, C).

The ability of TCR-like chimeric antigen receptors to exploit the CD58 and ICAM-1 interactions can be observed in a cellular system

We then validated this finding when the antigen was presented on APCs. To do so we used U87 target cells from which either or both of CD58 and ICAM-1 had been knocked out using CRISPR. We again measured the sensitivity of each receptor to these cells when pulsed with varying concentrations of peptide antigen.

In contrast to the D52N-CD8α-z CAR, we saw no difference in the magnitude of sensitivity lost between the TCR and each of the STAR and εTRuC when CD58 was knocked-out of the target cells (Figure 29, B-C). We did however observe a significant difference in loss of sensitivity between the D52N-CD8α-ε CAR and TCR in the absence of CD58 (Figure 29, D). We saw no significant difference in loss of sensitivity between TCR and STAR-T cells when the ICAM-1 interaction is abrogated (Figure 29, B). But both εTRuC and D52N-CD8α-ε T cells showed a smaller loss of sensitivity compared to the TCR when ICAM-1 is abrogated (Figure 29, C & D).

Overall, the TCR and STAR were impacted similarly by the abrogation of the CD58 and/or ICAM-1 interactions. The εTRuC exhibited worse sensitivity than the TCR (c.f. Figure 29, A for the TCR and εTRuC) and did not capitalise on ICAM-1 to the same extent (Figure 29, C). The ε-CAR displayed poor sensitivity and significantly less ability to capitalise on CD58 and ICAM-1 co-signalling (Figure 29, D) compared to the TCR. As previously, we note that when both ICAM-1 and CD58 are abrogated the ζ and ε chain CARs still show a 19 and 196-fold sensitivity defect respectively relative to the TCR (Figure 29, E). This would suggest that that other co-signalling receptors may be acting to further improve the sensitivity of the TCR in this system using APCs.

Taken together these results confirm our finding that in order to achieve TCR-like antigen sensitivity, synthetic receptors need to be able to exploit both the CD2/CD58 and LFA-1/ICAM-1 interactions.

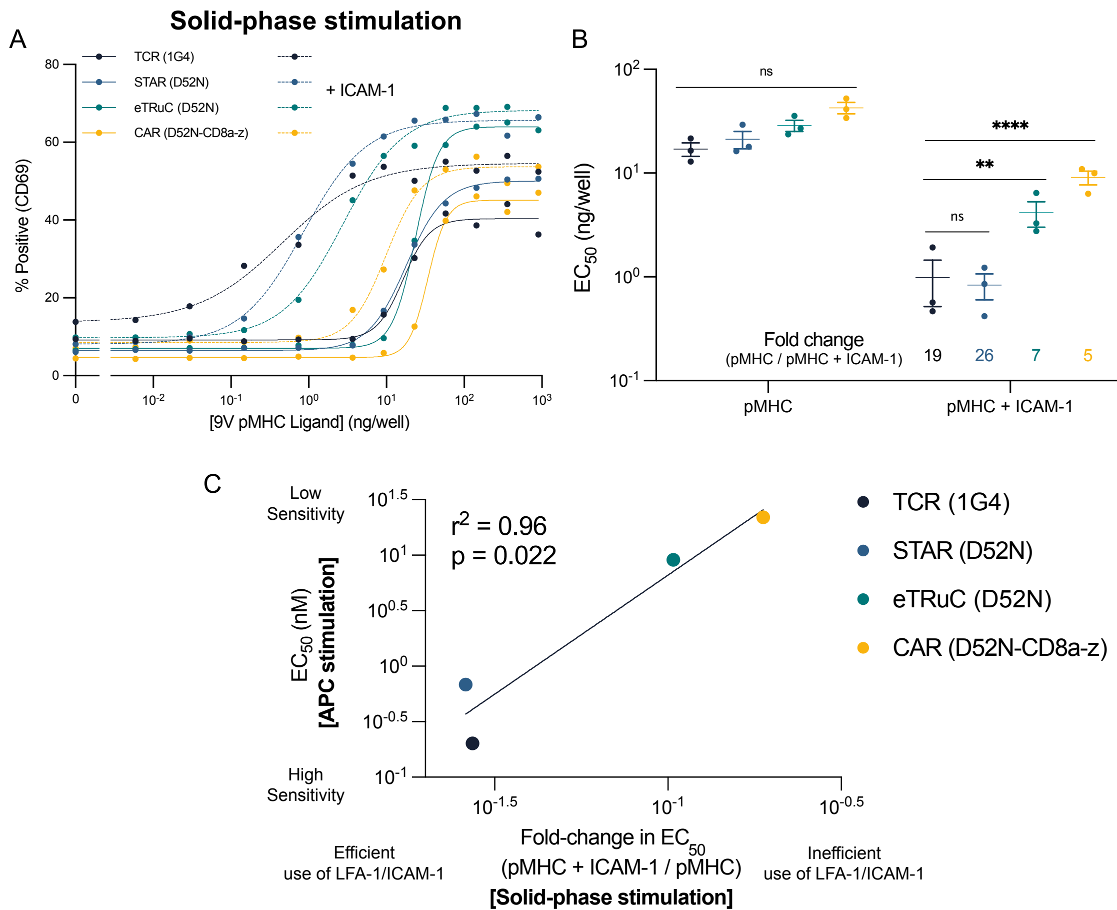


Figure 27: **The ability of TCR-like chimeric antigen receptors to recapitulate the sensitivity of the TCR depends on the efficiency with which they are able to exploit the LFA-1 adhesion interaction.** **(A)** T cells expressing the indicated antigen receptor were stimulated by a titration of purified pMHC alone (solid lines) or in combination with a fixed concentration of purified ICAM-1 (dashed lines). **(B)** Fitted EC_{50} values from two independent experiments. **(C)** The averaged EC_{50} values for CD69 upregulation from the APC stimulation assay (from Figure 28C) are plotted over the averaged fold-change in EC_{50} for CD69 induced by the addition of ICAM-1 from the solid-phase stimulation assay (from panel B). The EC_{50} values are compared using a one-way ANOVA on log-transformed values (B). Abbreviations: *** = p-value≤0.001, **** = p-value≤0.0001.

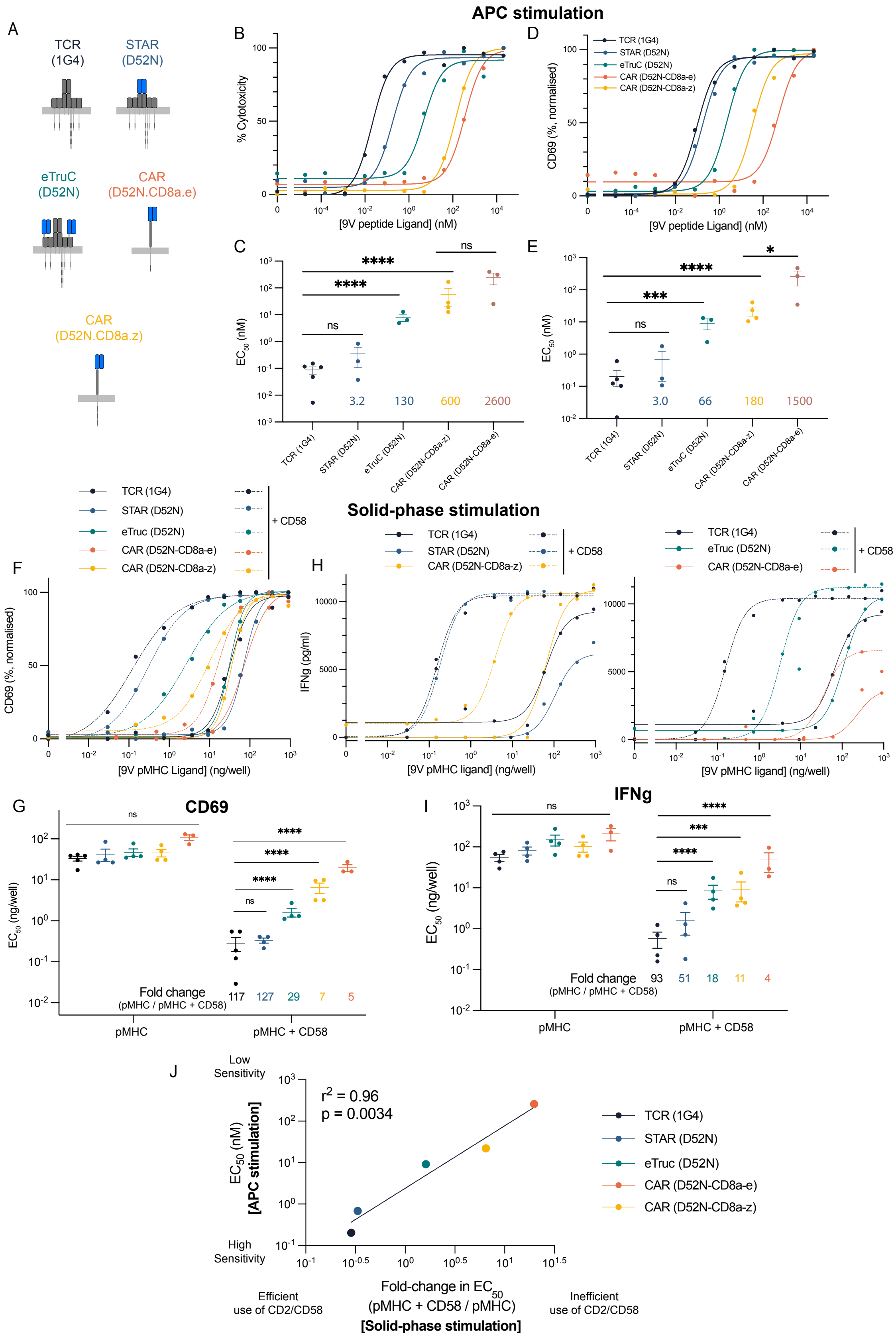

APC stimulation
A
TCR (1G4)
STAR (D52N)
eTruC (D52N)
CAR (D52N.CD8a.e)
CAR (D52N.CD8a.z)
B
% Cytotoxicity
[9V peptide Ligand] (nM)
D
CD69 (%, normalised)
[9V peptide Ligand] (nM)
C
E
EC50 (nM)
3.2
130
600
2600
3.0
66
180
1500
Solid-phase stimulation
F
CD69 (%, normalised)
[9V pMHC Ligand] (ng/well)
+ CD58
H
IFNg (pg/ml)
[9V pMHC ligand] (ng/well)
G
CD69
EC50 (ng/well)
Fold change
(pMHC / pMHC + CD58)
117
127
29
7
5
pMHC
pMHC + CD58
I
IFNg
Fold change
(pMHC / pMHC + CD58)
93
51
18
11
4
J
Low Sensitivity
High Sensitivity
r^2 = 0.96
p = 0.0034
EC50 (nM) [APC stimulation]
Fold-change in EC50 (pMHC + CD58 / pMHC) [Solid-phase stimulation]
Efficient use of CD2/CD58
Inefficient use of CD2/CD58

Figure 28: **The ability of TCR-like chimeric antigen receptors to recapitulate the sensitivity of the TCR depends on the efficiency with which they are able to exploit the CD2 adhesion interaction. (A)** Schematic of 'TCR-like' engineered antigen receptors. **(B-E)** T cells expressing the indicated antigen receptor were co-cultured with T2 target cells pulsed with different peptide antigen concentrations for 8 hours. Representative dose-response (top) and fitted EC_{50} values from at least 3 independent experiments (bottom) are shown for (B,C) cytotoxicity (measured by LDH release) and (D,E) CD69 upregulation. **(F-I)** T cells expressing the indicated antigen receptor were stimulated by a titration of purified pMHC alone (solid lines) or in combination with a fixed concentration of purified CD58 (dashed lines). Representative (F,H) dose-response curves and (G,I) fitted EC_{50} values from at least 3 independent experiments for (F,G) CD69 upregulation and (H,I) IFNγ production. **(J)** The averaged EC_{50} values for CD69 upregulation from the APC stimulation assay (from panel C) are plotted over the averaged fold-change in EC_{50} for CD69 induced by the addition of CD58 from the solid-phase stimulation assay (from panel G). The EC_{50} values are compared using a one-way ANOVA on log-transformed values (C,E,G,I). Abbreviations: * = p-value$\leq$0.05, ** = p-value$\leq$0.01, *** = p-value$\leq$0.001, **** = p-value$\leq$0.0001.

Adhesion receptors more efficiently enhance antigen engagement for the TCR compared to the εTRuC and CAR

The adhesion receptors CD2 and LFA-1 can improve sensitivity by increasing the effective on-rate (k_{on}), or by reducing the effective off-rate (k_{off}) of the antigen receptor with its antigen ligand. We hypothesised that CD2 and LFA-1 efficiently promoted antigen engagement for the TCR but not CARs. To test this hypothesis we used antigen receptor downregulation as a proxy for antigen receptor engagement, measuring the IC_{50} of receptor downregulation (the antigen concentration required for half of the receptor to be lost from the cell surface). These experiments were performed in the solid-phase system with antigen and co-signalling ligands presented as purified protein on plates.

We observed that the D52N-CD8α-z CAR appears to be upregulated following antigen stimulation (Figure 30, A). This is something which others have observed with CARs previously [16]. Consequently, we take the IC_{50} from the right of the bell-shaped curve which provides a lower limit on the true IC_{50}.

We found that when presenting antigen alone to T cells expressing either the TCR, εTRuC or D52N-CD8α-z CAR the IC_{50} of each receptor was the same (Figure 30, A-B). This would suggest that antigen engagement was also the same for each. In the presence of either CD58 or ICAM-1 however we observed that the εTRuC and CAR had significantly higher IC_{50} values compared to the TCR (Figure 30, B). Whilst the IC_{50} of the TCR decreased with the addition of CD58 or ICAM-1, there was only a small decrease for the εTRuC and no decrease for the CAR (Figure 30, B). If we take downregulation to be a proxy for engagement then this would suggest that the TCR was engaging antigen more efficiently in the presence of CD58 and ICAM-1 whilst the εTRuC and CAR were not.

Dynamics of CD2 and LFA-1 expression are similar for different antigen receptors

Inside-out signalling is well known for integrins like LFA-1 [17]. This is where intracellular signalling results in extracellular changes which then, for example, regulate the affinity of LFA-1 for its ligands. Intracellular signalling may also play a role in CD2 function. For example, it has been reported that CD2 influences rearrangement of the actin cytoskeleton (reviewed in [18]).

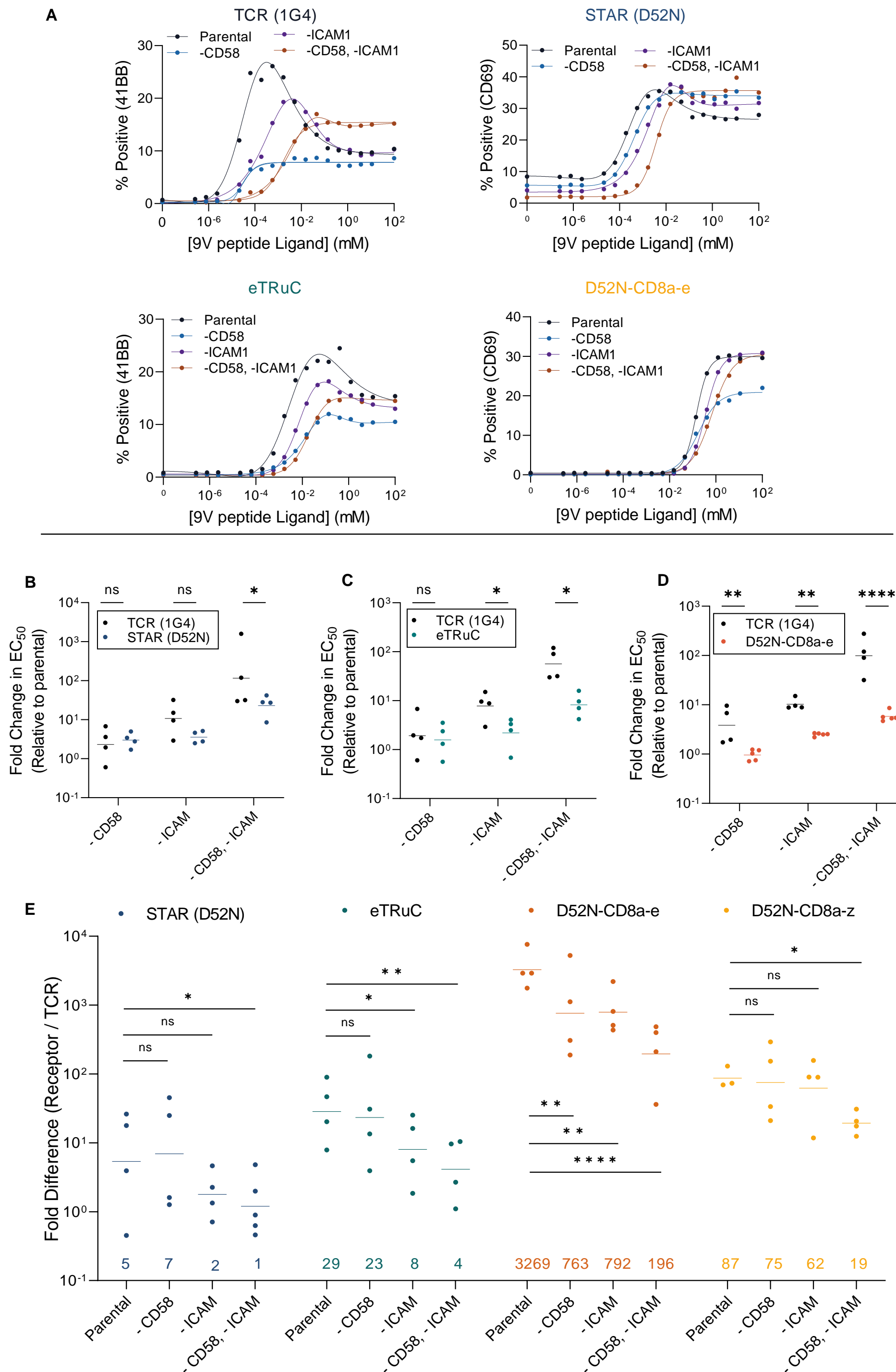


Figure 29: **The effect of abrogating the CD2 and LFA-1 adhesion interactions on sensitivity depends on the structure of the chimeric receptor.** **(A)** Representative curves showing the effect of knocking-out ICAM, CD58 or both from U87 cells on a variety of 'TCR-like' chimeric receptors. **(B-D)** Fold-change in EC_{50} relative to the parental U87 (top row) cell line. Data is shown for the TCR compared to (B) STAR, (C) εTRuC, and (D) D52N-CD8a-e (CAR). **(E)** Fold change in EC_{50} between the indicated receptor and the TCR when CD58, ICAM or both are knocked-out of U87 target cells. Geometric mean and mean fold changes indicated. Comparison between isotype and other conditions and between TCR and receptor by paired t-test with pooled SD. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001, **** = p-value≤0.0001.

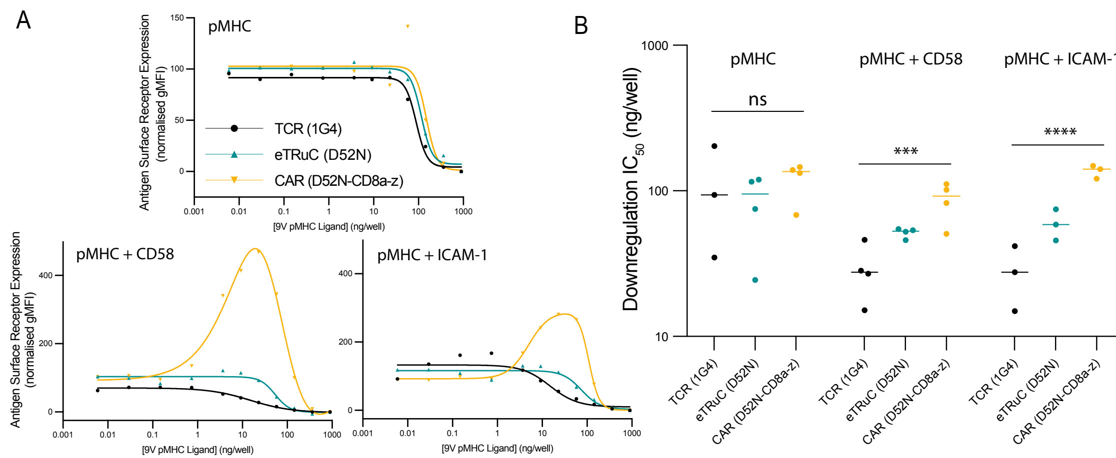


Figure 30: **Adhesion receptors more efficiently enhance antigen engagement for the TCR compared with the εTRuC and CAR. (A-B)** Effect of pMHC with or without CD58 or ICAM-1 on antigen receptor expression, as determined by pMHC tetramers. (A) Representative curves and (B) fitted IC_{50} values from at least 3 independent experiments. All data is normalised to surface expression without pMHC. The IC_{50} is determined to the right of the bell-shapes observed for the CAR with CD58 and ICAM-1. All comparisons are made using a one-way ANOVA on log-transformed values. Abbreviations: *** = p-value≤0.001, **** = p-value≤0.0001.

Consequently, one of the reasons these molecules may fail to increase CAR sensitivity is due to a defect in signalling between the CAR and either of CD2 and LFA-1. We measured the expression of both CD2 and LFA-1 in T cells expressing different chimeric receptors in order to determine if they showed any difference in regulation when these cells were stimulated with antigen.

We found no difference in the regulation of either CD2 (Figure 31, A) or LFA-1 (Figure 31, B) between the various TCR-like receptors tested here. Nor was there any evidence that either CD2 or LFA-1 were engaged differently between the various receptors (Figure 31, C-D).

CD4 T cells also exhibit deficient LFA-1 & CD2 engagement

We note that in the clinical use of CAR-T cells it is often the case that patients are given CAR-T cell products that contain a mixture of both $CD8^+$ and $CD4^+$ T cells. It would therefore be useful to know if $CD4^+$ CAR-T cells also fail to capitalise on the CD58 and ICAM-1 interactions.

We examined this using the same approach as for $CD8^+$ T cells; first assessing the sensitivity of $CD4^+$ T cells in a cellular system. Our results closely follow the sensitivity hierarchy we observed between the chimeric receptors in $CD8^+$ T cells. However in $CD4^+$ cells we were able to see a small but significant difference (3-fold) between the TCR and STAR (Figure 32, A). We also observed that the εTRuC became more TCR-like in its sensitivity — being only 4.2-fold worse than the TCR compared to 66-fold with $CD8^+$ T cells (c.f. Figure 28, E and 32, A).

To again verify that this difference in sensitivity is the result of a failure to capitalise on CD58 and ICAM-1 we measured the fold change in sensitivity when either of these ligands were presented, together with antigen, in our solid-phase system. We found that, in general, the sensitivity improvement conferred by CD58 or ICAM-1 was reduced compared to the same condition in $CD8^+$ T cells (Figure 32, B).

However the TCR and STAR still remained most able to capitalise on co-signalling. With an increase in sensitivity from CD58 of 20.2x and 18.4x respectively, together with an increase from ICAM-1 of 3.8 and 4.7x respectively (Figure 32, B). Whilst the εTRuC and CAR displayed less ability to capitalise on co-stimulation; 4.5 and 4- fold improvements from CD58, and 2.2 and 2.3 fold improvements from ICAM-1 respectively (Figure 32, B).

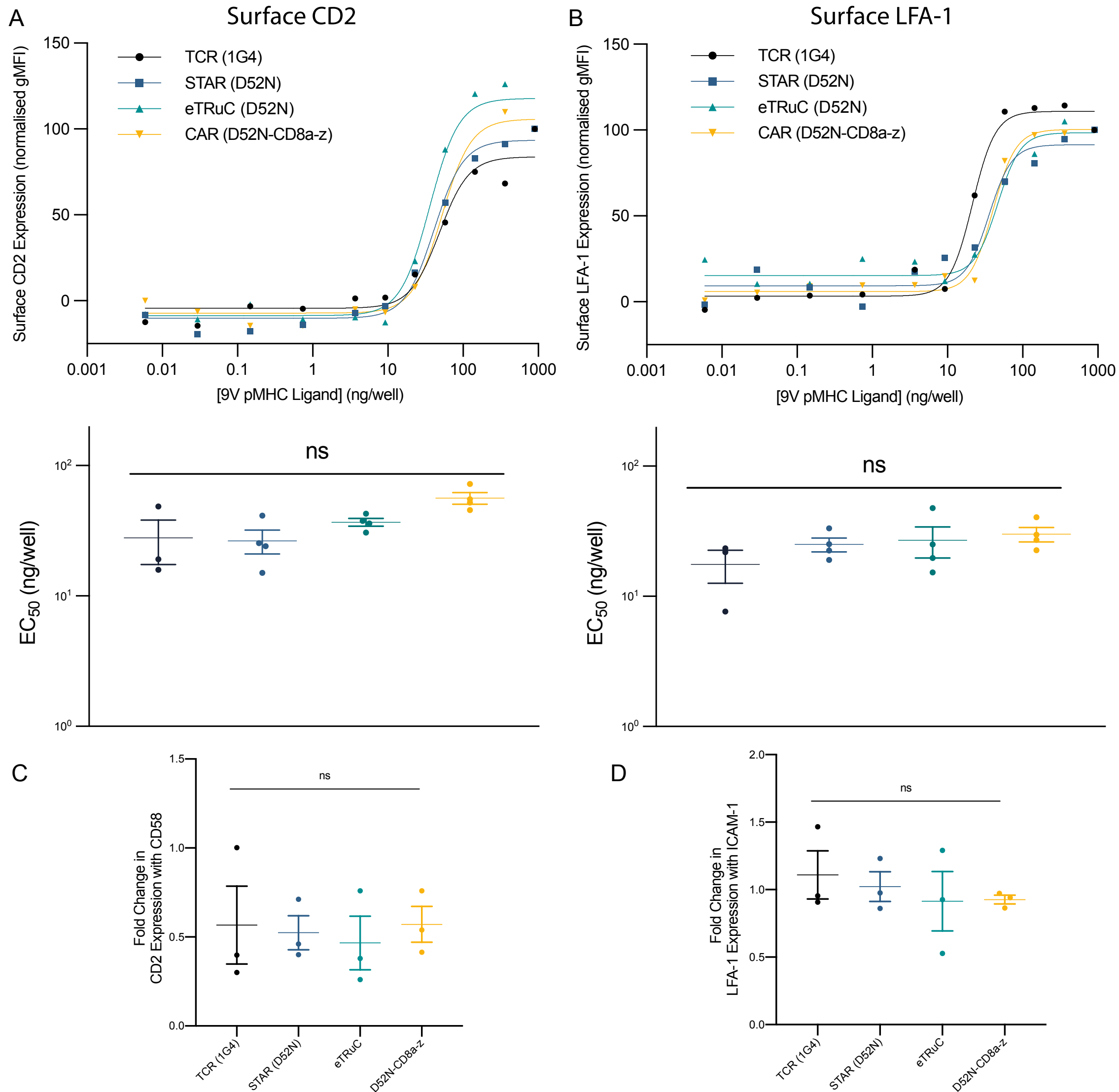


Figure 31: **Up-regulation of surface CD2 and LFA-1 is similar for different antigen receptors**. **(A)** Surface CD2 or **(B)** surface LFA-1 on T cells expressing the indicated antigen receptors stimulated in the solid-phase stimulation with antigen alone. The LFA-1 antibody clone used in the present work recognises the inactive conformation of LFA-1. Surface expression is normalised to the condition without antigen. The EC_{50} values are compared using a one-way ANOVA on log-transformed values. **(C,D)** The fold-change in (C) surface CD2 or (D) LFA-1 for T cells transduced with the indicated antigen receptor stimulated in the solid-phase stimulation with CD58 or ICAM-1, respectively in the absence of antigen.

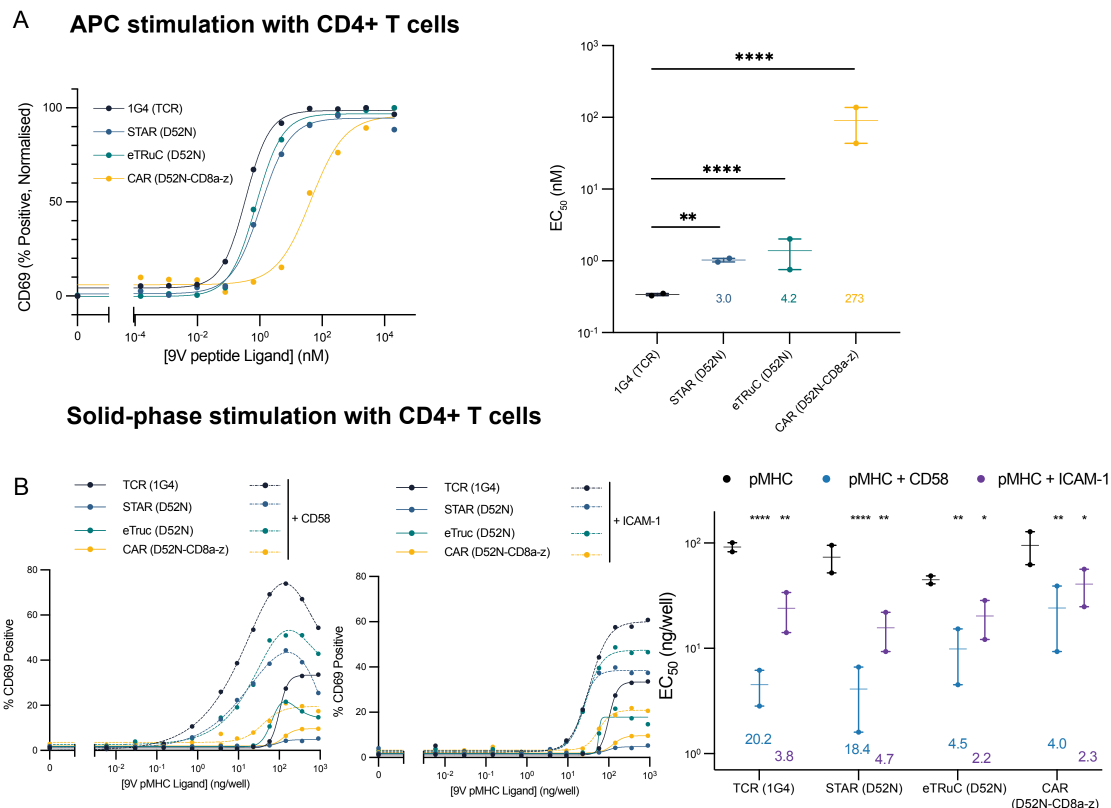


Figure 32: **CD4⁺ CAR-T cells exhibit reduced antigen sensitivity and inefficient exploitation of CD2 and LFA-1 compared to the TCR. (A)** CD4+ T cells expressing the indicated antigen receptor were stimulated by T2 cells pulsed with the indicated peptide concentration. Representative dose-response (left) and EC_{50} values (right) from N=2 independent samples. **(B)** CD4+ T cells expressing the indicated antigen receptor were stimulated by a titration of purified pMHC alone or in combination with a fixed concentration of purified CD58 or ICAM-1. Representative dose-response (left) and EC_{50} values (right) from N=2 independent samples. The EC_{50} values are compared using a one-way ANOVA on log-transformed values. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, **** = p-value≤0.0001.

Discussion

We have shown that two novel chimeric antigen receptors, εTRuCs and STARs, display improved sensitivity relative to traditional CARs, that a novel CAR using CD3ε exhibits reduced sensitivity, and that STARs are able to recapitulate the sensitivity of the T cell receptor (Figure 28, B-E).

The improved sensitivity of the STAR relative to CARs is consistent with the findings of *Liu et al.* [14], who show that a STAR is able to respond to an antigen low cell line whilst a CAR cannot. We extend this work by quantitating the size of the sensitivity defect and showing that TCR and STAR sensitivity are indistinguishable (Figure 28, B-E).

Taken together with our previous results (see chapter 1), we have shown that signalling chain variations (e.g, 1st generation, 2nd generation, ζ, ε) do not impact CAR sensitivity. This agrees with previous studies which suggest the CD3ε domain improves *in vivo* functionality by increasing persistence, with no effect on direct cytotoxicity [9, 10]. Though we recognise that here we

have used a ε chain only CAR, whilst in previous studies the ζ chain has been maintained in addition to the CD3ε domain. This means we cannot rule out any increase to sensitivity from having both the ε and ζ chain present — as might be suggested from the improved performance of the εTRuC relative to either CAR alone (see Figure 28, B-E).

We found that an εTRuC (fusing the CAR scFv with CD3ε) improves antigen sensitivity, but that this remains lower than that of the TCR by 66 and 130-fold when measured as CD69 expression and cytotoxicity respectively (see Figure 28, C & E). The only way to reproduce the antigen sensitivity of the TCR we found was to replace the TCR variable domains with those from a CAR (making a STAR) — we found no significant difference in the sensitivity of these two constructs (Figure 28, C & E).

That the εTRuC is deficient in sensitivity relative to the TCR despite having all the signalling machinery of the TCR, whilst the STAR is not deficient would suggest that the size of the receptor is also a key determinant of sensitivity — for example by ensuring that large phosphatases are excluded from the close contact region. This is consistent with reports that α and β TRuCs, which we would expect to be larger, perform worse than TRuCs formed from CD3 [13]. It is also consistent with our previous finding that the IgG1 hinge region, which we expect to be large compared to hinges derived from CD8α and CD28, reduced CAR sensitivity (chapter 1, Figure 11). We predict that the D52N scFv could add approximately 5 nm to the extent of CD3ε (Figure 33), but this may not make it larger than the TCRαβ chains, and we recognise that *Baeuerle et al.* [13] find that the TRuC does not interfere with TCR binding. This could suggest a similar or smaller extent, extension of TCRαβ or compression of the linker between CD3ε and the scFv.

We previously showed that ability to capitalise on the CD2/CD58 and LFA-1/ICAM-1 adhesion interactions is the means by which the TCR is able to achieve its dramatically increased sensitivity relative to traditional CARs (chapter 1). Here we investigated if this could explain the improved sensitivity of both εTRuCs and STARs, finding that this was the case. Ability to

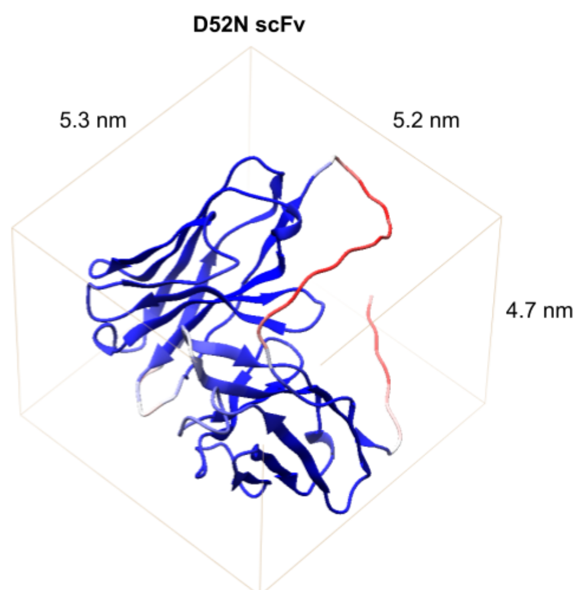


Figure 33: **The D52N scFv spans a distance of approximately 5 nm**. The AlphaFold [19] predicted fold of the D52N scFv is shown together with its minimal bounding box and the corresponding box dimensions.

capitalise on these adhesion interactions is correlated with sensitivity (see Figure 28J & 27C). Together these results localise the cause of the CAR sensitivity defect to an inefficient use of CD2 and LFA-1.

Our data supports a model whereby in the absence of co-signalling ligands the effective affinity (K_D) of both the CAR and TCR are similar, but in the presence of adhesion receptor ligands the effective affinity of the TCR is increased to a greater extent than that of the CAR (Figure 34). The means by which the effective affinity of the TCR could be increased may be either through signalling via the co-signalling receptor, or by the physical effects of co-signalling receptor binding (e.g., increased stability of the receptor/antigen interaction, increased local concentration of receptor &c).

We note that we observed much more modest effects of CD58 and LFA-1 on $CD4^+$ T cells than we did on $CD8^+$s for the TCR, STAR and εTRuC (Figure 32). We suggest this is because these receptors are now no longer able to capitalise on CD8 and that there is some synergy between the action of CD58/LFA-1 and CD8. Some evidence that this is the case was observed when we used HLA-A*02:01 with a mutation to prevent CD8 binding and observed a smaller improvement from CD58 in TCR-T cells (chapter 1, Figure 21).

We also note that the sensitivity of the εTRuC becomes more TCR like in $CD4^+$ cells, with the fold difference between the εTRuC and TCR being only 4-fold in $CD4^+$ compared to 66-fold in $CD8^+$ cells (c.f. Figure 32, A and 28, E). This may suggest that the CD8-MHC interaction antagonises εTRuC-antigen binding due to its proximity to CD3ε or a mismatch in size between the CD8–MHC and εTRuC–pMHC interactions.

In CD4 cells we also notice that the difference between the STAR and TCR, whilst remaining small (3-fold) becomes significant. This may be explained by small differences in transduction efficiency and expression (Figure 25) or given the small effect size it may require more experimental power to detect in $CD8^+$ T cells.

We propose a model (Figure 30) by which the CD2 and LFA-1 adhesion interactions increase the effective affinity of the TCR-pMHC interaction but not of the CAR-pMHC interaction, thereby increasing TCR, but not CAR, antigen sensitivity. Our model supports multiple mechanisms by which the effective affinity could be increased. One mechanism is that signalling between

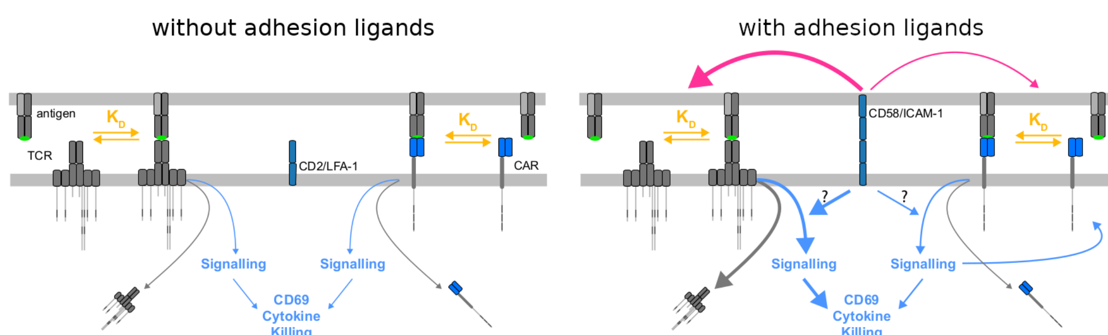


Figure 34: **A model for the impact of adhesion receptors on CAR and TCR signalling.** Schematic of a model showing similar performance of a TCR and a CAR in the absence of ligands for adhesion receptors (left panel) and superior enhancement of TCR versus CAR antigen engagement (red arrows) and signalling (blue arrows) by adhesion receptor ligands (right panel).

the TCR and the adhesion receptor could alter the affinity of the latter (as is the case with integrin inside-out signalling) which could then synergistically increase the effective affinity of the TCR, and that defective CAR signalling abrogates this sort of interaction. The other is that the physical properties of the adhesion interactions are advantageous to the TCR but not to the CAR. For example the size of the CD2–CD58 interaction may improve the effective affinity of TCR-pMHC interactions but not CAR-pMHC, owing to a mismatch in size.

It has previously been suggested, by *Harris et al.*, [20] that the CAR sensitivity defect can be explained by reduced ITAM phosphorylation in CARs. In agreement with the finding in *Harris et al.*, *Baeuerle et al* [13] also find poor LAT phosphorylation in CARs, and that the εTRuC construct shows better LAT phosphorylation when compared to a CAR. Our data here suggests that this cannot explain the whole of the CAR sensitivity defect as we observe that the εTRuC fails to recapitulate exactly the sensitivity of the TCR (Figure 28, B-E) despite having the entire complement of ITAMs present in the TCR complex.

This, together with our observation that the STAR, which completely matches the size of the TCR, is the only chimeric receptor capable of recapitulating TCR sensitivity leads us to suggest that the εTRuC is not able to achieve TCR sensitivity due to its increased size from having the scFv fused to CD3, and that receptor size has an important role in the ability of chimeric receptors to exploit co-stimulation. We also note that we have previously seen that CD58 may have an antagonistic effect on CAR sensitivity (chapter 1, Figure 17) which is also suggestive of a mismatched size.

Altogether this lead us to hypothesise that CARs fail to capitalise on co-signalling receptors due to a mismatch in the interaction distances of the CAR with antigen and the co-signalling receptors with their ligands. We explore this more fully in chapter 3.

References

[1] Nadia Martin-Blanco et al. "CD3ε recruits Numb to promote TCR degradation". In: *International immunology* 28.3 (2016), pp. 127–137.

[2] Matthew L Bettini et al. "Cutting edge: CD3 ITAM diversity is required for optimal TCR signaling and thymocyte development". In: *The Journal of Immunology* 199.5 (2017), pp. 1555–1560.

[3] Maria C Ramello et al. "An immunoproteomic approach to characterize the CAR interactome and signalosome". In: *Science signaling* 12.568 (2019), eaap9777.

[4] John S Bridgeman et al. "CD3ζ-based chimeric antigen receptors mediate T cell activation via cis-and trans-signalling mechanisms: implications for optimization of receptor structure for adoptive cell therapy". In: *Clinical & Experimental Immunology* 175.2 (2014), pp. 258–267.

[5] Timothy A Springer. "Adhesion receptors of the immune system". In: *Nature* 346.6283 (1990), pp. 425–434.

[6] Simon J Davis and P Anton Van Der Merwe. "The structure and ligand interactions of CD2: implications for T-cell function". In: *Immunology today* 17.4 (1996), pp. 177–187.

[7] Olivier Leupin et al. "Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes". In: *Current Biology* 10.5 (2000), pp. 277–280.

[8] Qian Xiao et al. "Size-dependent activation of CAR-T cells". In: *Science Immunology* 7.74 (2022), eabl3995.

[9] Wei Wu et al. "Multiple signaling roles of CD3ε and its application in CAR-T cell therapy". In: *Cell* 182.4 (2020), pp. 855–871.

[10] Frederike A Hartl et al. "Noncanonical binding of Lck to CD3ε promotes TCR signaling and CAR function". In: *Nature immunology* 21.8 (2020), pp. 902–913.

[11] Chuang Sun et al. "THEMIS-SHP1 recruitment by 4-1BB tunes LCK-mediated priming of chimeric antigen receptor-redirected T cells". In: *Cancer Cell* 37.2 (2020), pp. 216–225.

[12] Alexander I Salter et al. "Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function". In: *Science signaling* 11.544 (2018), eaat6753.

[13] Patrick A Baeuerle et al. "Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response". In: *Nature communications* 10.1 (2019), pp. 1–12.

[14] Yue Liu et al. "Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors". In: *Science Translational Medicine* 13.586 (2021), eabb5191.

[15] Jorge Mansilla-Soto et al. "HLA-independent T cell receptors for targeting tumors with low antigen density". In: *Nature Medicine* 28.2 (2022), pp. 345–352.

[16] Justin Eyquem et al. "Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection". In: *Nature* 543.7643 (2017), pp. 113–117.

[17] Navin Kumar Verma and Dermot Kelleher. "Not just an adhesion molecule: LFA-1 contact tunes the T lymphocyte program". In: *The Journal of Immunology* 199.4 (2017), pp. 1213–1221.

[18] Christian Binder et al. "CD2 immunobiology". In: *Frontiers in immunology* 11 (2020), p. 1090.

[19] John Jumper et al. "Highly accurate protein structure prediction with AlphaFold". In: *Nature* 596.7873 (2021), pp. 583–589. doi: 10.1038/s41586-021-03819-2.

[20] Daniel T Harris et al. "Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains". In: *The Journal of Immunology* 200.3 (2018), pp. 1088–1100.

Chapter 3

Investigating the effect of CD2 size on CAR-T cell sensitivity

Introduction

What is CD2?

CD2 is a co-stimulatory receptor found on the surface of T cells and a number of other immune cells (namely: NK cells, thymocytes and some dendritic cells [1]). In particular it is enriched in the uropod of the cell, along with other adhesion proteins, during the scanning of antigen presenting cells [2]. A member of the immunoglobulin (Ig) superfamily, the extracellular domain of CD2 consists of 2 Ig domains containing 3 glycosylation sites. The region away from the cell membrane is responsible for binding CD58 (LFA3); the main binding partner of CD2 in humans. CD2 is thought to bind CD58 'head-on' with a predicted total interaction distance of 14 nm [3]. This is similar to that of the TCR-pMHC interaction and the CD4-MHC interaction [4]. Although CD2 has a low affinity for CD58 (K_D =9 µM to 22 µM) [5] the affinity and avidity of the CD2-CD58 interaction may increase following activation [6]. CD2 is thought to be recruited to the site of TCR-pMHC interactions, the 'immune synapse', and there, along with other co-signalling molecules generate close-contacts that can exclude large phosphatases such as CD45 from the area around the TCR [7].

In addition to its extracellular exclusion and adhesion action, CD2 also contains a highly conserved intracellular tail. This is suggestive of a signalling function. Whilst the tail of CD2 contains no tyrosine-based motifs it does contain several signalling domains, including a number of SH3 binding domains. CD2 itself has been shown to associate with other SH3 domain containing proteins important in T cell signalling, such as, *Lck*. However, the importance of the signalling aspect of CD2 remains unclear as tail-deletion mutants of CD2 can still enhance TCR signalling [8].

Irrespective of the precise mechanism of action, CD2 is clearly a potent co-stimulator of T cell activation [9]. Further evidence for this comes from the evolution of strains of human cytomegalovirus to downregulate CD58 as a means of evading the immune response [10]. It is because of this potential as a co-stimulatory agent that the CD2-CD58 axis may have an important role to play in immunotherapy.

The importance of CD2-CD58 for immunotherapy

The sensitivity of CAR-T cells is critically important for their use in clinical applications. In phase 2 studies of a clinically approved CAR 25% of patients who completely responded to treatment later relapsed with cancers having undetectable levels of CD19 [11]. Similar issues have been observed with other targets for B cell malignancies, like CD22 [12]. This is thought to be because CAR-T cells require higher concentrations of antigen in order to become activated [13, 14].

Though the exact mechanism for the reduced sensitivity of CAR T cells is unknown, one of the ways in which T cells improve their sensitivity is through numerous co-signalling receptors on the cell surface [15]. We have shown in this thesis that two of these co-signalling receptors, LFA-1 and CD2, have a disproportionate impact on the sensitivity of TCR-T cells compared to CAR-T cells. The ligand for CD2 (CD58) increases TCR sensitivity 125 fold, but by less than 10 fold for CARs (chapter 1, Figure 11D). Equally, one of the ligands to LFA-1 (ICAM-1) increases TCR-T sensitivity by 22 fold whilst increasing CAR sensitivity by less than 5 fold (see chapter 1, Figure 11D).

The relevance of these molecules in immunotherapy depends on their expression on cancer cells. Since cancer is a diverse disease, cancer cell types have differing levels of CD58 expression. Previous studies have correlated immunotherapy response rate with CD58 expression; where cancers with low CD58 expression have correspondingly poor response rates [16]. This was also shown experimentally, with CD58 knockout cancer cells resulting in reduced lysis and IFNγ production by T cells, suggesting that CD58 expression by cancer cells can modulate T cell sensitivity [16].

This experimental study is also consistent with a single-cell RNA sequencing analysis of tumours taken from patients undergoing immune checkpoint therapy which found that low CD58 expression was a predictive biomarker for treatment resistance [17]. A related study using a CRISPR knockout screening library and subsequent sequencing of surviving cells after CAR-T therapy also identified CD58 as an indicator of treatment resistance [18]. Blocking of CD2 using antibodies was found in the same study to also promote resistance to CAR-T therapy, consistent with previous *in vivo* work showing that blocking antibodies to CD2 reduce the T cell response [19].

Taken together these studies demonstrate the importance of the CD2-CD58 axis in immunotherapies. We have shown that the sensitivity defect in CAR T cells can be explained by an inability to exploit the CD2-CD58 interaction (chapter 1). We also show that novel chimeric receptors, such as STARs [20], are able to achieve TCR-like sensitivity (chapter 2). This would suggest that there is some benefit to sensitivity from the structure of the TCR. We posit that the compact size of the TCR is important for capitalising on co-signalling receptor-ligand interactions.

We have chosen to focus on CD2-CD58 here as it conferred the largest sensitivity benefit to TCR-T cells in our assays. Additionally, CD58 is expressed widely in a range of tissues [21]; meaning that an approach to rescuing CD2-CD58 functionality in CAR-T cells could be applied against a range of targets.

A likely mechanism by which CD2 acts to increase sensitivity is by optimally aligning the T and antigen presenting cell membranes in close contact regions. Achieving a sensitivity improvement either by stabilising TCR-pMHC interactions for a sufficiently long period that kinetic proofreading can take place, or excluding large phosphatases like CD45 from the area around the receptor. Given this mechanism we propose that the reason CARs fail to exploit the CD2 interaction is due to a mismatch in size between CD2 and CARs which prevents these

optimal membrane alignments.

About this study

Given that the TCR-pMHC and CD2-CD58 interaction sizes are matched, we hypothesised that there is a mismatch in the size of the CAR-pMHC interaction to that of CD2-CD58. To explore if this mismatch could be the cause of our previously observed CAR antigen sensitivity defect we set out to increase the size of the CD2-CD58 interaction. We took two approaches to this, from both the antigen presenting cell (i.e., CD58) side and the T cell (i.e., CD2) side. Consistent with our predictions, we found an optimally elongated CD2-CD58 interaction can improve the sensitivity of CARs to antigen and that differently elongated CD2-CD58 interactions were required for different chimeric receptors.

Results

Estimating the size of CAR hinges

In the absence of crystal structures of CARs it is difficult to predict their extracellular reach. So far in this thesis we have considered 3 CARs, each of which has a different 'hinge' region taken from a variety of different proteins. These do not always match to the 'hinge' regions of the proteins in question, and may include parts of folded domains. A summary of the different hinges, together with a estimation[1] of their size is given in table 1.

Hinge	Extent in predicted or crystal structure of native protein	Number of amino acids	Estimated contour length
CD28	76 Å	40 AA	136 Å
CD8α	49 Å	45 AA	153 Å
IgG1	79 Å	264 AA	125 Å (folded)

Table 1: Estimated Size of Various CAR Hinges

We also used AlphaFold to predict the structure of the hinge regions of each CAR, which suggested that the CD28 and CD8α hinges fold in a disordered fashion. Whilst the IgG1 hinge forms two Ig domains with a short disordered tail. This results in a similar hierarchy as would be predicted from contour length alone. These predictions only serve as a rough estimation of hinge reach, since they lack the complete context of the remainder of the protein. We did not expand our prediction to the entire protein as AlphaFold (at the time of writing) does not predict post-translational protein modifications. A number of these, like glycosylation, are known to be key to determining the final size and extent of the folded protein.

Both CD8α and CD28 are glycoproteins, and have glycosylation sites in the regions utilised as CAR hinges. Using computational prediction [22], and a survey of the existing literature [23, 24, 25] we believe that the CD8α hinge has 5 glycosylation sites whilst the CD28 hinge has 2. There is also further evidence that CD8α forms an extended conformation [26]. It is unclear what impact these post-translational modifications would have on the hierarchy we estimate here, which is that the CD28 hinge CAR is the smallest, followed by the CD8α hinge and then the IgG1 hinge.

[1] Assuming an extent of 3.4 Å per amino acid.

Elongation of CD58 and ICAM-1 reduces their ability to increase antigen sensitivity

For our initial investigations into the effects of CD2-CD58 interaction distance on CAR-T sensitivity we used a variant of CD58 which had been elongated by the addition of 2 Ig domains from rat CD4 (Figure 35, A). As in our previous studies, these purified proteins were biotinylated and immobilised on streptavidin coated plates together with a titration of pMHC concentrations (see materials and methods — chapter 5) before T cells were added and their response, in terms of % CD69 positive cells, at each pMHC concentration was assessed (Figure 35, B-C).

We did this simultaneously for 'wild-type' CD58, elongated rat CD4-CD58 ('rCD4-CD58'), rat CD4 alone and for another adhesion molecule, ICAM-1. Again, in 'wild-type' and elongated variants.

We found that rat CD4 had no impact on CAR or TCR-T cell sensitivity, which was equivalent to antigen alone (Figure 35, D). Confirming that the presence of the rat CD4 domains in our elongated CD58 and ICAM-1 variants would not in itself explain any alteration in sensitivity. We found small, but statistically significant reductions in the improvement in sensitivity conferred by both rCD4-CD58 and rCD4-ICAM to CAR and TCR-T cells (Figure 35, E). This is consistent with previous work using elongated CD48 (the binding partner of CD2 in mice) [27, 28].

In addition to this smaller improvement in sensitivity conferred by the elongated adhesion ligands, we also observed that for CAR-T cells expressing 'traditional' CAR constructs (i.e., those consisting of a single chain variable fragment coupled to a extracellular hinge and intracellular signalling domain derived from TCRζ) elongation of CD58 increased the basal activation level of the CAR (c.f. Figure 35, B (left)). This was also the case for a more 'TCR-like' receptor, the eTRuC [29] (which consists of the scFv grafted onto one of the CD3 chains; see chapter 2), but not for a CAR whose intracellular signalling domain was derived from the intracellular part of CD3ε (Figure 35, F).

Together these results would suggest that elongating the CD2-CD58 interaction by increasing the size of CD58 leads to a modest reduction in antigen sensitivity. We hypothesised that the effect on sensitivity caused by elongating either CD58 or ICAM-1 may be due to their inconsistent orientations when immobilised on the plate manufacturer's dextrose matrix coupled with streptavidin. This would alter the effective interaction size of both the wild-type and elongated variants of each ligand, possibly preventing some receptor-ligand interactions from occurring as well as allowing large phosphatases into the area around the receptor (Figure 36).

In this assay we observe that elongated CD58 and ICAM-1 reduced improvement in antigen sensitivity for both the CAR and TCR compared to wild-type CD58/ICAM-1. This suggests that the elongated variants are largely mediating macroscopic adhesion between the T cell and the surface but not mediating the microscopic adhesion that would enable precise membrane alignment, the exclusion of large phosphatases and increased receptor-pMHC stability.

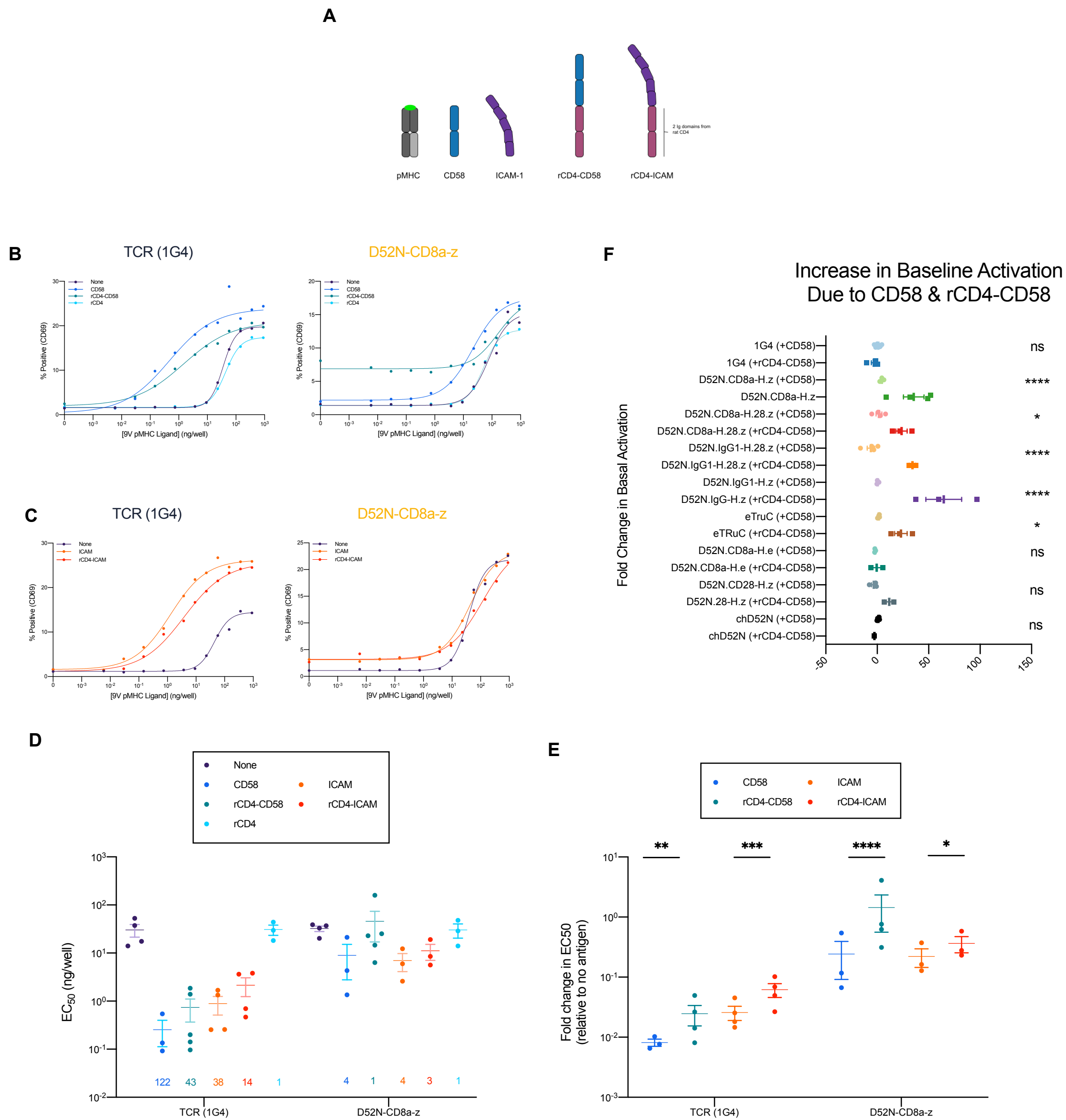


Figure 35: **Elongation of CD58 and ICAM-1 reduces their ability to increase antigen sensitivity (A)** The adhesion molecules CD58 and ICAM-1 are elongated by inserting two Ig domains from rat CD4 (rCD4-CD58 and rCD4-ICAM-1). **(B)** Representative dose-response curves showing the impact on sensitivity of both CD58 and rCD4-CD58 on TCR-T and CAR-T cells. **(C)** Representative dose-response curves showing the impact on sensitivity of both ICAM-1 and rCD4-ICAM-1 on TCR-T and CAR-T cells. **(D)** EC_{50} values measured for $n \geq 3$ independent donors. (**E**) Fold changes in EC_{50} relative to the antigen alone condition. Comparison between conditions with or without ligand elongation by paired t-tests using pooled SD. (**F**) Fold change in basal activation ([9V pMHC Ligand] = 0 ng/well) in the presence of CD58 or elongated CD58 for a the indicated antigen receptors. Paired t-tests between conditions indicated. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001 **** = p-value≤0.0001.

Elongating CD2 reveals an optimal CD2-CD58 interaction size for chimeric receptors

To precisely elongate the CD2/CD58 interaction, we turned our attention to a cellular system whereby we elongated CD2 recognising CD58 directly on APCs. To do this we used an increasing number of amino acids from the extracellular domain of CD43 to elongate CD2 (Figure 37, B). We used CD43 because this mucin-like molecule has previously been shown to adopt a highly extended conformation with an average extent of 0.2 nm per amino acid [30]. We produced a panel of elongated CD2 molecules with an N-terminal FLAG tag with different number of polypeptides from CD43 (Figure 37, C).

Our estimates of CAR sizes put the CD8α hinge size as approximately 7 nm longer than the TCR (table 1) so we initially focused on elongating CD2 by approximately this length. Elongating with 4, 8, 20, 40 or, as a control, all 234 amino acids from the extracellular region of CD43 (Figure 37, B). Given an extent of 0.2 nm per amino acid these correspond approximately to the sizes of elongated CD2 in table 2.

To test each of these CD2 sizes we first engineered Jurkat T cells using CRISPR to knockout

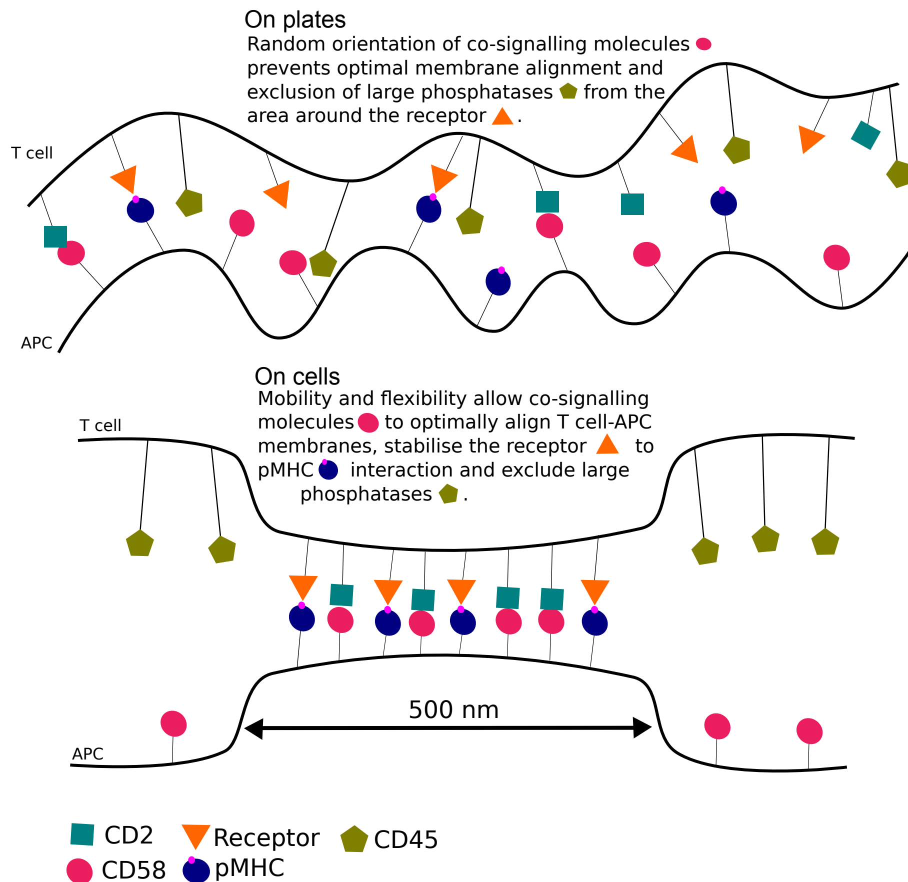
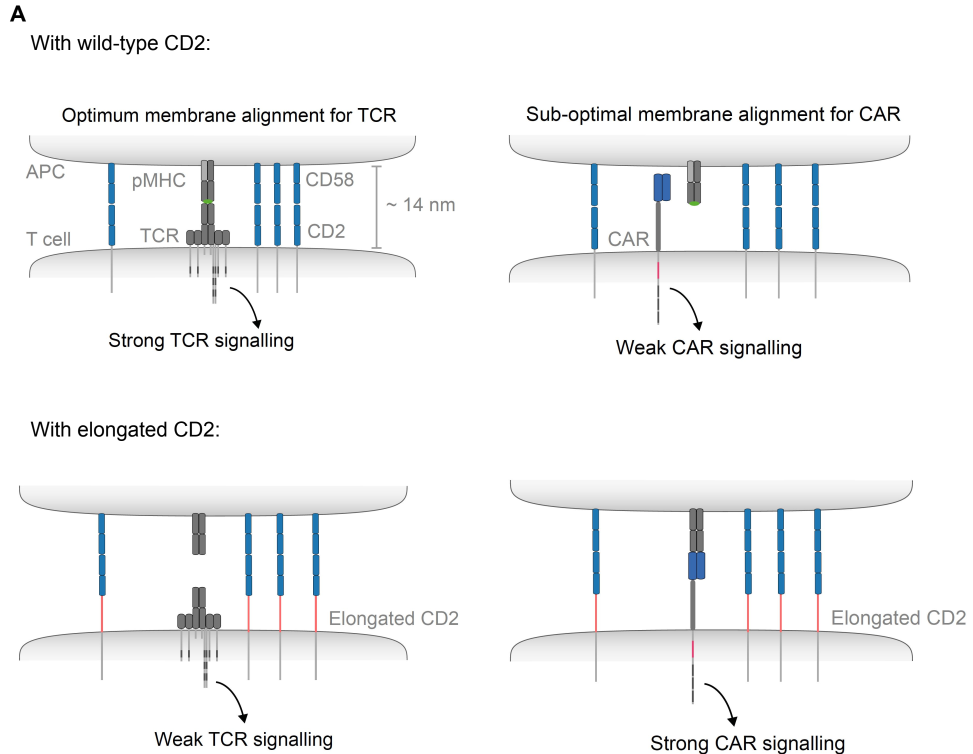
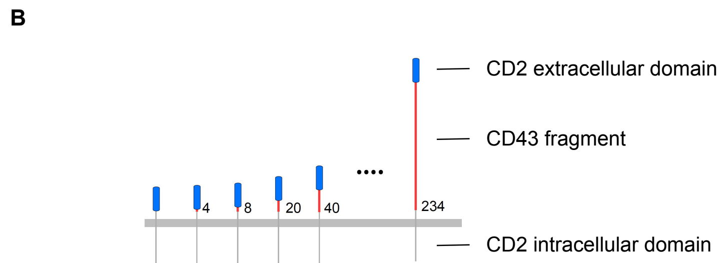


Figure 36: **Random orientation of immobilised proteins prevents optimal membrane alignment in a solid-phase system.** On plates the random orientation of co-signalling molecules prevents optimal membrane alignment and the exclusion of large phosphatases from the area around the receptor whilst in a cellular system mobility allows co-signalling molecules to optimally align T cell-APC membranes, stabilise the receptor-pMHC interaction and exclude large phosphatases.





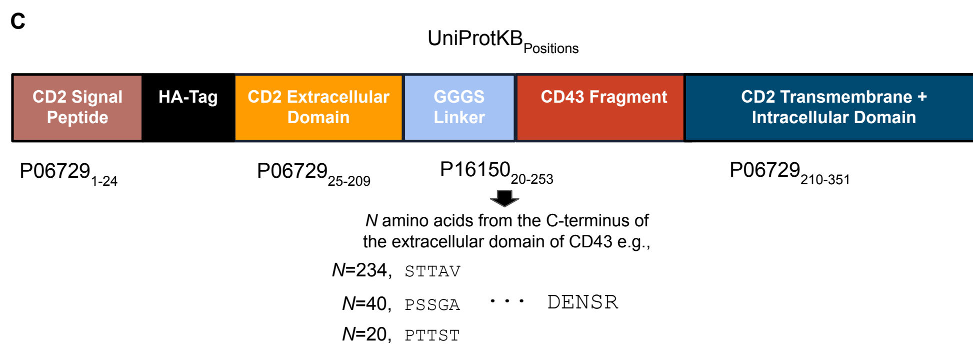


Figure 37: **A strategy for exploring the impact of CD2 size on the ability of CAR-T cells to exploit the CD2-CD58 adhesion interaction. (A)** Mis-alignment of cell membranes is suggested to lead to reduced impact of CD58 on CAR-T cell sensitivity which can be rescued by identifying the optimal CD2 extent. **(B)** CD2 is elongated using fragments of CD43, a T cell surface protein thought to form a extended glycosylated confirmation with a known average extent per amino acid (0.2 nm) [30]. **(C)** Elongated CD2 is produced by inserting fragments of CD43 from the C-terminal end between the extracellular and transmembrane domains of CD2.

Size of CD43 Fragment (AA)	Predicted reach of CD2-CD43 (nm)
0	7.5
4	8.3
8	9.1
20	11.5
40	15.5
234	54.3

Table 2: Predicted reach of recombinant CD2-CD43 based on number of amino acids taken from CD43. Estimated size of CD2 is 7.5 nm

CD2. This was done by lentivirally transducing them with a cassette expressing both a CD2 guide RNA and Cas9. These cells were subsequently sorted to gain a pure CD2 knockout population. After sorting for pure CD2 knockouts we then transduced, again lentivirally, an antigen receptor such as the 1G4 TCR or the D52N-CD8α-z CAR. Finally sorting one more time to get a pure receptor expressing population. These cells then served as the base into which we transduced our various CD2-CD43 variants (Figure 38, A).

To test the behaviour of T cells with each of CD2 variants we performed a dose-response assay using U87 target cells. Briefly: U87 cells (a glioblastoma cell line) were seeded one day before the experiment, then on the day of the experiment the U87 cells were 'pulsed' with a titration of antigen concentrations and incubated with Jurkats expressing one of the CD2-CD43 variants for 4 hours. The % of cells positive for CD69 at each antigen dose was then assessed by flow cytometry.

In TCR expressing T cells transduced with this panel of CD2 variants we found that wild-type CD2 increased sensitivity by approximately 10-fold. But that adding just 4 amino acids from CD43 dramatically reduced sensitivity and 8 amino acids from CD43 completely abrogated the effect of CD2. Further increases in the size of the CD43 fragment further reduced sensitivity, resulting an a sensitivity lower than the condition without CD2 (Figure 38, A).

We next determined the impact of this panel of elongated CD2 receptors on antigen sensitivity mediated by the D52N-CD8α-z CAR. As before, we transduced the CAR into $CD2^-$ Jurkat T cells and sorted to obtain a pure $CD2^-$ CAR^+ population. We then transduced different elongated CD2 molecules into these cells and assessed antigen sensitivity.

We found that wild-type CD2 conferred a modest (<< 10)-fold improvement in T cell sensitivity compared to the TCR, consistent with our previous findings (see chapter 1). But, remarkably, increasing the size of the CD43 fragment up to 40 amino acids increased the sensitivity of the CAR-T cells. At this point, as with the TCR, further elongation of the CD2 molecule reduces sensitivity beyond that of $CD2^-$ Jurkat T cells (Figure 39, A).

To determine if further improvement in antigen sensitivity for the CAR can be achieved between the variants containing 40 and 234 amino acids, we used 80, 120 and 160 amino acids from CD43. Repeating our screen using CAR-T cells expressing these new CD2 variants we again observed no significant improvement in sensitivity from wild-type CD2. Moreover each of the variants under test decreased sensitivity beyond that of the $CD2^-$ condition (Figure 39, B). We concurrently performed the same screen with TCR expressing cells and found that these constructs reduced sensitivity to the same extent as elongation by the full length of CD43 (Figure 38, C).

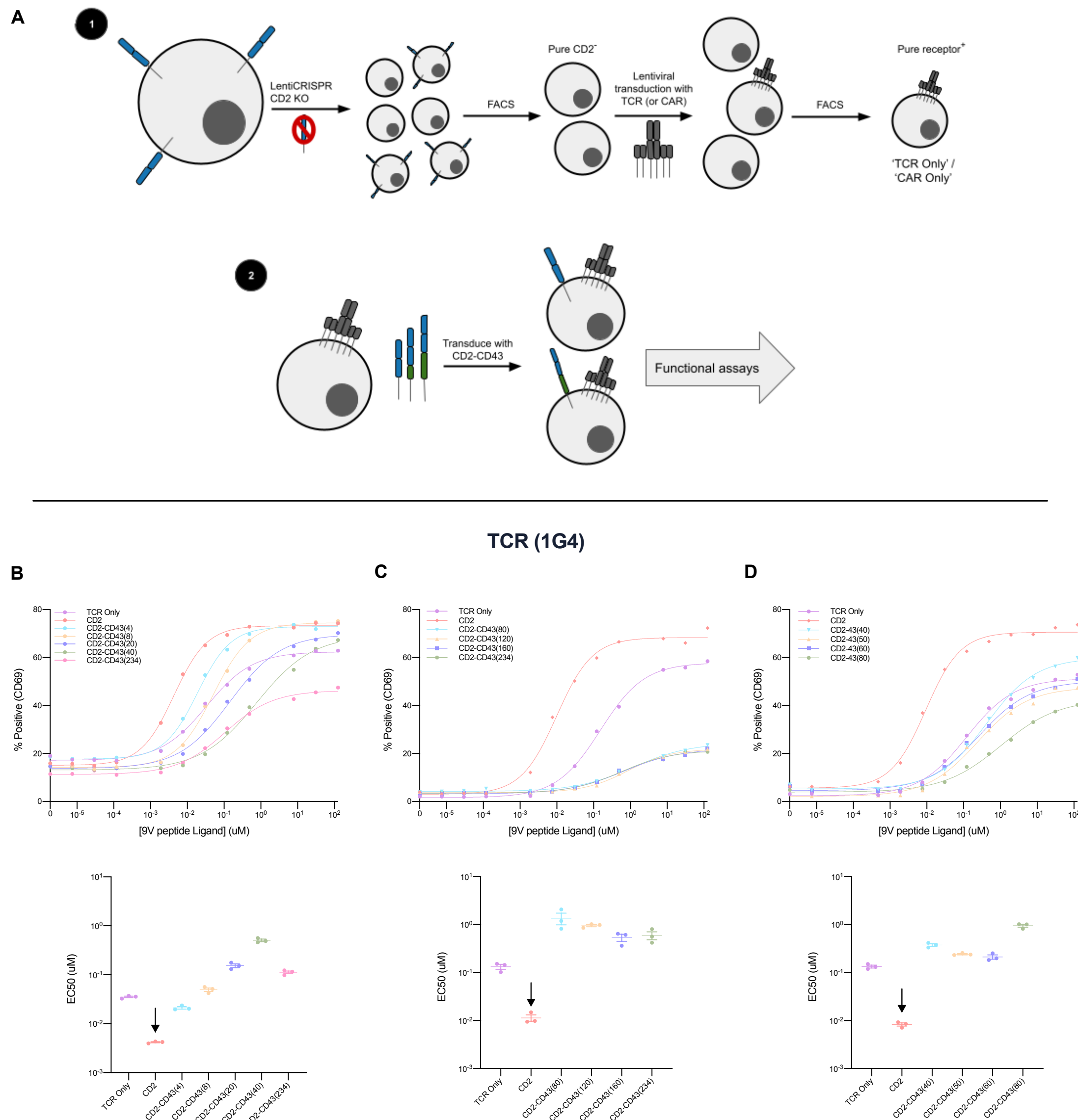


Figure 38: **A systematic screen of elongated CD2 identified wild-type CD2 as optimising antigen sensitivity for the TCR. (A)** Jurkat T cells expressing either a CAR or TCR and a elongated CD2 variant are produced by using CRISPR to remove endogenous CD2 before transduction of an antigen receptor (e.g. TCR or CAR) and subsequently with different CD2 molecules before being tested in functional experiments using U87 cells as APCs. **(B, C, D)** Representative dose-response curves (top) and extracted EC_{50} values (bottom). The data was generated by transducing one of three sets of elongated CD2 molecules into Jurkat T cells (one set in each of B, C and D). Each EC_{50} value is the result of n = 3 independent experiments. Optimal CD2 size identified with an arrow.

On the basis of these screens it could be determined that the 'optimal' CD2 size for D52N-CD8α-z CAR-T cells is between 40 and 80 amino acids of CD43. Consequently we designed a further two constructs to span this gap, using 50 and 60 amino acids from CD43. We found that the 50 and 60 amino acid variants also decreased CAR-T cell sensitivity to a level similar to that of the CAR only condition (Figure 39, C). We thereby concluded that the 'optimal' size of CD2 for the CD8α hinge CAR is that which was elongated with 40 amino acids from CD43.

Again, we screened the same set of constructs in TCR expressing T cells and found that these CD2 sizes also did not improve sensitivity relative to the TCR only condition (Figure 38, C).

In summary, we found that wild-type CD2 improves the sensitivity of TCR-T cells by 10-fold whilst improving the performance of CAR T cells by only 2-fold (Figure 39, D); consistent with the earlier work in this thesis (chapter 1). We note that even with the optimal CD2 size the D52N-CD8α-z CAR only achieves a sensitivity equal to that of the TCR in the absence of CD2 (Figure 39, E).

Given that the magnitude of the sensitivity improvement is the same between the TCR and wild-type CD2 and the CAR with CD2-CD43(40) (Figure 39, F) we suggest that it would not be possible to further increase the sensitivity of the CAR. It appears that it is possible to rescue the function of CD2 in CAR-T cells by optimising the extracellular reach of CD2 but not to match sensitivity to that of the TCR. This suggests that the TCR benefits from other mechanisms that further increase its sensitivity.

Different chimeric antigen receptors have different optimal sizes of CD2

Having identified the optimally sized CD2 molecule for a CAR with a particular hinge, we hypothesised that CARs with alternative hinge regions (and consequently different extracellular reaches) would have different optimal CD2 sizes. To this end we used the same CD2-CD43 constructs as described, but this time screened their behaviour in Jurkat cells expressing CARs with different hinge regions: from CD28 (D52N-CD28-z, D52N-CD28-28z) and IgG1 (D52N-IgG1-z).

We anticipated that the CD28 hinge CARs would have a smaller optimal CD2 size, consistent with our previous estimation that the CD8α hinge would be longer than the CD28 one. Performing the same screen with the D52N-CD28-28z CAR, which has both a CD28 hinge and CD28 co-signalling domain, we see that increasing the size of the CD43 fragment improves T cell sensitivity (Figure 40, A-B). However, this time, the point at which elongation of CD2 starts to antagonise T cell sensitivity is after 20 amino acids of CD43 rather than 40. This is in line with our size predictions.

As a further validation we tested both first and second generation CD28 hinge CARs (i.e., with and without a CD28 co-signalling domain) where we would expect that the same CD2-CD43 size would be optimal for both. Although due to variation in the data it is harder to discern that elongations up to 20 amino acids in size improve CD28 hinge CAR-T sensitivity over that of wild-type CD2, we do observe that in both generations of CD28 hinge CAR the point at which further elongation of CD2 reduces sensitivity is the same. Similarly, we performed the exact same screen with an IgG1 hinge CAR, which we expect to be longer than both the CD28 and CD8α hinge CARs. To date we have shown that the optimally sized CD2 for this CAR is $\geq$ 40 amino acids.

A number of newer CARs have been devised which are more TCR-like in their structure. These include the eTRuC [29], where the scFv of a CAR is fused with CD3ε, and STARs [20], in which the variable regions of the CAR scFv are engrafted into the variable regions of a TCR. A fuller explanation of each of these constructs is provided in chapter 2. As might be expected from their 'TCR-like' structure we find that they, like the TCR, are able to capitalise more efficiently on the CD2-CD58 interaction; unlike 'conventional' CARs (see chapter 2 for details). As a consequence of this we would expect them to have an optimal CD2 size which is closer to that of the TCR than to any of the CARs.

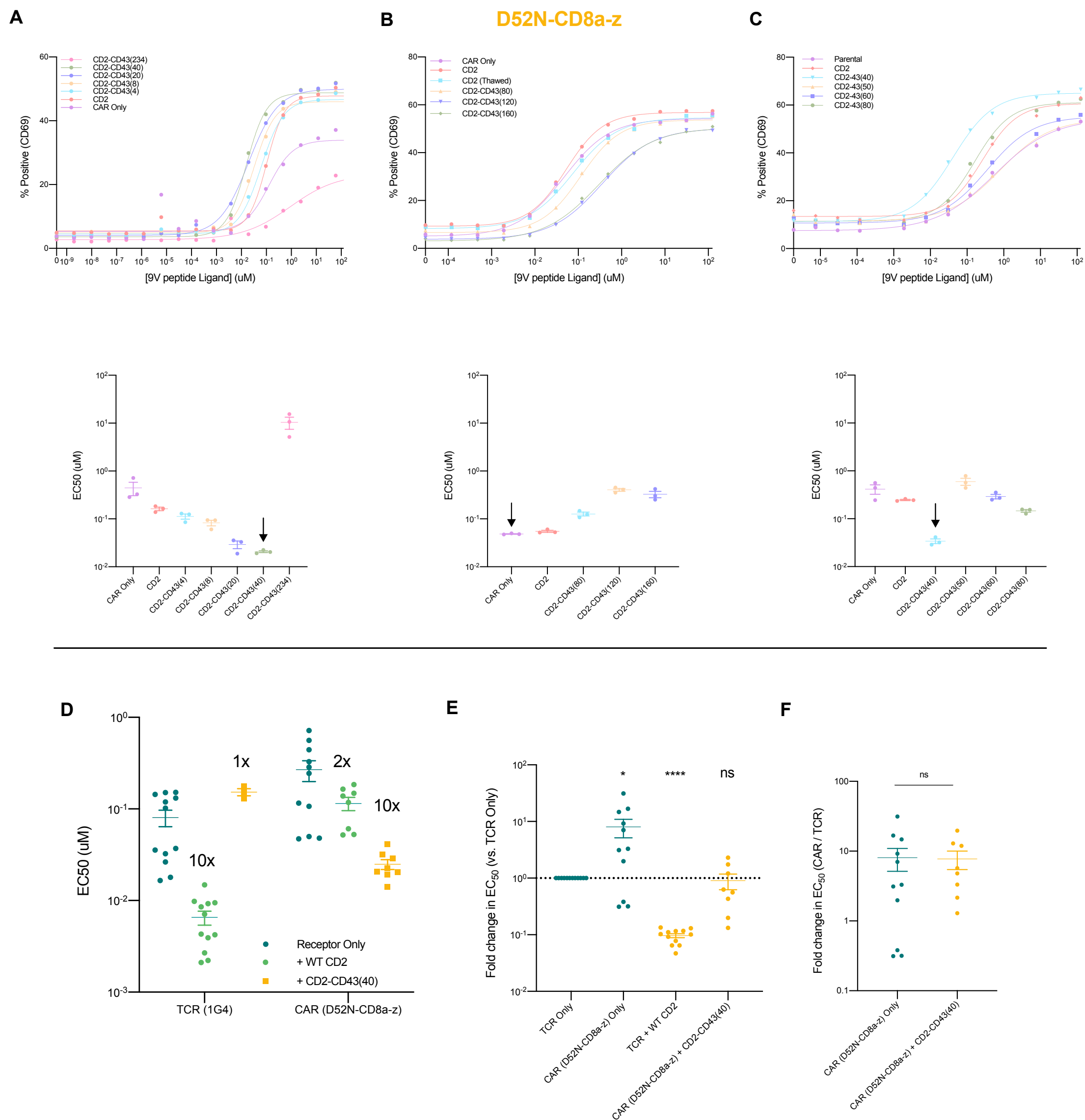


Figure 39: **A systematic screen of elongated CD2 identified CD2-CD43(40) as optimising antigen sensitivity for the CD8α hinge CAR. (A, B, C)** Representative dose-response curves (top) and extracted EC_{50} values (bottom). The data was generated by transducing one of three sets of recombinant CD2 molecules into Jurkat T cells (one set in each of A, B and C) and n = 3 independent experiments were performed on each set. Optimal CD2 size identified with an arrow. **(D)** Sensitivity, as EC_{50} values, plotted for TCR and a reference CAR (D52N-CD8a-z) when Jurkats with CD2 knocked-out ('Receptor Only') are transduced with the TCR optimal CD2 size ('WT CD2') or the optimal CD2 size for the CAR ('CD2-CD43(40)'). Fold change to receptor only condition indicated. **(E)** Fold change in sensitivity compared to Jurkats expressing only TCR and no CD2 showing CAR-T cells perform worse than TCR T-cells in the absence of the CD2-CD58 interaction, and that in the presence of CAR optimal CD2 CAR sensitivity is rescued only to that of the TCR without CD2. **(F)** Fold difference between TCR and CAR in conditions indicated, magnitude of effect of CD2-CD58 on CAR and TCR-T cells is similar but TCR-T cells remain more sensitive than CAR-Ts.

For the STAR, which is predicted to have a similar structure to the TCR, we find that the optimal size of CD2 is that of wild-type CD2 — the same as the TCR. For the eTRuC on the other hand we find that the optimal CD2 size matches that of the CD28 hinge CAR, at 20 amino acids of CD43 (Figure 40, A-B). Both the TCR and STAR constructs favour wild-type CD2 whilst all the other constructs we screened required a different size of CD2 to benefit from an improvement in sensitivity (Figure 41, A).

Effect of elongated CD2 also applies to T2 cells

So far in this study we have been assessing sensitivity using U87s, a glioblastoma cell line, as a surrogate APC. CARs, like T cells, recognise antigen on a diverse range of target cells. We therefore wanted to see if our results generalised to other cell lines with differing expression of CD58.

To do this, we repeated the screen of the CD8α hinge CAR and TCR with the previously identified optimal CD2 size for the CAR (CD2-CD43(40), wild-type CD2, full length CD2-CD43 and the CD2 size identified as the transition point at which further elongation decreases sensitivity [CD2-CD43(80)]. This time we used T2 cells, a lymphoma derived cell line, as target cells. Screening in the same way as previously, we saw that the improvements conferred by wild-type CD2 to TCR-T cells were reduced (<< 10-fold). But elongated CD2-CD43 variants still decreased sensitivity. Again, beyond that of the no CD2 condition, and consistent with the screen against U87 target cells (Figure 42, C).

Correspondingly, we again observed that CD8α hinge CAR-T cells see almost no benefit from wild-type CD2, but we were able to see an improvement in sensitivity when CD2 is elongated by 40 amino acids of CD43. As before, further elongation reduces CAR-T sensitivity (Figure 42, C). Taken together these results show that the impact of CD2 size on CAR-T cell sensitivity generalises to other cell lines.

CD2 increases macroscopic conjugate formation independent of its extracellular size

We have proposed that the improvement in antigen sensitivity mediated by elongated CD2 molecules is a result of optimal membrane alignment. Another possible explanation however is that, being an adhesion molecule, CD2 simply increases the bulk number of cell-cell contacts and thereby increases sensitivity when viewed at a population level (in this study we consider the % of cells which are CD69 positive). To control for this possibility we explored the effects of elongated CD2 on macroscopic conjugate formation.

To do this, we compared the ability of T cells to form conjugates with different target cells by loading the T cell and target cells with different dyes in order to gate for T cell/APC conjugates by flow cytometry (Figure 43, A).

We observed that the addition of wild-type CD2 or elongated variants increased conjugate formation compared to T cells lacking CD2 (Figure 43, B-C). This was the case for both TCR and CAR expressing Jurkat T cells. A reduction in conjugate formation when blocking CD2 has been observed by others [31], consistent with this result. Taken together, this provides additional evidence that elongated CD2 molecules can function to increase macroscopic adhesion; but are only able to improve antigen sensitivity when optimally sized for the antigen or antigen receptor.

Figure 40: **Different chimeric antigen receptors have different optimal sizes of CD2.** (**A**) Representative curves showing the effect of elongating CD2 with fragments of CD43 on T cell sensitivity for various CAR constructs, as indicated. (**B**) Extracted EC_{50} values from dose-response curves shown in A (n=3).

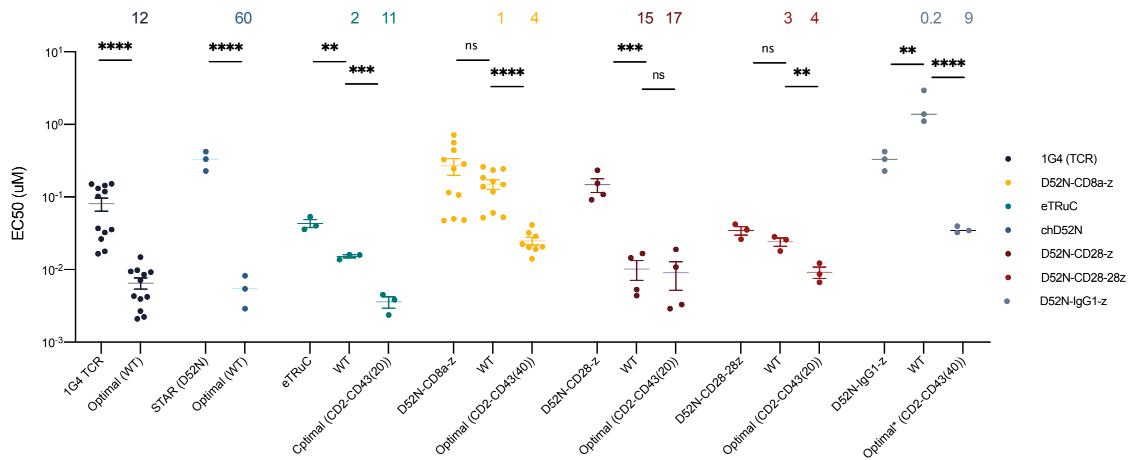


Figure 41: **Elongated CD2, but not wild-type CD2 increases the antigen sensitivity of CARs. (A)** Sensitivity plotted for all screened receptors without CD2, with wild-type CD2, and with the optimal CD2 size identified for that particular construct. Pairwise comparison between receptor only and wild-type CD2, and wild-type CD2 and optimal CD2 by Sidak's multiple comparisons test. Fold changes given between the receptor only condition and those indicated. **(B)** Mean (and standard error of the mean) of fold change in EC_{50} to the TCR only condition (dashed line) when the optimal CD2 size for each construct is used. Multiple t-test between TCR and indicated conditions with Dunnett's correction for multiple comparisons. **(F)** Fold difference between each construct at the TCR in each of the indicated conditions. Abbreviations: * = p-value≤0.05, ** = p-value≤0.01, *** = p-value≤0.001 **** = p-value≤0.0001.

Optimal CD2 size is not determined by surface expression

We noted some variation in the surface expression of the elongated CD2 receptors (Figure 44, A) so it was possible that antigen sensitivity was simply determined by surface expression rather than size of CD2. To assess this we plotted EC50 over the surface expression of CD2, finding across a range of chimeric receptors that this is not the case (Figure 44, B).

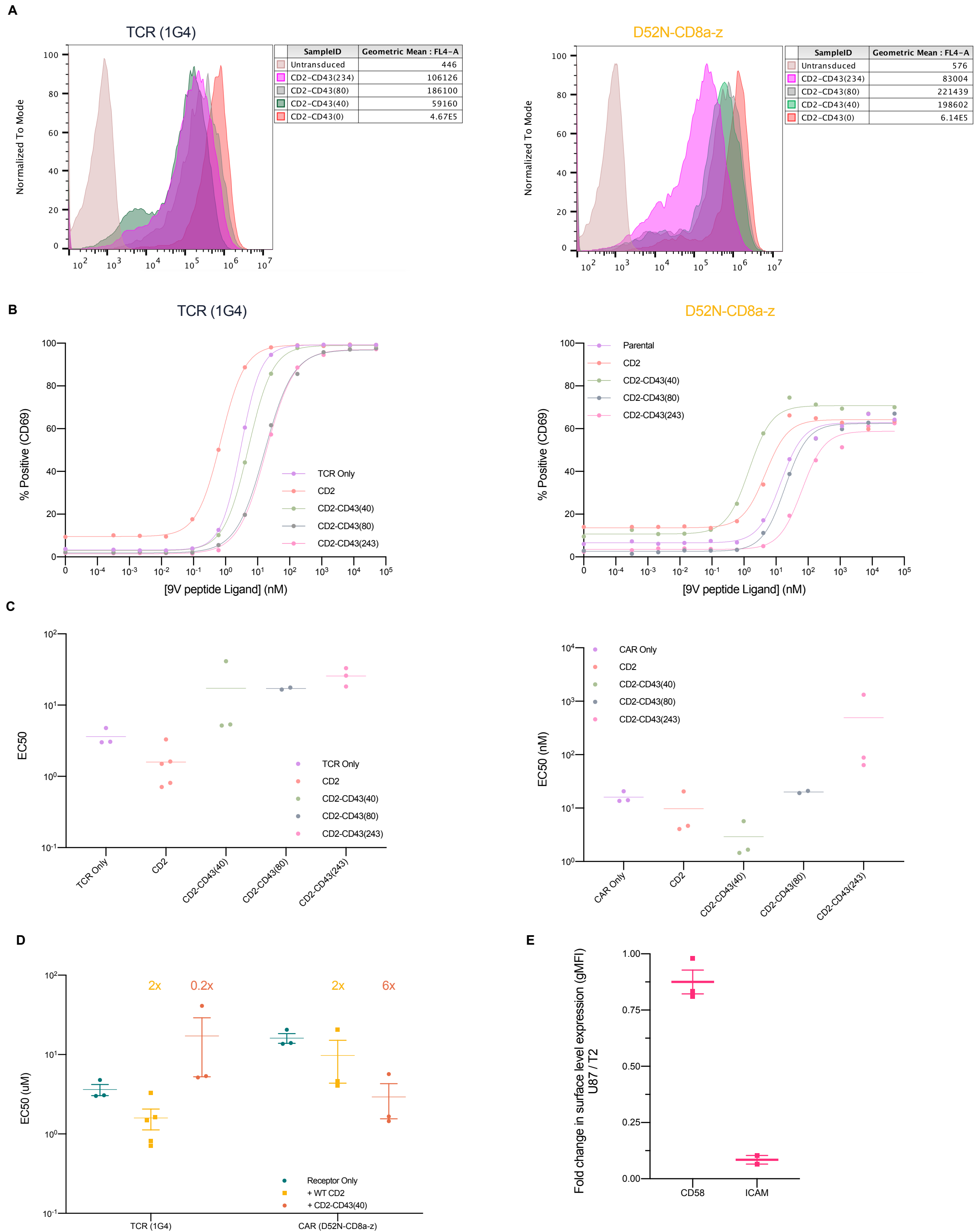


Figure 42: **Effect of elongated CD2 also applies to T2 cells.** (**A**) Representative histograms showing expression of elongated CD2 on TCR and CAR transduced T cells respectively. (**B**) Representative dose-response curves showing sensitivity of TCR (respectively CAR) T cells when transduced with different elongations of CD2. **(C)** EC_{50} values extracted from $n = 3$ dose-response assays for TCR and CAR-T cells. **(D)** EC_{50}values when either the CAR or TCR optimal sized CD2 is used. **(E)** Fold difference in surface expression of CD58 and ICAM-1 between T2 and U87 cells.

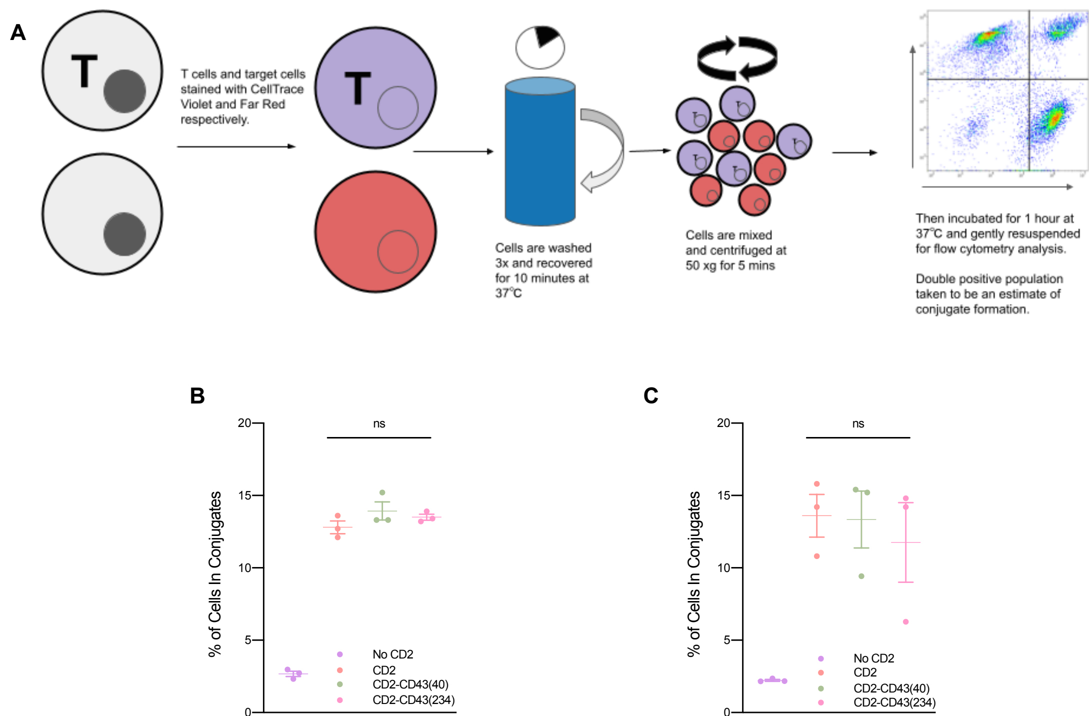
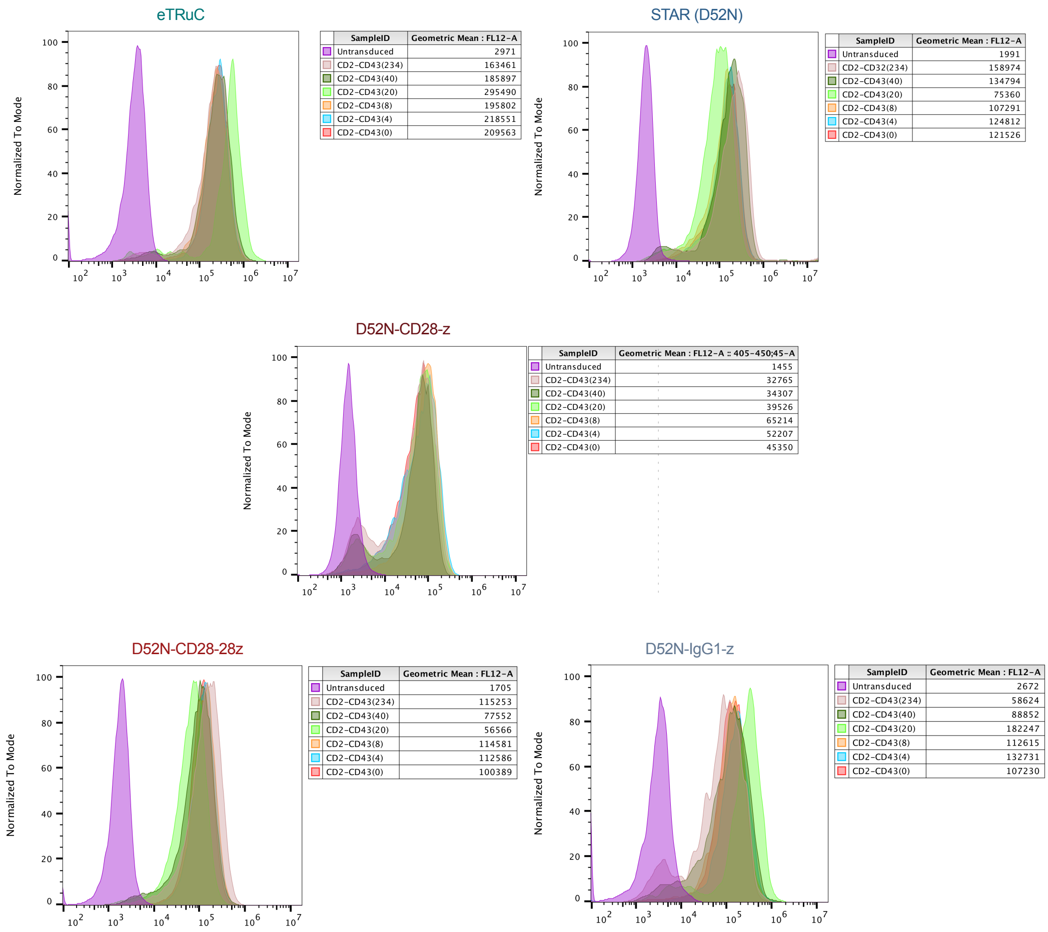


Figure 43: **CD2 increases T cell-APC macroscopic conjugate formation independent of its extracellular size. (A)** Jurkat T cells expressing the TCR or CAR (D52N-CD8α-z) transduced with different CD2 molecules are loaded with CellTrace violet dye before being incubated with APCs loaded with CellTrace far-red dye. Conjugate formation is then assessed as percentage of double positives by flow cytometry. **(B)** Proportion of Jurkat cells expressing the D52N-CD8a-z CAR in conjugates with U87 cells when expressing different variants of elongated CD2. **(C)** As (B), but with T2 target cells.

A



B

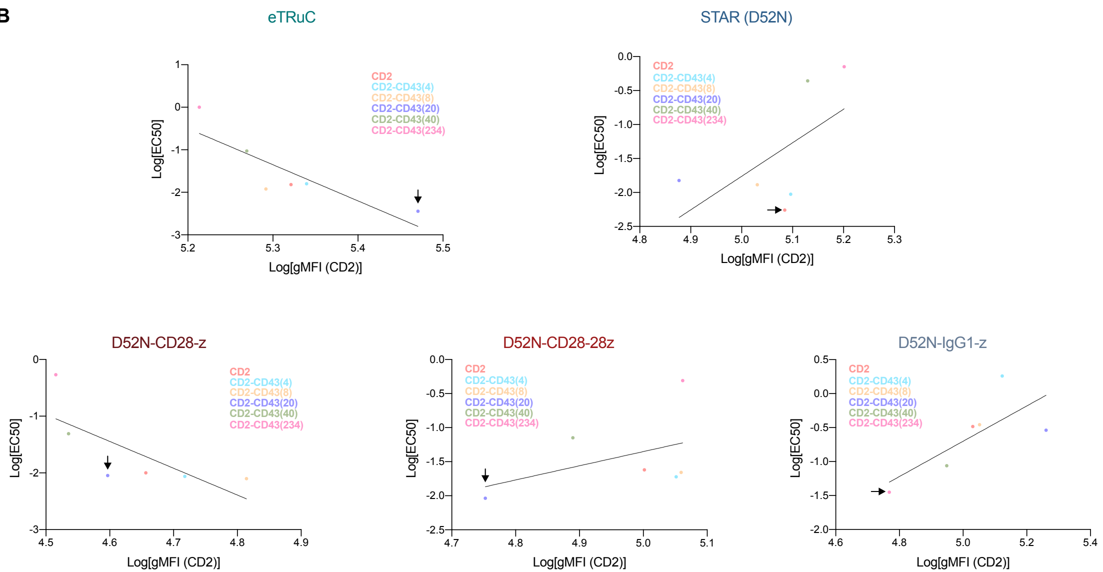


Figure 44: **Optimal CD2 size is not determined by surface expression.** **(A)** Histograms showing the expression of elongated CD2 constructs on the surface of Jurkat cells transduced with the indicated constructs together with gMFI of CD2 expression. **(B)** For the indicated constructs sensitivity ($log[EC_{50}]$) is plotted against expression levels (log[gMFI]) of differently sized CD2 molecules (coloured). The optimal, i.e, most sensitive, CD2 size is indicated with an arrow.

Discussion

In this study we have shown that differences in the extracellular size of CARs and the TCR can lead to differences in sensitivity between these two receptors. To do this we took the adhesion molecule CD2, whose interaction distance with its binding partner CD58 is known to match that of the TCR-pMHC interaction distance, and elongated it using fragments of CD43 (for which an average extent per amino acid has been previously determined). In doing so we observed that elongated CD2 reduces TCR sensitivity but contrastingly increases CAR sensitivity. We then show that different optimal CD2 sizes exist for different chimeric receptors (c.f. eTRuC vs. D52N-CD8α-z).

Taken together our data support a hypothesis where optimal alignment of the T-cell and APC membranes by CD2 increases T cell sensitivity either by stabilising the receptor-antigen interaction, increasing the effective k_{on} or excluding large phosphatases from the area around the receptor [32, 33, 34].

This suggests in turn that the mechanism for the previously observed sensitivity defect in CAR T cells compared to TCR-T cells [13, 14] is that the larger size of CARs prevents them from associating with other co-signalling molecules in an 'optimal' signalling environment, failing to efficiently engage antigen or having unstable antigen interactions. Or some combination of all of these.

We note that a previous study found that CARs more efficiently recruit antigen at low doses than do TCRs [14]. This would suggest that CARs are able to efficiently engage antigen. However in this system the only co-stimulatory molecules present were ICAM-1 and CD80 (B7.1). The latter has been shown to have limited adhesion ability, requiring CD2-CD48 (the CD2 binding partner in mice) to form interactions [35]. We would suggest that this means CD28-CD80 does not markedly constrain the intermembrane distance in this assay.

We also think that the former would create a sufficiently large (an estimated 40 nm [36]) intermembrane distance that a bulky CAR would still be able to efficiently engage antigen. Moreover, the same study also finds that a CAR with a shorter hinge region actually loses sensitivity; suggesting that the large CAR size is actually beneficial in this set-up. Consequently this experiment cannot rule out the idea that CAR size may prevent optimal antigen engagement.

Our data also suggests that the primary role for CD2 is that of modulating the physical dimensions of the T cell-APC interaction, rather than it having a signalling role. Though the two are linked, since mechanical exclusion effects are likely to be modulated by the T cells ability to 'sculpt' the immune synapse. For instance by actin remodelling; something which is associated with the CD2 intracellular region (reviewed in [5]).

We note that CAR T cells have previously been shown to form 'non-classical' immune synapses [37] suggesting that, for instance, some molecules are not excluded, or are not as effectively excluded, from the synapse than in TCR-T cells. A future set of experiments exploring how the CAR-T cell immune synapse changes in the presence of elongated adhesion molecules using

confocal microscopy may shed some light on the specific mechanism by which CD2 enhances sensitivity — whether from exclusion effects or improved receptor engagement. A related experiment using electron microscopy to measure the size of the intermembrane distance in CAR and TCR T cells in the absence of CD2 would also provide further evidence in support of our hypothesis.

Another matter for future work will be to verify that the currently identified 'optimal' CD2 size for the D52N-IgG1-z CAR is in fact the overall optimal size, or if an as yet unscreened CD2-CD43 variant increases sensitivity further for this construct. We also acknowledge that some variability in the data for the D52N-CD28-z CAR prevents us from strongly concluding that CD2-CD43(20) is the optimal size for this receptor and would like to repeat this series of experiments.

We also recognise that a limitation of this work is that it was performed in an immortalised cell line (Jurkats). These have been shown to differ in their behaviour relative to primary human T cells [38]; in particular in their expression and function of costimulatory receptors and also in the range of cytokines they release. We believe it would therefore be worthwhile to replicate these results in primary human T cells.

Our data also show that gradually elongating CD2 towards an optimal size likewise gradually increases the sensitivity before sharply reducing sensitivity again once the optimal threshold has passed. This is consistent with previous models showing that 2D affinity exhibits a maximal value at the point of optimal separation of a receptor and its ligands and is asymmetric about this point [39]. This asymmetry being the result of the ability of the receptor-ligand complexes to tilt at distances smaller than the optimum, but which would need to stretch at larger distances (the ability for which is constrained by the flexibility of the cell membrane and any anchoring forces, such as those facilitated by the actin cytoskeleton, applied to the receptor or ligand) (Figure 45).

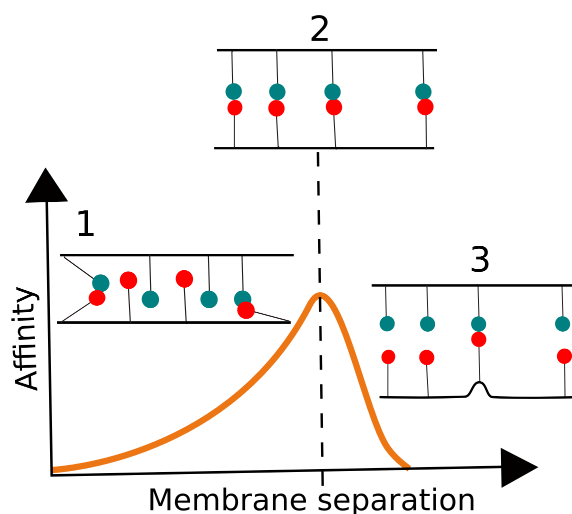


Figure 45: **A model [39] predicts asymmetric affinity for receptor-ligand binding about an optimal separation distance.** Asymmetry in receptor-ligand affinity about an optimum binding distance arises from the ability of the receptor-ligand complexes to tilt at distances smaller than the optimum, but which must stretch at larger distances. The latter, stretching, action being more unfavourable and the ability for which is constrained by the flexibility of the cell membrane and any anchoring forces applied to the receptor or ligand.

Whilst we observe that elongating CD2 to the optimal size rescues the functionality of CD2 it does not necessarily improve the sensitivity of the CAR over that of the TCR. This may suggest that other effects stemming from the other structural differences between CAR and TCR remain at play. Here we should add that the ability of the TCR to associate with the CD8 co-receptor is not responsible, as the Jurkat T cells used for this study lacked CD8.

However, other differences, such as effective ITAM multiplicity or the role of the CD3 chains, which have been explored by others previously [40, 41] may still lead to the remaining observed sensitivity defect. Additionally, of course, we have focused on matching the size of a single co-stimulatory molecule (CD2) to that of the CARs. But it may remain the case that other molecules which improve the sensitivity of the TCR still remain mismatched for the CARs.

We also note that it has recently been shown that CD2 can interact with CD58 on the same T cell, as well as other T cells. Here we only engineered CD2 such that it was optimal for APC targets, and so the effect of this set of interactions on CARs remains unexplored. The same study suggests that the ability for self-stimulation via CD2 depends on interactions between CD2, *Lck* and the TCR-CD3 complex which, like our findings, supports a spatial arrangement model for the action of CD2.

Another recent study elongated the CAR rather than CD2; they found that elongating the CAR reduced CD45 exclusion and correspondingly reduced activation. That study also found that blocking CD2 or LFA-1 had no effect on CD45 exclusion which might suggest that the CD2 and LFA-1 interactions do not set the intermembrane distance. This could be explained by antigen density and the high affinity of the CAR–antigen interaction. We suggest that at high antigen concentrations the higher affinity of the CAR–antigen interaction makes it the dominant interaction for setting intermembrane distance size and so the contribution of CD2 and LFA-1 become less prominent.

One of the proposed advantages of engineering the CD2-CD58 interaction, and part of the rationale for this work, is the widespread expression of CD58 on a range of tissue types (see the Human Protein Atlas [21]). Enabling the sensitivity of CAR-T cells to be improved against a range of target cells. This has important implications for immunotherapy as the reduced sensitivity of CAR-T cells can lead to antigen-escape associated relapse.

An obvious caveat to this, as shown in the data presented here, is that the relative effect of CD2-CD58 varies with different cell lines (c.f. the sensitivity improvement with CD58 on U87s and T2s in Figures 38 and 42 respectively). In particular we see a reduced impact on sensitivity in T2 cells despite these cells having higher CD58 levels than U87s (Figure 42, E). We note that despite the higher CD58 levels they conversely display reduced ICAM-1 levels (Figure 42, E). This may explain the reduced impact of CD58 as it is believed that ICAM-1 binds first, creating the initial T cell-antigen presenting cell contact, before CD58 is able to bind. In other words, there is a synergy between the impact of ICAM-1 and CD58. It is unclear if small differences in sensitivity observed *in vitro* would have a large effect *in vivo.*

We have shown that different optimal sizes of CD2 exist for different chimeric receptors (c.f. eTRuC vs. D52N-CD8α-z) consistent with our estimation that different chimeric receptors have different extracellular reaches. The interaction distance of a chimeric receptor with its antigen is also dependent on the epitope to which it binds. For example, the distance between an anti-CD19 CAR and CD19 is likely to be larger than between the anti-pMHC CARs here and pMHC; due to the comparatively shorter size of CD19. This is likely to necessitate a different optimal CD2 length even if the hinge region of the CAR is kept constant. The screening assay presented here allows for quick identification of the optimal CD2 size and we expect that it

could also be used to optimise CD2 for clinically relevant CARs.

Overall our data supports a hypothesis that CAR-T cells fail to capitalise on co-stimulation via the CD2-CD58 interaction because the size of the CAR is mismatched to the size of the CD2-CD58 interaction. Therefore in the presence of wild-type CD2 CARs are either unable to engage with their antigen, do not benefit from the exclusion of large phosphatases such as CD45, or are segregated into an area of the close contact region which is suboptimal for signalling (i.e., having a lower concentration of other co-signalling molecules).

References

[1] Keith Crawford et al. "CD2 engagement induces dendritic cell activation: implications for immune surveillance and T-cell activation". In: *Blood* 102.5 (2003), pp. 1745–1752.

[2] Elena V Tibaldi, Ravi Salgia, and Ellis L Reinherz. "CD2 molecules redistribute to the uropod during T cell scanning: implications for cellular activation and immune surveillance". In: *Proceedings of the National Academy of Sciences* 99.11 (2002), pp. 7582–7587.

[3] Jia-huai Wang et al. "Structure of a heterophilic adhesion complex between the human CD2 and CD58 (LFA-3) counterreceptors". In: *Cell* 97.6 (1999), pp. 791–803.

[4] P Anton van der Merwe et al. "Topology of the CD2–CD48 cell-adhesion molecule complex: implications for antigen recognition by T cells". In: *Current Biology* 5.1 (1995), pp. 74–84.

[5] Christian Binder et al. "CD2 immunobiology". In: *Frontiers in immunology* 11 (2020), p. 1090.

[6] De-Min Zhu et al. "Mechanisms of cellular avidity regulation in CD2–CD58-mediated T cell adhesion". In: *ACS chemical biology* 1.10 (2006), pp. 649–658.

[7] P Anton van der Merwe. "A subtle role for CD2 in T cell antigen recognition". In: *The Journal of experimental medicine* 190.10 (1999), pp. 1371–1374.

[8] Philippe Moingeon et al. "CD2-mediated adhesion facilitates T lymphocyte antigen recognition function". In: *Nature* 339.6222 (1989), pp. 312–314.

[9] Martin F Bachmann, Marijke Barner, and Manfred Kopf. "CD2 sets quantitative thresholds in T cell activation". In: *The Journal of experimental medicine* 190.10 (1999), pp. 1383–1392.

[10] Eddie CY Wang et al. "Suppression of costimulation by human cytomegalovirus promotes evasion of cellular immune defenses". In: *Proceedings of the National Academy of Sciences* 115.19 (2018), pp. 4998–5003.

[11] Robbie G Majzner and Crystal L Mackall. "Tumor antigen escape from CAR T-cell therapy". In: *Cancer discovery* 8.10 (2018), pp. 1219–1226.

[12] Terry J Fry et al. "CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy". In: *Nature medicine* 24.1 (2018), pp. 20–28.

[13] Daniel T Harris et al. "Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains". In: *The Journal of Immunology* 200.3 (2018), pp. 1088–1100.

[14] Venugopal Gudipati et al. "Inefficient CAR-proximal signaling blunts antigen sensitivity". In: *Nature Immunology* 21.8 (2020), pp. 848–856.

[15] Jesús A Siller-Farfán and Omer Dushek. "Molecular mechanisms of T cell sensitivity to antigen". In: *Immunological reviews* 285.1 (2018), pp. 194–205.

[16] Patricia Ho et al. "The CD58: CD2 axis is co-regulated with PD-L1 via CMTM6 and governs anti-tumor immunity". In: *bioRxiv* (2022).

[17] Livnat Jerby-Arnon et al. "A cancer cell program promotes T cell exclusion and resistance to checkpoint blockade". In: *Cell* 175.4 (2018), pp. 984–997.

[18] Xin Yan et al. "CD58 loss in tumor cells confers functional impairment of CAR T cells". In: *Blood Advances* (2022).

[19] Francisco Sanchez-Madrid et al. "Three distinct antigens associated with human T-lymphocyte-mediated cytolysis: LFA-1, LFA-2, and LFA-3." In: *Proceedings of the National Academy of Sciences* 79.23 (1982), pp. 7489–7493.

[20] Yue Liu et al. "Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors". In: *Science Translational Medicine* 13.586 (2021), eabb5191.

[21] Mathias Uhlén et al. "Tissue-based map of the human proteome". In: *Science* 347.6220 (2015), p. 1260419.

[22] Stephen E Hamby and Jonathan D Hirst. "Prediction of glycosylation sites using random forests". In: *BMC bioinformatics* 9.1 (2008), pp. 1–13.

[23] Jenny S Wong et al. "Stalk region of β-chain enhances the coreceptor function of CD8". In: *The Journal of Immunology* 171.2 (2003), pp. 867–874.

[24] Bruce Y Ma et al. "CD28 T cell costimulatory receptor function is negatively regulated by N-linked carbohydrates". In: *Biochemical and biophysical research communications* 317.1 (2004), pp. 60–67.

[25] Anne Marie Moody et al. "Developmentally regulated glycosylation of the CD8$\alpha\beta$ coreceptor stalk modulates ligand binding". In: *Cell* 107.4 (2001), pp. 501–512.

[26] Jean Philippe Boursier et al. "Evidence for an extended structure of the T-cell co-receptor CD8 alpha as deduced from the hydrodynamic properties of soluble forms of the extracellular region." In: *Journal of Biological Chemistry* 268.3 (1993), pp. 2013–2020.

[27] Martin K Wild et al. "Dependence of T cell antigen recognition on the dimensions of an accessory receptor–ligand complex". In: *The Journal of experimental medicine* 190.1 (1999), pp. 31–42.

[28] Oren Milstein et al. "Nanoscale increases in CD2-CD48-mediated intermembrane spacing decrease adhesion and reorganize the immunological synapse". In: *Journal of Biological Chemistry* 283.49 (2008), pp. 34414–34422.

[29] Patrick A Baeuerle et al. "Synthetic TRuC receptors engaging the complete T cell receptor for potent anti-tumor response". In: *Nature communications* 10.1 (2019), pp. 1–12.

[30] Jason G Cyster, David M Shotton, and Alan F Williams. "The dimensions of the T lymphocyte glycoprotein leukosialin and identification of linear protein epitopes that can be modified by glycosylation." In: *The EMBO journal* 10.4 (1991), pp. 893–902.

[31] Qian Xiao et al. "Size-dependent activation of CAR-T cells". In: *Science Immunology* 7.74 (2022), eabl3995.

[32] Simon J Davis and P Anton Van Der Merwe. "The structure and ligand interactions of CD2: implications for T-cell function". In: *Immunology today* 17.4 (1996), pp. 177–187.

[33] Simon J Davis et al. "The nature of molecular recognition by T cells". In: *Nature immunology* 4.3 (2003), pp. 217–224.

[34] Michael L Dustin et al. "Identification of self through two-dimensional chemistry and synapses". In: *Annual review of cell and developmental biology* 17.1 (2001), pp. 133–157.

[35] Shannon K Bromley et al. "The immunological synapse and CD28-CD80 interactions". In: *Nature immunology* 2.12 (2001), pp. 1159–1166.

[36] Timothy A Springer. "Adhesion receptors of the immune system". In: *Nature* 346.6283 (1990), pp. 425–434.

[37] AJ Davenport et al. "Chimeric antigen receptor T cells form nonclassical and potent immune synapses driving rapid cytotoxicity". In: *Proceedings of the National Academy of Sciences* 115.9 (2018), E2068–E2076.

[38] Rebekah R Bartelt et al. "Comparison of T cell receptor-induced proximal signaling and downstream functions in immortalized and primary T cells". In: *PloS one* 4.5 (2009), e5430.

[39] Guang-Kui Xu et al. "Binding constants of membrane-anchored receptors and ligands: a general theory corroborated by Monte Carlo simulations". In: *The Journal of chemical physics* 143.24 (2015), 12B613_1.

[40] John R James. "Tuning ITAM multiplicity on T cell receptors can control potency and selectivity to ligand density". In: *Science signaling* 11.531 (2018), eaan1088.

[41] Frederike A Hartl et al. "Noncanonical binding of Lck to CD3ε promotes TCR signaling and CAR function". In: *Nature immunology* 21.8 (2020), pp. 902–913.

Chapter 4

Discussion

Summary

The reduced sensitivity of CAR-T cells limits their effectiveness as a therapy because it can lead to the escape of antigen-low target cells; creating the subsequent possibility for relapse to occur. We sought here to identify the reason for the reduced sensitivity of CAR-T cells, and to propose a means to overcome it.

First, we confirmed previous reports showing that CARs required 100-1000 fold more antigen compared to the TCR in order to become activated [1, 2] (Figure 11). We then tested if this could be attributed, at least in part, to co-signalling receptor-ligand interactions; which have previously been reported to increase the sensitivity of the T cell receptor. We found that whilst CD2 and LFA-1 do function in CAR-T cells they do not increase their sensitivity to the same extent as they do for TCR-T cells (Figure 14).

Our results show a 125 and 22-fold increase in TCR antigen sensitivity by CD2 and LFA-1 respectively, but a less than 5 fold increase in CAR antigen sensitivity when antigen and co-signalling ligands are presented as purified proteins. We confirmed this difference in effect using APCs (Figure 16). Altogether we suggest that this failure to exploit the CD2 and LFA-1 co-signalling molecules is the predominant mechanism for the reduced sensitivity of CARs compared to the TCR.

CARs differ from the TCR both in terms of their signalling machinery and overall structure; either of which could be the root cause of their sensitivity defect. This lead us to assess the sensitivity of novel synthetic receptors whose architecture is more similar to that of the TCR. We found that synthetic receptors which contain the full complement of signalling chains found in the TCR-CD3 complex show improved sensitivity relative to the traditional CAR format, and that those which recapitulate the structure of the TCR are also able to recapitulate its sensitivity (Figure 28). We then showed that the improved sensitivity of these novel receptors is predicted by their ability to exploit co-signalling interactions via CD2 and LFA-1 (Figure 28 & 27).

Given that the full complement of TCR signalling machinery is insufficient for achieving TCR-like sensitivity, we hypothesised that a mismatch in size between the co-signalling receptor–ligand interaction and the antigen receptor–antigen interaction was the cause of the failure to exploit co-signalling molecules. We confirmed this was the case by creating elongated chimeras of the CD2 co-signalling receptor; whose interaction distance with its binding partner (CD58) is

usually matched to that of the TCR-pMHC interaction. We show that elongating CD2 increases CAR sensitivity but not TCR sensitivity (Figure 38 & 39) and that different synthetic receptors require different optimal CD2 sizes (Figure 40).

Mechanisms for the improvement in CAR sensitivity from elongated CD2

There are a number of mechanisms by which the larger size of the CAR–antigen interaction compared to that of the TCR–pMHC might reduce CAR-T cell sensitivity. Firstly, it is well established that T cells form a specialised junction with APCs called the immune synapse [3] in which the spatial and temporal arrangement of various receptors is key to their function. For instance allowing the segregation of phosphatases away from various signalling domains.

CD2 appears to initially co-localise with the TCR before segregating into microclusters and this spatial arrangement amplifies TCR signalling [4]. The mismatch between the CAR–antigen interaction and the CD2–CD58 interaction might cause the CAR and CD2 to become segregated. This could prevent efficient signalling by also segregating the intracellular signalling molecules which interact with the cytoplasmic tails of these two proteins. At the same time as being segregated from smaller molecules, the larger extracellular reach of the CAR can reduce the efficiency of their segregation from large phosphatases such as CD45 [5].

Adhesion molecules like CD2 provide the energy needed to bring the T cell and antigen presenting cells sufficiently close together that antigen recognition can occur. Whilst CD2–CD58 creates the optimal intermembrane distance for TCR–pMHC binding, this can create suboptimal conditions for CAR–antigen binding. For pMHC antigens (as in our case), a large local concentration of bound (wildtype) CD2 would require larger CARs to enter a less extended conformation, or to change their orientation relative to the membrane in order to bind. This is predicted to be more unfavourable [6] than if the membranes were at a larger separation, such as that provided by our elongated CD2, and this energetic penalty would result in a reduced level of overall CAR engagement.

The correct alignment of the T cell and APC membranes may also be important for cooperative binding of both the antigen receptor and various co-signalling molecules present on the T cell surface. Co-operative binding is predicted between TCR–self-pMHC and TCR–foreign-pMHC interactions [7]. When the CD2 and CAR are matched in the dimensions of their corresponding interactions then binding of either promotes the engagement of the other by creating local regions of the membrane that are at the optimal distance for efficient binding [8].

Contrastingly, when CD2 and the CAR are mismatched in size their binding may create a locally 'rough' membrane which, in aggregate, reduces the amount of the membrane that is at the optimal binding distance not only for the CAR and CD2 but also for the various other co-signalling receptors to engage — potentially reducing the engagement of them all. Matched CD2 and CAR interaction distances may also promote stability of the CAR–antigen interaction by sharing the force load from the compression of the glycocalyx (which would oppose receptor–antigen binding) over more molecules.

In all likelihood it is not a single mechanism, but rather several of these effects that lead to improved antigen sensitivity when CAR–antigen and CD2–CD58 interaction distances are matched. Possible investigations into the precise mechanism of action for CD2 are discussed in the section on further work.

Implications

Our findings have important implications for the field of adoptive cell therapy. They highlight the importance of co-signalling molecules and, in particular, our results show the importance of adhesion molecules in T cell sensitivity. Being able to increase T cell sensitivity is of critical importance in preventing the relapse of liquid tumours. These relapses occur because poor sensitivity leads to antigen-low cells evading killing by CAR-T cells, a process called 'antigen escape' [9]. Although there are also situations where it is in fact desirable to have low antigen sensitivity. This is the case when antigen is expressed at high levels on cancer cells but at low levels on healthy tissue. In such a situation, highly sensitive CAR-T cells would lead to 'on-target off-tumour toxicity'.

What is needed therefore, is a means to tune the sensitivity of CAR-T cells. Our results suggest a new approach for tuning the sensitivity of CAR-T cells by exploiting the interplay between CARs and adhesion molecules. For increasing the sensitivity, and preventing relapse, we propose engineering shorter CARs which are able to capitalise on adhesion interactions from CD2 and LFA-1 or to simultaneously endow these cells with optimally sized co-signalling receptors. A proposed approach for reducing sensitivity, and thereby controlling on-target off-tumour toxicity, is to alter the affinity of the CAR [10]. Here we would suggest using a longer hinge region when reduced sensitivity is desired.

Another approach which has been suggested for tuning the sensitivity of CAR-T cells is the use of gene circuits whereby low affinity receptors are used to filter out cells with low antigen density (as they require a high density to overcome the low affinity) and trigger the upregulation of high affinity receptors that can induce killing and proliferation [11]. This is a more complex approach as the cells must be carefully controlled to eliminate any 'leaky expression' — that is, cells which express the CAR constitutively, as these could still cause toxicity. In addition the two-step receptor cascade delays the onset of tumour killing. It is not clear if this would have an impact *in vivo* where the cells can migrate. Finally this approach does not eliminate the need to alter the affinity of the CAR, indeed careful engineering of multiple scFvs is required as the low affinity (Notch) receptor and high affinity CAR have to be carefully tuned in order to set the sensitivity threshold.

Our approach has several advantages. First, as the antigen binding region is not modified, the risk of inadvertently altering the specificity of the CAR is reduced. Second, the advantage of this approach over those which require the structure of the TCR–CD3 complex (such as the HIT/STAR [12, 13]) is that it is applicable to a wider range of cell types, including those which do not express the CD3 chains. Indeed there has been recent interest in engineering other immune cells, like macrophages [14] and NK cells [15] in the same way as we engineer CAR-T cells today.

Tuning the cells by matching the CAR size to the co-signalling receptor size as we have suggested may also increase the applicability of CAR-T cell therapy for solid tumours; an area where they have traditionally performed poorly. In particular it creates the possibility of targeting large antigens with a high sensitivity, creating more options for targeting CAR-T cells to tumour cells. For example, it creates the possibility of targeting large glycoproteins that are deferentially glycosylated on cancer cells. MUC-1 for instance, which extends 200 nm from the cell surface [16], and has been proposed as a selective CAR target [17].

One of the reasons that CAR-T cells perform poorly in solid tumours is their poor persistence and tumour infiltration [18]. Increased antigen sensitivity could help overcome poor infiltration as it increases the chance that even a few infiltrating CAR-T cells would be able to locate their

target antigen. Poor persistence may also be a result of CAR-T cells migrating away after failing to locate their cognate antigen, and increasing the sensitivity might therefore allow CARs to recognise their target and subsequently persist in the tumour.

Improving the sensitivity of CARs also has the potential to reduce toxicity, as it may mean that fewer, more sensitive cells, can be given to patients. This would lead to a corresponding reduction in the amount of cytokines produced and thus a reduced risk of cytokine release syndrome.

Finally, CAR-T exhaustion, another factor limiting their efficacy is thought to be due to persistent antigen stimulation [19]. This arises when the antigen presenting cells are not efficiently cleared and can happen if the CAR-T cells are not sensitive enough that they can clear all the antigen presenting cells. It follows that improving sensitivity may also reduce CAR-T cell exhaustion by removing this persistent stimulation.

Although we have identified that CARs fail to capitalise on co-signalling molecules, and that this is due to a mismatch in the size of these interactions compared to the CAR–antigen interaction, we have not elucidated the precise mechanism behind this. To do so will be the topic of further work.

Further work

There are a number of possible mechanisms which could lead to the failure of CARs to exploit co-signalling molecules:

- CARs fail to co-localise with co-signalling molecules in the close-contact region leading to inefficient signalling.
- Binding of either the co-signalling receptor or the CAR prevents the effective engagement of the other.
- Inherently inefficient signalling by CARs prevents optimal co-signalling receptor behaviour.

These could be addressed with further experiments. We propose using electron microscopy to measure the intermembrane distance between T cells and APCs when the T cells express either the CAR or a TCR, with and without co-stimulation. This would allow us to ascertain which of these interactions is dominant in setting the size of the close-contact region. Additional light microscopy could be used to determine if CARs and co-signalling molecules are able to co-localise in the close contact region, or if they are segregated. This would help determine if it is the composition of co-signalling molecules in the local area of the receptor that results in enhanced sensitivity.

When comparing our cellular and solid-phase assays we note that the TCR still displays superior sensitivity compared to CARs even when the CD2 and LFA-1 interactions are blocked. This suggests that there are other co-signalling molecules which are able to compensate for the loss of CD58 and ICAM-1. Identifying these molecules may improve our understanding of T cell biology as well as provide further targets for the rational engineering of optimal co-signalling molecules (as we did in chapter 3). We also note that in this study we have considered the impacts of co-signalling molecules primarily in isolation, it remains to be explored whether there is any synergy or cooperativity between groups of co-signalling molecules.

Despite recent strides in our ability to predict protein structures, it still remains a challenge to use this information to inform the rational design of CARs. Partially this stems from the

use of largely unstructured linkers or hinge regions in the design of many CARs. Consider the predicted structure of the εTRuC shown in Figure 46, the linker region is predicted with low confidence, suggesting it is unstructured and the relative positioning of the scFv domain (residues 1-251) relative to extracellular region of CD3ε (residues 304-407) has a high error (see Figure 46, 'predicted alignment error'). The final conformation is additionally constrained by the other TCR chains and the presence of the cell membrane, whose impacts cannot be predicted.

Given that we have shown here the importance of receptor size in determining sensitivity we suggest that future CAR designs would benefit from tighter control over their extent and conformation so that better inferences can be made about their behaviour. Recent advances in the field of *de novo* protein engineering, such as self-stabilising helices [20] may prove useful in this regard.

Our results contribute to the existing knowledge on the mechanism of CD2, suggesting that the spatial constraints imposed by its interaction with its ligand are key to its function. There are several mechanisms for CD2 function that are related to its size which could be investigated further. For one, it has recently been proposed that part of the mechanism of the action for CD2 is that it shields the antigen receptor from forces. There is evidence that T cells generate forces whilst scanning APCs for antigen and that these forces are experienced by the TCR and other T cell surface molecules (such as LFA-1) [24, 25, 26].

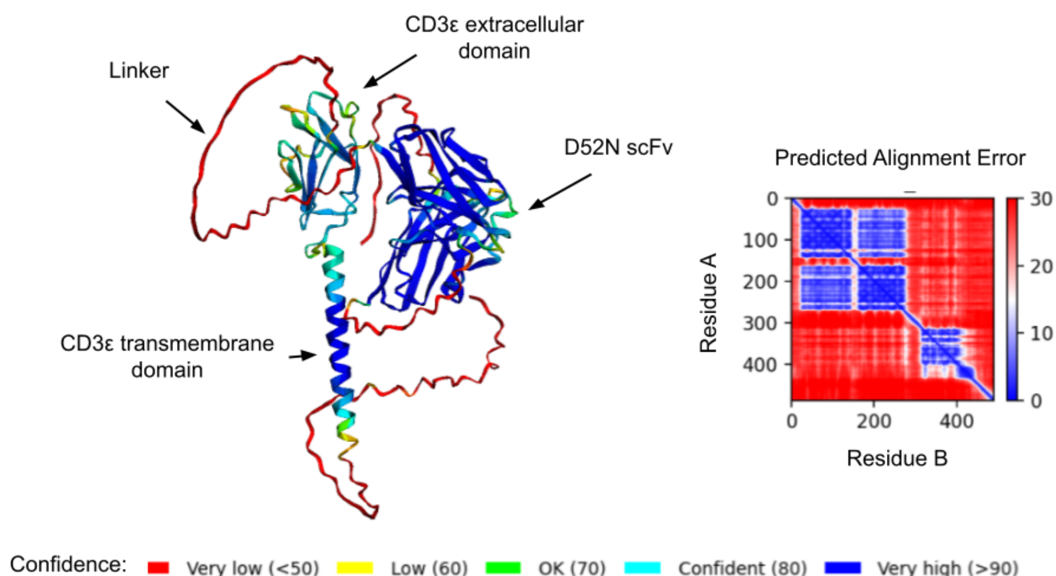


Figure 46: **The conformation of CARs in situ is hard to determine due to the use of long unstructured regions**. The eTRuC is shown here as the scFv linked to the CD3ε chain in isolation as predicted by AlphaFold [21, 22]. The linker between the scFv and ε-chain are predicted to be unstructured as indicated by their very low confidence score (confidence score corresponds to lDDT [23]). The predicted alignment error (right) is the predicted error in the distance between the residues (x, y) when the y axis residue is assumed to be in the correct position.

For example, TCR binding requires that the large molecules of the glycocalyx (e.g., CD45, CD43, &c.) are compressed to allow the TCR and pMHC to come into contact with each other. This creates a force which opposes TCR-pMHC binding and increases the receptor off-rate (k_{off}) [27]. It has been proposed that the TCR can be shielded from these forces by adhesion molecules such as CD2 and LFA-1 taking some of the strain; thus reducing the receptor off-rate. A model shows that increasing the force on the TCR–pMHC interaction reduces antigen sensitivity, but that a force shielding effect can only partially explain the enhanced sensitivity that occurs as a result of the CD2 and LFA-1 interactions [28]. Actual measurements of the forces experienced by the TCR, or indeed other antigen receptors such as CARs, in the presence of CD2–CD58 remain to be performed.

The (2D) affinity of a receptor–ligand interaction is dependent on the average membrane separation. When the membrane separation is comparable to the length of the receptor–ligand complex then the affinity is maximal [6]. Consequently, the larger the fraction of the cell membrane which is at a separation within the binding range of the receptor–ligand interaction the more likely it is that a binding interaction will occur. Because receptor–ligand binding increases the fraction of the membrane which is at the correct distance this binding facilitates the formation of further receptor–ligand complexes in a co-operative fashion [8].

This also applies between pairs of receptors and their ligands when they have similar interaction distances. For example CD2–CD58 and TCR–pMHC, which share an interaction distance of approximately 14 nm [29], are likely to display a this cooperativity. That is, binding of CD2 is likely to promote further TCR binding. This is supported experimentally by the observation that CD2 enhances TCR downmodulation, which is suggestive of engagement [30] and the observation that whilst wild-type CD2 is able to increase antigen recognition, the elongation of the CD2–CD48 interaction in mice inhibits antigen recognition [31].

The relationship between elongation of CD2–CD58 and the biophysical parameters of the TCR–pMHC or CAR–antigen interactions is unknown, and it may prove instructive to model how the on-rate (k_{on}) of the antigen receptor varies based on the changes in intermembrane distance. Particularly as knowing this relationship would allow for a new means of precisely tuning receptor affinity.

Several studies have found that CARs appear to have defective signalling, showing reduced ITAM [2], and LAT [32], phosphorylation. This may be a result of an inability to localise with co-signalling molecules that recruit *Lck*. CD2, for instance, has been shown to recruit *Lck* [33] and is suggested to link *Lck* with the TCR-CD3 complex [34]. Conversely, the defective signalling may be a cause, rather than a consequence of failing to capitalise on co-signalling. It will be important to disentangle these aspects if we are to rationally engineer better receptors. More generally, our finding that receptor and ligand size is important must be squared with the obvious signalling capacity of the various co-signalling receptors. It is likely these are linked and, as with all proteins, the function of co-signalling molecules follows their form.

Conclusion

Overall the work in this thesis shows that it is possible to engineer chimeric receptors which have the same sensitivity as the TCR, and that achieving this level of sensitivity requires an ability to exploit the adhesion molecules CD2 and LFA-1. In particular we found that the ability to exploit the CD2-CD58 interaction is determined by the size of the chimeric receptor and, consequently, that it is possible to engineer chimeric adhesion molecules which increase the sensitivity of CARs.

Although we have identified a possible reason why CD2 fails to improve CAR sensitivity as much as it does TCR sensitivity, the precise biological mechanism remains an open question. The answer to which could, perhaps, be left as an exercise for the reader.

References

[1] Daniel T Harris et al. "Comparison of T cell activities mediated by human TCRs and CARs that use the same recognition domains". In: *The Journal of Immunology* 200.3 (2018), pp. 1088–1100.

[2] Venugopal Gudipati et al. "Inefficient CAR-proximal signaling blunts antigen sensitivity". In: *Nature Immunology* 21.8 (2020), pp. 848–856.

[3] Michael L Dustin. "The immunological synapse". In: *Cancer immunology research* 2.11 (2014), pp. 1023–1033.

[4] Philippos Demetriou et al. "A dynamic CD2-rich compartment at the outer edge of the immunological synapse boosts and integrates signals". In: *Nature immunology* 21.10 (2020), pp. 1232–1243.

[5] Qian Xiao et al. "Size-dependent activation of CAR-T cells". In: *Science Immunology* 7.74 (2022), eabl3995.

[6] Thomas R Weikl et al. "Binding equilibrium and kinetics of membrane-anchored receptors and ligands in cell adhesion: Insights from computational model systems and theory". In: *Cell Adhesion & Migration* 10.5 (2016), pp. 576–589.

[7] Bartosz Różycki and Thomas R Weikl. "Cooperative Stabilization of Close-Contact Zones Leads to Sensitivity and Selectivity in T-Cell Recognition". In: *Cells* 10.5 (2021), p. 1023.

[8] Thomas R Weikl et al. "Adhesion of membranes via receptor–ligand complexes: Domain formation, binding cooperativity, and active processes". In: *Soft Matter* 5.17 (2009), pp. 3213–3224.

[9] Robbie G Majzner and Crystal L Mackall. "Tumor antigen escape from CAR T-cell therapy". In: *Cancer discovery* 8.10 (2018), pp. 1219–1226.

[10] Esther Drent et al. "A rational strategy for reducing on-target off-tumor effects of CD38-chimeric antigen receptors by affinity optimization". In: *Molecular therapy* 25.8 (2017), pp. 1946–1958.

[11] Rogelio A Hernandez-Lopez et al. "T cell circuits that sense antigen density with an ultrasensitive threshold". In: *Science* 371.6534 (2021), pp. 1166–1171.

[12] Zelig Eshhar et al. "Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the gamma or zeta subunits of the immunoglobulin and T-cell receptors." In: *Proceedings of the National Academy of Sciences* 90.2 (1993), pp. 720–724.

[13] Yue Liu et al. "Chimeric STAR receptors using TCR machinery mediate robust responses against solid tumors". In: *Science Translational Medicine* 13.586 (2021), eabb5191.

[14] Michael Klichinsky et al. "Human chimeric antigen receptor macrophages for cancer immunotherapy". In: *Nature biotechnology* 38.8 (2020), pp. 947–953.

[15] Guozhu Xie et al. "CAR-NK cells: A promising cellular immunotherapy for cancer". In: *EBioMedicine* 59 (2020), p. 102975.

[16] Poshmaal Dhar and Julie McAuley. "The role of the cell surface mucin MUC1 as a barrier to infection and regulator of inflammation". In: *Frontiers in cellular and infection microbiology* 9 (2019), p. 117.

[17] Claudia Rossig et al. "Carbohydrate targets for CAR T cells in solid childhood cancers". In: *Frontiers in oncology* (2018), p. 513.

[18] Faroogh Marofi et al. "CAR T cells in solid tumors: challenges and opportunities". In: *Stem cell research & therapy* 12.1 (2021), pp. 1–16.

[19] Taku Kouro, Hidetomo Himuro, and Tetsuro Sasada. "Exhaustion of CAR T cells: potential causes and solutions". In: *Journal of Translational Medicine* 20.1 (2022), pp. 1–10.

[20] Gabriella Collu et al. "Chimeric single α-helical domains as rigid fusion protein connections for protein nanotechnology and structural biology". In: *Structure* 30.1 (2022), pp. 95–106.

[21] John Jumper et al. "Highly accurate protein structure prediction with AlphaFold". In: *Nature* 596.7873 (2021), pp. 583–589. doi: 10.1038/s41586-021-03819-2.

[22] Milot Mirdita et al. "ColabFold: making protein folding accessible to all". In: *Nature Methods* (2022), pp. 1–4.

[23] Valerio Mariani et al. "lDDT: a local superposition-free score for comparing protein structures and models using distance difference tests". In: *Bioinformatics* 29.21 (2013), pp. 2722–2728.

[24] Julien Husson et al. "Force generation upon T cell receptor engagement". In: *PloS one* 6.5 (2011), e19680.

[25] Victor Pui-Yan Ma et al. "The magnitude of LFA-1/ICAM-1 forces fine-tune TCR-triggered T cell activation". In: *Science advances* 8.8 (2022), eabg4485.

[26] Janett Göhring et al. “Temporal analysis of T-cell receptor-imposed forces via quantitative single molecule FRET measurements”. In: *Nature communications* 12.1 (2021), pp. 1–14.
[27] Jun F Allard et al. “Mechanical modulation of receptor-ligand interactions at cell-cell interfaces”. In: *Biophysical Journal* 102.6 (2012), pp. 1265–1273.
[28] Johannes Pettmann et al. “Mechanical forces impair antigen discrimination by reducing differences in T cell receptor off-rates”. In: *bioRxiv* (2022).
[29] P Anton van der Merwe et al. “Topology of the CD2–CD48 cell-adhesion molecule complex: implications for antigen recognition by T cells”. In: *Current Biology* 5.1 (1995), pp. 74–84.
[30] Martin F Bachmann, Marijke Barner, and Manfred Kopf. “CD2 sets quantitative thresholds in T cell activation”. In: *The Journal of experimental medicine* 190.10 (1999), pp. 1383–1392.
[31] Martin K Wild et al. “Dependence of T cell antigen recognition on the dimensions of an accessory receptor–ligand complex”. In: *The Journal of experimental medicine* 190.1 (1999), pp. 31–42.
[32] Patrick A Baeuerle et al. “Synthetic TRuC receptors engaging the complete T cell receptor for potent antitumor response”. In: *Nature communications* 10.1 (2019), pp. 1–12.
[33] Bin Li et al. “Cis interactions between CD2 and its ligands on T cells are required for T cell activation”. In: *Science Immunology* 7.74 (2022), eabn6373.
[34] Arshad Muhammad et al. “Sequential cooperation of CD2 and CD48 in the buildup of the early TCR signalosome”. In: *The Journal of Immunology* 182.12 (2009), pp. 7672–7680.

Chapter 5

Materials & methods

Peptides

Peptides were synthesised at a purity of >95% (Peptide Protein Research, UK). 9V refers to a peptide derived from NY-ESO$_{157-165}$ (SLLMWITQV), 4A is derived from the same sequence (SLLAWITQV), and SL9 refers to a peptide from HIV p17 GAG$_{77-85}$ (SLYNTVATL).

Protein production

HLA-A*02:01 heavy chain (UniProt residues 25–298) with a C-terminal BirA tag and β$_2$-microglobulin were expressed as inclusion bodies in *E.coli*, refolded *in vitro* as described in [1] together with the relevant peptide variants, and purified using size-exclusion chromatography on a Superdex S75 column (GE Healthcare, USA) in HBS-EP buffer (10 mM M HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v Tween-20). Purified pMHC was biotinylated using the BirA enzyme (Avidity, USA).

His-tagged, soluble extracellular domain (ECD) of human CD58 was produced either in Freestyle 293F suspension cells (Thermo Fisher) or adherent HEK 293T cells. His-tagged, soluble versions of the ECD of human ICAM1, 41BBL, CD70 and CD86 were produced using adherent HEK 293T cells. Freestyle 293F suspension cells were transfected using Freestyle MAX reagent, as previously reported [2]. Adherent HEK 293T cells were transfected using Roche X-tremeGENE HP transfection reagent following the manufacturer's protocol. In both cases the resulting supernatant was filtered with a 0.45 μm filter and proteins were then purified using Ni-NTA agarose columns. Biotinylation was either performed *in vitro* after purification, or *in situ* by co-transfection (final proportion 10%) of a secreted BirA and adding 100 μM D-biotin to the growth media. Further purification and excess biotin removal was performed by size exclusion chromatography in HBS-EP.

D52N chains were produced as inclusion bodies in E. coli and refolded in vitro as described in [3], except that inclusion bodies were solubilised in 20 mM Tris-HCl (pH 8.0), 8 M urea, 2 mM DTT, refolding buffer contained 150 mM Tris-HCl (pH 8.0), 3 M urea, 200 mM Arg-HCl, 0.5 mM EDTA, 0.1 mM PMSF, and the refolding mixture was dialysed against 10 mM Tris-HCl (pH 8.5). The D52N dimer was purified on anion-exchange chromatography on a HiTrap Q column, followed by size-exclusion chromatography on a Superdex S200 column (both from GE Healthcare).

All purified proteins were aliquoted and stored at −80 °C until use.

Lentiviral production

HEK 293T cells were seeded in DMEM supplemented with 10% FBS and 1% pen-strep in 6-well plates to reach 60–80% confluency on the following day. Cells were transfected with 0.25 µg pRSV-Rev (Addgene, #12253), 0.53 μg pMDLg/pRRE (Addgene, #12251), 0.35 μg pMD2.G (Addgene, #12259), and 0.8 μg of transfer plasmid using 5.8 µL X-tremeGENE HP (Roche). Media was replaced after 16 hours and supernatant harvested after a further 24 hours by filtering through a 0.45 µm cellulose acetate filter. Supernatant from one well of a 6-well plate was used to transduce 1 million T cells.

T cell production

Human $CD8^+$ T cells were isolated from leukocyte cones purchased from the National Health Service's (UK) Blood and Transplantation service. Isolation was performed using negative selection. Briefly, blood samples were incubated with Rosette-Sep Human $CD8^+$ enrichment cocktail (Stemcell) at 150 µL/mL for 20 minutes. This was followed by a 3.1 fold dilution with PBS before layering on Ficoll Paque Plus (GE) at a 0.8:1.0 ficoll to sample ratio. Ficoll-Sample preparation was spun at 1200 x*g* for 20 minutes at room temperature. Buffy coats were collected, washed and isolated cells counted. Cells were resuspended in complete RMPI (RPMI supplemented with 10% v/v FBS, 100 Units/mL penicillin, 100 µg/mL streptomycin) with 50 U/mL of IL-2 (PeproTech) and CD3/CD28 Human T-activator Dynabeads (Thermo Fisher) at a 1:1 bead to cell ratio. At all times isolated human $CD8^+$ T cells were cultured at 37 °C and 5% CO_2.

1 million cells in 1 mL of media were subsequently transduced on the following day using lentivirus encoding for the various constructs (e.g., TCR), per the section on lentiviral transduction. On days 2 and 4 post-transduction, 1 mL of media was exchanged and IL-2 was added to a final concentration of 50 U/mL. Dynabeads were magnetically removed on day 5 post-transduction. T cells were further cultured at a density of 1 million cells/mL and supplemented with 50 U/mL IL-2 every other day. T cells were used between 10 and 16 days after transduction.

APC stimulation (co-culture with T2 cells)

T2 cells were stained with 5 µM Tag-It Violet (BioLegend) following the manufacturer's protocol and then 60000 cells were seeded in a volume of 100 µL per well in a V-bottom 96 well tissue culture plate. T2 cells were then incubated with 100 µL of peptide dilution prepared to the desired concentration in complete RPMI for 1 hour at 37 °C. T2 cells were then washed, resuspended in 100 µL of complete RPMI and transferred to a flat-bottom 96 well tissue culture plate.

Primary T cells were counted and re-suspended in fresh media such that there were 30000 receptor positive cells per 100 µL. This volume was then added to the T2 cells transferred previously.

As controls for the LDH assay additional wells were prepared in triplicate containing only 30000 T cells for each construct, or only 60000 T2 cells. Both with media to the same final volume as the co-cultured cells. Triplicate wells serving as volume correction and media controls were also prepared.

Plates were then spun at 50 x*g* for 2 minutes and incubated for 8 hours at 37 °C. After this period plates were spun again at 50 x*g* for 2 minutes and a fraction of supernatant was removed for assessing LDH release. LDH release was assessed using CyQUANT LDH Cytotoxicity Assay Kits (Thermo Fisher) following the manufacturers protocol. EDTA was added to the remaining supernatant (final concentration 2.5 µM) and cells were detached by pipetting.

Cells were stained for CD69 (Clone FN50, dilution 1:200) as well as with pMHC tetramers (dilution 1:500). Stained cells were either analysed immediately or fixed with 1% formaldehyde in PBS and analysed on the following day.

T cells were discriminated from T2 cells by the absence of Tag-It Violet stain. Single T cells were identified on the basis of size and subsequent analysis performed on this population.

Solid-phase plate stimulation

Pierce Streptavidin Coated High Capacity 96 well plates (Thermo Fisher) were washed with PBS and dilutions of biotinylated pMHC in PBS were added to each well in a 50 µL volume and incubated for 90 minutes at room temperature. Subsequently, plates were washed again with PBS and biotinylated accessory molecules (CD58, ICAM-1, CD86, CD70, 41BBL) were added at a fixed dose of 250 ng/well in 50 µL. Plates were again incubated for 90 minutes and then washed with PBS.

T cells were counted, washed in media and 75000 cells in 200 µL were dispensed per well, plates were spun for 2 minutes at 50 x*g* and then incubated for 24 hours at 37 °C. Following this incubation a portion of supernatant was removed and stored for performing ELISAs. EDTA was added to the remaining supernatant (final concentration 2.5 mM) and cells were detached by pipetting. Collected cells were stained for CD45 (Clone HI30, dilution 1:200), CD69 (Clone FN50, dilution 1:200), 4-1BB (Clone 4B4-1, dilution 1:200) and with tetrameric PE-conjugated pMHC. Cells were analysed either immediately or 1 day later, following fixation with 1% formaldehyde in PBS. In a separate assay cells were alternatively stained for CD2 (Clone TS1/8, dilution 1:200), LFA-1 (Clone HI111, dilution 1:200) and tetrameric PE-conjugated pMHC.

Generating U87 knockout cell lines

U87 cells (a kind gift of Vincenzo Cerundolo) were used to generate genetic knockouts for CD58, ICAM1, or both using CRISPR Cas9 RNP transfection. To generate CD58 KO cells, 50,000 U87 cells were seeded in a 24-well plate and transfected the next day using Lipofectamine CRISPRMAX Cas9 Transfection agent (Thermo Fisher), annealed crRNA:tracrRNA (TrueGuide CRISPR758411_CR, `GTCAATGCACAAGTTAGTGT`, Thermo Fisher; A35506 for tracrRNA, Thermo Fisher), and TrueCut Cas9 Protein v2 (Thermo Fisher, A36496) according to manufacturer's instructions. Cells were sorted by FACS and this mixed population was used for all experiments. Sorted CD58 KO cells or WT U87 cells were used to generate CD58/ICAM1 double KO cells or ICAM1 KO cells, respectively using the same protocol as above. Specifically, cells were transfected with crRNA:tracrRNA (TrueGuide CRISPR845351_CR, `GCTATTCAAACTGCCCTGAT`, Thermo Fisher) and subsequently sorted by FACS. Accutase (Biolegend 423201) was used to dissociate cells before screening or sorting with anti-CD58 (TS2/9, Invitrogen 12-0578-42) or anti-ICAM1 (HA58, Biolegend 353114) to prevent potential digestion of CD58 or ICAM1 by trypsin. All cell lines showed similar expression of HLA-A2 by flow cytometry (clone BB7.2, Biolegend 343306).

APC stimulation (co-culture with U87 cells)

25000 U87 cells were seeded in a tissue culture treated flat-bottom 96 well plate and grown overnight. On the following day the media was removed from these cells and they were incubated with peptides prepared to the appropriate concentration in complete DMEM (DMEM supplemented with 10% v/v FBS, 100 Units/mL penicillin, 100 μg/mL streptomycin) for 1 hour at 37 °C.

If blocking antibodies were used then the appropriate amount of T cells were incubated for 30 minutes prior to addition to the U87 cells with either anti-IgG1 κ Isotype control (BioLegend, Clone MOPC-21), anti-CD58 (BioLegend, Clone TS2/9) or anti-ICAM1 (eBioscience, Clone HA58) at a concentration of 10 μg/mL. Alternatively, both anti-CD58 and anti-ICAM1 together at a concentration of 5 μg/mL each (total antibody concentration 10 μg/mL).

Peptide containing media was then removed and 50,000 T cells per well were added. The co-culture was then spun for 2 minutes at 50 x*g* , and incubated for 4 hours at 37 °C. After this period a fraction of supernatant was removed for cytokine ELISAs and stored at −20 °C. EDTA was added to the remaining supernatant (final concentration 2.5 μM) and cells were detached by pipetting.

Cells were stained in PBS 1% BSA for CD45 (Clone HI30, dilution 1:200), CD69 (Clone FN50, dilution 1:200) and 4-1BB (Clone 4B4-1, dilution 1:200) as well as with PE-conjugated tetrameric pMHC (dilution 1:500). Stained cells were either analysed immediately or fixed with 1% formaldehyde in PBS and analysed on the following day.

T cells were discriminated from U87 cells by CD45 staining and/or an assessment of size and complexity. Single T cells were identified on the basis of size and subsequent analysis performed on this population.

Flow cytometry

Tetramers were produced by mixing refolded monomeric biotinylated pMHC and streptavidin-PE (Biolegend) at a 1:4 molar ratio. Streptavidin-PE was added in 10 steps with a 10 minute incubation at room temperature between each addition. 0.05–0.1% sodium azide was added for preservation and tetramers were kept for up to 3 months at 4 °C.

Samples were analysed using a BD LSR Fortessa X-20 (BD Biosciences) or CytoFLEX LX (Beckman Coulter) flow cytometer and data analysis was performed using FlowJo v10 (BD Biosciences).

Electroporation of 868 TCR

868 TCR alpha and beta chains were amplified using PCR, adding a T7 promoter at the 5′ end. The resulting PCR product was 'cleaned up' using a NucleoSpin Gel and PCR clean-up kit (Macherey-Nagel). Capped and Poly(A) tailed mRNA was produced from this PCR product using a mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (ThermoFisher). mRNA was collected by lithium chloride precipitation, quality checked by gel electrophoresis and stored in single use aliquots at −80 °C.

For electroporation, T cells are collected and washed 3x with Opti-MEM (Gibco) and resuspended at a concentration of 25×10^6 cells/mL. 5×10^6 cells with 2 μg per million cells of each of the RNA for the TCRα, β and ζ chains. Cells were then aliquoted in 200 μL into an electroporation cuvette (Cuvette Plus 2mm gap BTX). Electroporation is performed using an ECM

830 Square Wave electroporation system (BTX) at 300 V for 2 ms. Cells are then transferred to pre-warmed complete RPMI at a density of 1×10^6 cells/mL. Electroporated cells are used in assays 24 hours later.

Immobilisation Assay

Following a plate stimulation assay, after cells were collected, plates were washed 3 times with PBS 0.05% TWEEN 20 ('PBST') and then stained with anti-HLA-A,B,C (clone W6/32, dilution 1:1000) in PBS for 2 hours at room temperature. Plates were then washed 3x with PBST and stained with secondary goat anti-mouse IgG IRDye 800CW (LI-COR) in PBS for a further 2 hours. Finally plates were washed one more time with PBST and then imaged using a LICOR Odyssey Sa (LI-COR). Integrated intensity per well is reported.

ELISAs

Invitrogen Uncoated ELISA kits for IFNγ (Thermo Fisher) were used following the manufacturer's protocol. Supernatants were either used immediately for ELISAs post-harvesting or stored at −20 °C for up-to 2 weeks. Supernatants were diluted using an empirically determined ratio before use in an ELISA so that quantities of assessed cytokines fell within the linear range of the kits.

Surface Plasmon Resonance

D52N–pMHC interactions were analysed on a Biacore T200 instrument (GE Healthcare Life Sciences) at 37°C and a flow rate of 30 µl/min. Running buffer was HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v Tween-20). Streptavidin was coupled to CM5 sensor chips using an amino coupling kit (GE Healthcare Life Sciences) to near saturation, typically 10,000–12,000 response units (RU). Biotinylated pMHCs (47 kDa) were injected into the experimental flow cells (FCs) for different lengths of time to produce desired immobilisation levels (300–1000 RU). FC1 and FC3 were used as reference FCs for FC2 and FC4, respectively. Biotinylated ECD of CD58 (24 kDa + ~25 kDa glycosylation) was immobilised in the reference FCs at levels matching those of pMHCs. Excess streptavidin was blocked with two 40 s (D52N STAR) or 60 s (D52N scFv) injections of 250 µM biotin (Avidity). Before injections of purified D52N, the chip surface was conditioned with eight injections of the running buffer. Dilution series of D52N were injected simultaneously in all FCs starting from the lowest concentration, which was injected again after the highest concentration to confirm stability of pMHC on the chip surface. The duration of injections (20 or 180 s) was the same for conditioning and D52N injections. After every 2 or 3 D52N injections, buffer was injected to generate data for double referencing. In addition to subtracting the signal from the reference FC (single referencing), all D52N binding data were double referenced versus the average of the closest buffer injections before and after D52N injection to correct for small differences in signal between flow cells. D52N binding versus D52N concentration was fitted with the following model: $B = B_{max} \cdot \frac{[D52N]}{K_D+[D52N]}$, where B is the response (binding) and B_{max} is the maximal binding.

Production of recombinant CD2-CD43

Elongated CD2 variants were produced by fusing the ectodomain of CD2 (UniProtKB: $P06729_{25\text{-}209}$) with that of CD43 (UniProtKB: $P16150_{20\text{-}253}$) using a short linker sequence (GGGS). From the full-length sequence consisting of the entire CD43 ectodomain shorter variants were produced using site directed mutagenesis leaving a number of amino acids from

the C terminal end of the CD43 domain. E.g., CD2-CD43(20) contains the CD2 ectodomain fused to the 20 amino acids proximal to the C terminal end of the CD43 ectodomain.

APC stimulation (Jurkat T cells with chimeric CD2-CD43 co-cultured with U87 cells)

Jurkat TCR$\alpha^-\beta^-$ cells were a gift from Simon J. Davis (Oxford) and were cultured in 'complete media' (RPMI 1640 supplemented with 10% FBS (v/v) and penicillin-streptomycin (100 U/mL, 100 µg/mL respectively)) at 37 °C and 5% CO2.

The endogenous CD2 was knocked out via lentiviral transduction of an expression cassette encoding both a CRISPR CD2 guide RNA and Cas9. The expression cassette was sourced from Horizon Discovery.

25,000 U87 cells were seeded in a tissue culture treated flat-bottom 96 well plate and grown overnight. On the following day the media was removed from these cells and they were incubated with peptides prepared to the appropriate concentration in 'complete DMEM' (DMEM supplemented with 10% (v/v) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin) for 1 hour at 37 °C.

Peptide containing media was then removed and 50,000 T cells per well were added. The co-culture was then spun for 2 minutes at 50 xg, and incubated for 4 hours at 37 °C. After this period a fraction of supernatant was removed for cytokine ELISAs and stored at −20 °C. EDTA was added to the remaining supernatant (final concentration 2.5 $\mu mol/dm^3$) and cells were detached by pipetting.

Cells were stained in PBS 1% BSA for CD45 (Clone HI30, dilution 1:200), CD69 (Clone FN50, dilution 1:200) and 4-1BB (Clone 4B4-1, dilution 1:200) as well as with PE-conjugated tetrameric pMHC (dilution 1:500). Stained cells were either analysed immediately or fixed with 1% formaldehyde in PBS and analysed on the following day.

T cells were discriminated from U87 cells by CD45 staining and/or an assessment of size and complexity. Single T cells were identified on the basis of size and subsequent analysis performed on this population.

Conjugation assays

Jurkat or primary human $CD8^+$ T cells were tagged with CellTrace Violet (ThermoFisher) whilst target cells were tagged with CellTrace Far Red (ThermoFisher) following the manufacturers instructions. Labelled cells were then mixed in equal quantities (usually 1 mio of each), centrifuged at 50 xg for 5 minutes and incubated for 1 hour at 37 °C. Cells were then gently resuspended and analysed by flow cytometry. Samples of target or T cells alone were used to set gates so that double positive cells were identifiable — the percentage of these events out of the total is reported as the % conjugate formation.

Sequences

D52N scFvs with the following sequence were produced by Absolute Antibody Ltd.

D52N scFv:
EVQLLESGGGLVQPGGSLRLSCAASGFTFSTYQMSWVRQAPGKGLEW
VSGIVSSGGSTAYADSVKGRFTISRDNSKNTLYLQMNSLRAEDTAVY
YCAGELLPYYGMDVWGQGTTVTVSSAKTTPKLEEGEFSEARVQSELT

QPRSVSGSPGQSVTISCTGTERDVGGYNYVSWYQQHPGKAPKLIIHN
VIERSSGVPDRFSGSKSGNTASLTISGLQAEDEADYYCWSFAGGYYV
FGTGTDVTVLG

The D52N-IgG1 CARs contain a 'HNG spacer sequence' derived from the IgG1 hinge region, described in [4], and spliced with a spacer region from the CH2-CH3 regions of IgG1 as described in [5].

HNG Spacer:
DPAEPKSPDKTHTCPPCP

The 1G4 TCR α and β chains are joined by a P2A linker peptide with an additional spacer and furin cleavage site, as described in [6]. The sequence is given below.

Furin-P2A:
GSRAKRSGSGATNFSLLKQAGDVEENPGP

Independent experiments and data analysis

To produce independent measurements of EC_{50} for a given antigen receptor, we produced a new batch of lentivirus and transduced T cells isolated from a new human blood donor. Although we observed variations in the transduction efficiency, the surface level of each antigen receptor was always at the same level or higher compared to the TCR.

Statistical analysis was performed using Prism (GraphPad Software) or Excel (Microsoft). Curve fitting was performed using the robust nonlinear regression function in Prism or MATLAB (MathWorks) and the EC_{50} extracted from the fitted curves. Data was excluded from analysis if the computed fit was reported as 'ambiguous' in Prism, if the fit did not converge in 1000 iterations, or if the computed EC_{50} was outside of the tested ligand concentration.

References

[1] Adnane Achour et al. "Murine class I major histocompatibility complex H-2Dd: expression, refolding and crystallization". In: *Acta Crystallographica Section D: Biological Crystallography* 55.1 (1999), pp. 260–262.

[2] Johannes Pettmann et al. "The discriminatory power of the T cell receptor". In: *Elife* 10 (2021), e67092.

[3] Jonathan M Boulter et al. "Stable, soluble T-cell receptor molecules for crystallization and therapeutics". In: *Protein engineering* 16.9 (2003), pp. 707–711.

[4] Aysha Patel. "Development of T cell based therapeutic strategies for childhood cancer neuroblastoma". PhD thesis. UCL (University College London), 2017.

[5] Norihiro Watanabe et al. "Fine-tuning the CAR spacer improves T-cell potency". In: *Oncoimmunology* 5.12 (2016), e1253656.

[6] S Yang et al. "Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition". In: *Gene therapy* 15.21 (2008), pp. 1411–1423.

Thanks!

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So, if you can, consider giving blood.

* There is a Swedish saying: 'Ingen nämnd, ingen glömd' — 'No one mentioned, no one forgotten'. But since I have already made the error of mentioning people, I can only apologise to those I have forgotten. You certainly have my thanks.