Engineering T cells with enhanced ligand discriminatory powers



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A thesis submitted for the degree of

Doctor of Philosophy

Trinity 2024

Acknowledgements

I would like to start by especially thanking Omer, who has guided and supported me throughout my project. He has taught me how to ask the right scientific questions and design experiments. I am also very grateful for all the opportunities he has provided me for personal growth, including managing scientific collaborations, supervising students and supporting me in any opportunity I wanted to get involved in. I would also like to thank Anton, for providing frequent feedback on the scientific direction of my project throughout the whole process.

Next, I would like to thank the Interdisciplinary Bioscience DTP team, for funding my project and supporting me in my scientific and career development. The DTP has provided me with a really valuable network of friends and colleagues across different departments at the University of Oxford.

I am very thankful for all the people that have provided feedback and insights into my project. I want to thank Simon Davis and David Cole for agreeing to examine this thesis. I would also like to thank Bass Hassan, Audrey Gérard, Michael Dustin and Paresh Vyas for assessing my project at the proposal, transfer and confirmation of status stages.

I also want to thank the entire team at the Dushek lab. I really enjoyed the daily lunches, frequent coffee breaks and conversations across lab benches. We are a very collaborative lab and I have always been supported when I needed to learn anything new. I especially want to thank Jake, a computer scientist-turned-biologist, who was my lab mentor when I joined the lab with zero immunology experience and taught me just about every technique I know.

Finally, I want to thank my family and Jamie, for always supporting me.

Contributions

This work is the result of collaborative projects in the Dushek lab and therefore it includes the work of others. All the work in this thesis is my own work except for the following exceptions detailed below.

Mikhail Kutuzov performed the surface plasmon resonance experiments with the NY-ESO-1 altered peptide ligands panel (Chapter 2) and with the c259 TCR self-peptides panel (Chapter 4). I performed the data analysis to determine the K_D values from the SPR instrument data.

Anna Huhn and Mikhail Kutuzov developed the high-throughput surface plasmon resonance method and performed affinity-measurements experiments with the NY-ESO-1 positional scanning library (Chapter 4). Anna Huhn performed the data analysis to determine the K_D values. I subsequently fit these K_D values to target cell killing data from my experiments.

Abstract

T cells play a key role in mediating immune responses against infected and cancer cells. They recognise peptides presented on major histocompatibility complexes (pMHCs) via their T cell receptors (TCRs). To mediate effective immune responses, T cells must discriminate between self-peptides and foreign or tumour peptides presented on MHC molecules. Additionally, T cells express a wide variety of co-signalling receptors that influence their decision to activate. However, the precise molecular mechanisms that regulate T cell ligand discrimination remain unclear.

In this thesis, we quantify the impact of different T cell co-signalling receptors on ligand discrimination by CRISPR/Cas9 knock-out in primary human T cells. We find that the adhesion receptors CD2 and LFA-1 increase T cell sensitivity to peptides regardless of their affinity, and therefore do not impact ligand discrimination. In contrast, the inhibitory receptor CD5 increases ligand discrimination by selectively reducing sensitivity to lower affinity peptides. Conversely, the CD8 co-receptor decreases ligand discrimination by selectively increasing sensitivity to lower affinity peptides.

Adoptive T cell therapy using T cells engineered with novel TCRs targeting tumour-specific peptides is a promising immunotherapy strategy. However, these TCR-T cells can cross-react with off-target peptides, leading to severe autoimmune toxicities. Our results suggest that knocking-out CD8 could be exploited to increase the safety of these therapies. Typically, both CD8+ cytotoxic and CD4+ helper T cells are engineered with the same tumour-reactive TCR. We compared the ligand discrimination of these two T cell subsets engineered with the same pMHC-I restricted TCR and found that CD4+ helper T cells display enhanced discrimination due to their incompatible CD4 co-receptor, which cannot bind the target pMHC-I. Finally, we demonstrate that engineering cytotoxic T cells with a CD8-to-CD4 switch leads to an even higher increase in ligand discrimination. Moreover, CD8-to-CD4 switch cytotoxic T cells display reduced cross-reactivity to large peptide libraries. Thus, these findings could be useful for developing safer TCR-T cell therapies with reduced risk of off-target cross-reactivities.

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List of Abbreviations

$\beta_2 m$ . . . . . β_2-microglobulin

ACT . . . . . adoptive cell therapy

AICD . . . . . activation-induced cell death

AIRE . . . . . autoimmune regulator

APL . . . . . altered peptide ligand

APC . . . . . antigen presenting cell

BCR . . . . . b cell receptor

BFP . . . . . biomembrane force probe

BSA . . . . . bovine serum albumin

CAR . . . . . chimeric antigen receptor

Cas9 . . . . . CRISPR associated protein 9

CD . . . . . cluster of differentiation

CDR . . . . . complementarity determining region

CLIP . . . . . class-II Ii peptide

CLP . . . . . common lymphoid progenitor

CRISPR . . . clustered regularly interspaced short palindromic repeats

cTEC . . . . . cortical thymic epithelial cells

DAG . . . . . diacylglycerol

DAMP . . . . damage associated molecular pattern

DN . . . . . double negative

DNA . . . . . deoxyribonucleic acid

DP . . . . . double positive

EC . . . . . effective concentration

ELISA . . . . . enzyme-linked immunosorbent assay

ER . . . . . endoplasmic reticulum

FBS . . . . . fetal bovine serum

GPCR . . . . . g-protein coupled receptor

HBV . . . . . hepatitis B virus

HLA . . . . . human leukocyte antigen

HPV . . . . . human papillomavirus

HSC . . . . . hematopoietic stem cell

IFN . . . . . interferon

Ig . . . . . immunoglobulin

Ii . . . . . invariant chain

IL . . . . . interleukin

IP_3 . . . . . inositol triphosphate

ITAM . . . . . immunoreceptor tyrosine-based activation motif

JAM-A . . . . junctional adhesion molecule A

KO . . . . . knock-out

KP . . . . . kinetic proofreading

KS . . . . . kinetic segregation

LAT . . . . . linker for activation of t cells

LFA . . . . . lymphocyte function-associated antigen

MAGE . . . . melanoma associated antigen

MAIT . . . . . mucosal associated invariant t cell

MBP . . . . . myelin basic protein

MHC . . . . . major histocompatibility complex

MIIC . . . . . MHC-II endosomal compartment

mTEC . . . . . medullary thymic epithelial cell

NHEJ . . . . . non-homologous end joining

PAMP . . . . . pathogen associated molecular pattern

PE . . . . . phycoerythrin

pMHC . . . . . peptide-MHC

PRR . . . . . pattern recognition receptor

RAG . . . . . recombination-activating gene

RNA . . . . . ribonucleic acid

RSS . . . . . recombination signal sequence

sgRNA . . . . single guide RNA

SPR . . . . . surface plasmon resonance

TAP . . . . . transporter associated with antigen processing

TCR . . . . . t cell receptor

TdT . . . . . terminal deoxynucleotidyl transferase

TIL . . . . . tumour infiltrating lymphocyte

TLR . . . . . toll like receptor

Treg . . . . . regulatory t cell

WT . . . . . wild-type

ZAP-70 . . . . zeta-chain-associated protein kinase 70

Chapter 1

Introduction

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1.1 The adaptive immune response

The immune system protects its host against pathogens as well as cancer cells. Immune cells have diverse cell surface receptors that enable them to detect and respond to different antigens. The immune system can be broadly classified into the innate immune system and the adaptive immune system.

Innate immune cells express germline encoded receptors or pattern recognition receptors (PRRs) that recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) [1, 2]. PAMPs are evolutionarily conserved molecular motifs in pathogens that are absent in the host cells, whilst DAMPs are signals produced by the host as a result of tissue damage. PRRs can bind to different types of macromolecules, such as proteins, nucleic acids, lipids or carbohydrates. For example, TLR5 recognises flagellin, a highly conserved protein component of the gram negative bacterial flagellum [3] and TLR2 recognises extracellular DNA and histones, which are a sign of cell death [4]. Upon ligand binding, the signal is transduced and a cellular response is rapidly triggered, such as phagocytosis or secretion of cytokines. Furthermore, all innate immune cells of the same type express the same PRRs, which enables them to surveil the body for activation signals and rapidly trigger a response.

In contrast, adaptive immune cells express antigen receptors that are encoded in the germline as gene segments that are somatically recombined to assemble different antigen receptors in each cell. Therefore, each adaptive immune cell has a unique antigen specificity and after becoming activated can be clonally expanded. This results in a slower initial immune response, but one that is highly tailored to specific pathogen antigens. The two main subsets of adaptive immune cells are T cells and B cells, which express variable T cell receptors or B cell receptors (BCRs), respectively. TCRs recognise short peptides presented on MHC molecules, whilst BCRs can recognise any folded protein as well as other macromolecules, such as lipids or carbohydrates [5–7]. Additionally, TCRs are only expressed at the cell

surface, whilst BCRs can either be expressed at the cell membrane or secreted in a truncated form or antibody.

Following their activation, adaptive immune cells can elicit different effector responses. For instance, B cells produce secreted antibodies that can neutralise or opsonise pathogens for phagocytosis. T cells can be categorised into cytotoxic T cells, which directly mediate cell death, and helper T cells, which produce cytokines to recruit and activate other immune cells. Even though the primary adaptive immune response is slow due to the time-delay required for clonal expansion, long-lived memory cells are created, which enable a much faster secondary response upon future exposures to the same antigen [8].

There are complex interactions between the innate and adaptive immune responses, as they do not operate in isolation from each other [9]. For instance, innate immune cells use their PRRs to detect pathogens and produce cytokines that recruit adaptive immune cells [10]. Additionally, professional antigen presenting cells (APCs), such as dendritic cells and macrophages, recognise pathogens via their PRRs and subsequently phagocytose them to present pMHC antigens to T cells and activate them.

1.2 TCR recognition of pMHC

Most T cells express $\alpha\beta$ TCRs that recognise peptides presented on MHC molecules, but they can also recognise other ligands presented on MHC-like molecules, such as lipids presented on CD1a [11] or metabolites presented on MR1 [12]. Additionally, a small subset of T cells express semi-invariant $\gamma\delta$ TCRs that bind antigens independently of MHC [13]. In this chapter, we will discuss the recognition of pMHC antigens by conventional $\alpha\beta$ T cells.

1.2.1 MHC processing

The immune system is not able to directly detect the presence of pathogenic antigens within the intracellular environment of infected cells or cells that have phagocytosed pathogens. MHC molecules first appeared in jawed fish over 500 million years ago and they play an essential role in adaptive immunity by presenting peptides derived from intracellular or phagocytosed proteins on the cell surface [14]. T cells must deploy distinct effector functions depending on whether a pathogenic peptide is presented by an infected cell or alternatively, by an immune cell that has phagocytosed pathogens. As a result, there are two classes of MHC molecules, MHC-I and MHC-II, that activate different T cell subsets, cytotoxic T cells and helper T cells, respectively [15]. Cytotoxic T cells express the CD8 co-receptor, which binds MHC-I and helper T cells express the CD4 co-receptor, which binds MHC-II.

All nucleated cells in the body express MHC-I molecules to present peptides derived from their cytosol, which enables cytotoxic T cells to directly kill infected or tumour cells. In contrast, MHC-II molecules are only expressed in professional antigen presenting cells, such as B cells, dendritic cells and macrophages, and they present peptides from extracellular antigens that have been endocytosed. This enables helper T cells to stimulate other cells of the immune system, rather than eliminate the antigen presenting cell. Virally infected or tumour cells can lose MHC-I expression to evade recognition by T cells [16, 17]. However, natural killer cells and T cells

can recognise abnormal cells that lack MHC molecules and eliminate them [18, 19].

MHC-I molecules are heterodimers comprised of a polymorphic alpha chain and a non-polymorphic invariant beta chain or beta-2 microglobulin (β_{2m}). The alpha chain contains a transmembrane domain and its α_1 and α_2 domains form the peptide binding groove [20]. In contrast, MHC-II molecules are heterodimers of polymorphic alpha and beta chains, which both contain transmembrane domains. The peptide binding groove is formed by the α_1 and β_1 domains [21] (Figure 1.1).

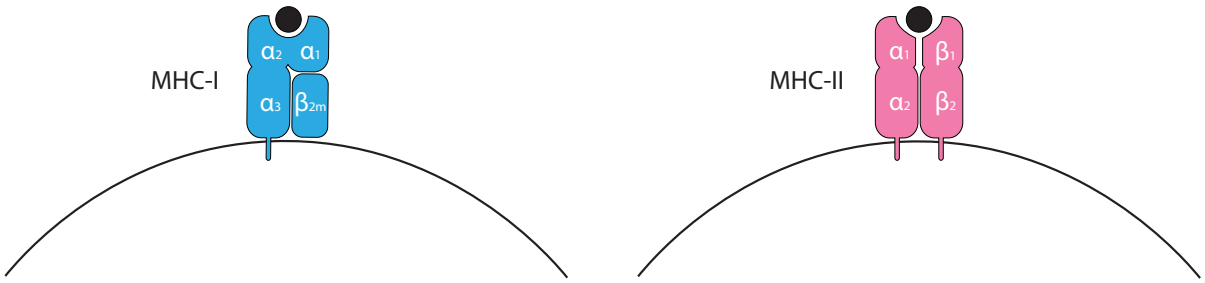


Figure 1.1: Schematic of MHC molecules.
(Left) MHC-I molecules are composed of a polymorphic alpha chain and an invariant beta chain. (Right) MHC-II molecules are composed of polymorphic alpha and beta chains.

Each MHC molecule displays different peptide binding preferences, but they are highly degenerate and each can bind a large peptide repertoire [22–24]. There are multiple genes that encode MHC molecules in humans. HLA-A, HLA-B and HLA-C encode MHC-I molecules and HLA-DP, HLA-DQ and HLA-DR encode MHC-II molecules. Additionally, these genes are co-dominantly expressed, increasing the total diversity of MHC molecules expressed in each individual and the total repertoire of peptides that they can present [25]. Up to 6 different MHC-I molecules can be expressed per individual if they are heterozygous for the 3 MHC-I genes. For MHC-II, each individual can express up to 12 different molecules, since the alpha and beta chains from different alleles can pair to form additional combinations. The genes encoding MHC molecules are some of the most polymorphic in the human genome, with thousands of different alleles reported [26], which ensures a higher protection against pathogens at the population level [27].

Cytosolic proteins are degraded by proteasomes and loaded into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). TAP preferentially binds peptides that are between 8-12 amino acids in length [28] and with hydrophobic or basic amino acids at the C-terminus [29]. Therefore, the peptide binding preferences of TAP shape the peptides that enter the ER, and consequently, which peptides are ultimately presented on MHC-I molecules on the cell surface. Empty MHC-I molecules are stabilised in the ER by chaperones such as Tapasin, Calreticulin and ERp57 until they bind a peptide with high affinity [15]. Different MHC alleles display different levels of dependence on Tapasin for peptide loading, which in turn influences the repertoire of presented peptides [30]. Once the pMHC-I complex is assembled, the chaperones are released and it is trafficked to the cell surface [15].

MHC-II molecules are synthesised in the ER and they associate with the invariant chain (Ii), which blocks the peptide binding groove. Subsequently, they are transported to MHC-II endosomal compartments (MIICs) for peptide loading. The invariant chain is digested and a smaller class-II Ii peptide (CLIP) remains bound at the peptide groove [15]. Exogenous proteins are internalised by phagocytosis or endocytosis and they are trafficked to the MIIC, where they are degraded by proteases. HLA-DM is a non-polymorphic chaperone that holds MHC-II open to exchange CLIP for an exogenous peptide. In an analogous role to Tapasin, HLA-DM helps shape the repertoire of peptides presented on MHC-II molecules [31]. Once the MHC-II molecule binds a peptide with high affinity, HLA-DM is released and the pMHC-II complex is transported to the cell surface [15].

In addition to these canonical peptide loading pathways, dendritic cells can cross-present exogenous peptides on MHC-I molecules to directly activate cytotoxic T cell clones. The molecular mechanisms of cross-presentation are not fully understood, but it has been proposed that exogenous peptides are translocated to the cytosol and then transported to the ER for loading on MHC-I or alternatively, MHC-I

molecules are transported to endocytic compartments to be loaded with exogenous peptides [32].

1.2.2 The TCR complex

The TCR is a multimeric complex composed of six distinct protein chains. The TCR$\alpha\beta$ dimer associates non-covalently with a CD3$\epsilon\gamma$ dimer, a CD3$\epsilon\delta$ dimer and a CD3$\zeta\zeta$ dimer in 1:1:1:1 stoichiometry [33, 34] (Figure 1.2). The TCR$\alpha\beta$ dimer mediates recognition of the pMHC ligand, whilst the associated CD3 chains contain the signalling motifs necessary for signal transduction.

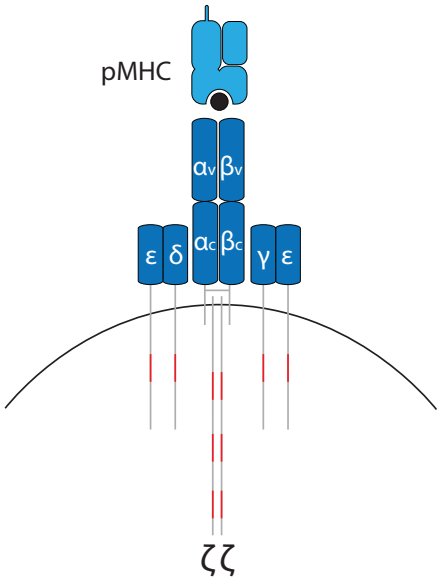


Figure 1.2: Schematic of the TCR.
The TCR is composed by 4 dimers. The alpha and beta dimer contains variable (α_v/β_v) and constant (α_c/β_c) regions. They associate with the invariable CD3 gamma, delta, epsilon and zeta chains. The CD3 chains contain ITAM domains (shown in red) that are phosphorylated upon activation. The grey line between alpha and beta chains represents a cysteine bond.

The extracellular domains of the α and β subunits are composed of a germline encoded constant domain and a variable domain generated by somatic recombination. The variable regions bind the pMHC ligand and the constant regions associate with the other components of the TCR complex. The constant domains are composed of an immunoglobulin (Ig)-like domain, a connecting peptide, a transmembrane domain and a short intracellular tail [35]. The TCR$\alpha\beta$ dimer is associated via a

disulfide bridge [36, 37] and the basic residues in the $\alpha\beta$ transmembrane domains form electrostatic interactions with acidic residues in the transmembrane domains of the CD3 chains [34, 38, 39].

The CD3ϵ, CD3γ and CD3δ subunits are composed of one Ig-like domain, a connecting peptide, a transmembrane domain and an intracellular domain containing one immunoreceptor tyrosine-based activation motif (ITAM). The CD3ζ subunits, or CD247, are composed of a short extracellular domain, a transmembrane domain and an intracellular tail containing three ITAMs. ITAMs are common signalling motifs across many immune receptors, including the BCR or innate PRRs like Dectin-1 [1]. They are composed of two repeats of the consensus sequence YxxL/I, separated by 6-8 amino acids and they are typically phosphorylated upon receptor binding to activate downstream signalling pathways [40].

1.2.3 TCR signalling

T cell activation is initiated by binding of the TCR to a pMHC molecule, which triggers a signalling cascade (Figure 1.3). Firstly, the Lck kinase phosphorylates the ITAMs in the TCR CD3 intracellular chains. Subsequently, ZAP-70 binds the phosphorylated ITAMs via its SH2 domains and is phosphorylated and activated by Lck. ZAP-70 is also auto-phosphorylated in-trans by nearby ZAP-70 molecules. Upon activation, ZAP-70 phosphorylates multiple signalling molecules such as linker for activation of T cells (LAT) [41, 42].

Phosphorylation of tyrosine residues in LAT results in the formation of signalling condensates, where multiple SH2 domain-containing proteins are recruited and activate diverging downstream signal transduction pathways [43, 44]. Adapter molecules such as SLP-76 associate with LAT and recruit more signalling molecules [45, 46]. For instance, recruitment and activation of PLCγ1 leads to production of diacylglycerol (DAG) and inositol triphosphate (IP_3), which results in an increase in intracellular calcium. The recruitment of VAV1 induces a rearrangement of the cytoskeleton, which is required to form conjugates with the antigen presenting

cell and polarise the secretion of cytokines towards it [41, 47]. These downstream pathways culminate in the activation of three key transcription factors, NF-κB, AP-1 and NFAT. Collectively, the activation of these transcription factors result in the regulation of hundreds of genes to induce T cell effector functions, such as proliferation and the production of cytokines [42].

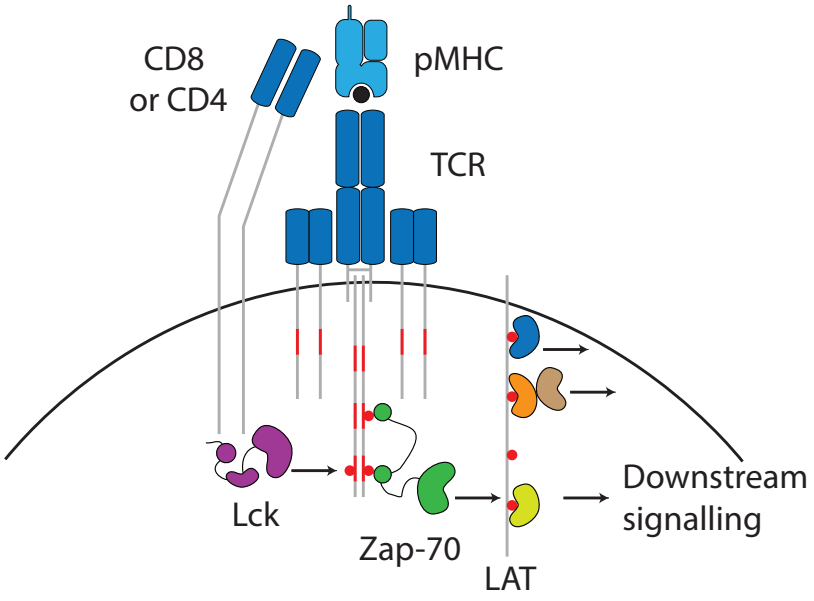


Figure 1.3: Schematic of the TCR proximal signalling pathway.
Upon ligand binding, the TCR ITAMs (shown as red lines) are phosphorylated by Lck. Zap-70 binds phosphorylated ITAMs (phosphate group shown as red circles) and phosphorylates LAT. Different effector proteins bind the phosphorylated LAT residues which activates downstream signalling pathways.

1.2.4 TCR triggering

The signalling steps that transduce TCR/pMHC binding to T cell activation are well understood. However, the precise mechanisms that transduce extracellular ligand binding into the first intracellular signalling step, phosphorylation of the CD3 ITAM motifs, are still under debate. Some triggering models propose that the TCR has an intrinsic ability to detect ligand binding and facilitate phosphorylation of its ITAM motifs by Lck. Alternatively, it has been proposed that triggering is a cellular phenomenon that relies on the spatial reorganisation of signalling molecules at the cell membrane after pMHC binding.

Clustering

It has been proposed that TCRs aggregate upon pMHC binding, which triggers their activation. According to this model, the clustering of TCRs increases the local concentration of signalling molecules, such as the Lck kinase, which initiates the phosphorylation of CD3 ITAMs. The clustering hypothesis is supported by findings that anti-CD3 antibodies can induce T cell activation [48, 49]. However, even though enforced aggregation of TCRs by antibodies can induce signalling, it is unclear if clustering is required for TCR triggering when interacting with pMHC on an antigen presenting cell. Following T cell activation, TCR clusters are observed [50], but this might be a consequence of T cell signalling rather than the cause of triggering. More recently, a single-molecule imaging study has shown that a single TCR bound to a single pMHC molecule can become phosphorylated and recruit ZAP-70 without forming TCR clusters [51]. Therefore, these findings suggest TCR clustering is not required for triggering.

Conformational change

Alternatively, it has been suggested that the TCR might undergo conformational changes upon pMHC binding that lead to triggering. Crystallography studies of the TCR$\alpha\beta$ ectodomains showed no large structural rearrangements upon peptide binding [52–54]. Only small adjustments at the TCR binding interface were observed, with no structural changes in the TCR$\alpha\beta$ constant domains. Furthermore, the structures of the same TCR bound to agonist and non-agonist peptides were very similar, except for small alterations in the CDR loops to accommodate the different peptide sequences [52]. Therefore, this suggests that the TCR$\alpha\beta$ domains do not undergo a conformational switch upon ligand binding. Given the extensive sequence and structural diversity of the TCR ligand-binding domains, it would be difficult to evolve a conserved mechanism of triggering through conformational change. Instead, it has been proposed that ligand binding could induce alterations of the structural organisation between the TCR and its associated CD3 chains [55, 56].

A cryo-EM structure of the fully assembled TCR$\alpha\beta$-CD3 complex also revealed no major structural rearrangements between the unligated and the pMHC bound states [57]. However, a more recent cryo-EM structure determined in a more native-like environment using lipid nanodiscs revealed larger structural changes upon ligand binding [58]. The unligated TCR$\alpha\beta$-CD3 structure displayed a compact closed conformation, which was extended when bound to pMHC, accompanied by movements in the transmembrane helices. However, the authors tested mutations in the TCR that would prevent ectodomain opening and found only a small impact on T cell activation [58]. Therefore, this suggests that these dynamic changes in the TCR structure might not be required for triggering.

Some studies have suggested that basic rich residues in the CD3 intracellular tails associate with the negatively charged plasma membrane, preventing phosphorylation of their ITAM residues. Upon binding to a pMHC ligand, the CD3 intracellular tails dissociate from the membrane and phosphorylation is possible [59–61]. However, directly resolving the structure of the dynamic intracellular CD3 tails has not been possible. Additionally, experiments using phosphatase inhibitors show that ITAM phosphorylation can occur even in unligated TCRs, which suggests that membrane decoupling is not a required step for triggering [62].

Kinetic segregation

The kinetic segregation model proposes that the TCR is a passive structure that does not undergo any conformational changes upon ligand binding. Instead, triggering is regulated by the relative proportion of kinases and phosphatases surrounding the TCR [63] (Figure 1.4).

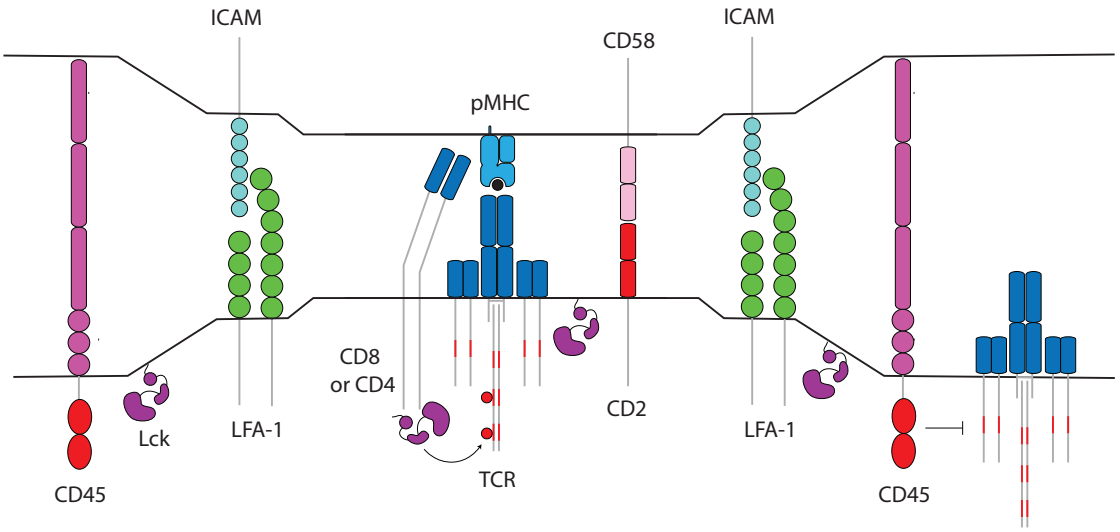


Figure 1.4: Organisation of molecules at close contacts.
Molecules organise at close contacts based on their size. The TCR/pMHC interaction and the CD2/CD58 interaction span ∼15 nm. The adhesion molecule LFA-1 binds to ICAMs with a larger interaction size. The CD45 phosphatase is excluded from the close contacts due to its large extracellular size. Since Lck can diffuse throughout the T cell membrane, TCRs in the close contact can be phosphorylated, due to the exclusion of phosphatases..

In the resting state, the abundance of phosphatases at the membrane, such as CD45, prevents the phosphorylation of the TCR. The adhesion molecule CD2 binds CD58 on target cells and creates close contacts between the T cell and the APC spanning approximately 15 nm [64]. Upon ligand binding, the TCR/pMHC interaction spans a similar distance. Therefore, ligated TCRs accumulate at the close contacts, and their diffusion is slowed while they remain bound to pMHC. Due to the much larger size of its extracellular domain, CD45 is excluded from these close contacts and phosphorylation of the TCR can proceed. Finally, upon ligand unbinding, the TCR diffuses from the close contacts and is quickly dephosphorylated. Even though a similar re-organisation of molecules is observed in mature immune synapses [65], close contact formation is sufficient to induce TCR triggering before the immune synapse is assembled.

Supporting the kinetic segregation model, studies have shown that truncating the extracellular domains of the CD45 and CD148 phosphatases prevents their exclusion from close contacts, inhibiting TCR triggering [66]. Similarly, chimeric Lck molecules with large extracellular domains were unable to phosphorylate the TCR and initiate signalling, as they were excluded from the close contacts [67].

It has also been suggested that the lateral size of close contacts must be limited to less than 200 nm to prevent the spontaneous activation of unligated TCRs [68]. If an individual close contact has a larger radius, any unligated TCR entering this area could become phosphorylated and trigger signalling before diffusing out. A recent study demonstrated that increasing the size of close contacts by 10 fold resulted in the activation of over 60% of T cells even in the absence of pMHC [69]. Therefore, this result is incompatible with the conformational change and aggregation models, which require pMHC binding for triggering.

1.3 Generation of the T cell repertoire

All adaptive immune cells—B cells, T cells, and natural killer cells—are generated by progressive differentiation from hematopoietic stem cells (HSCs). In the bone marrow, HSCs differentiate into common lymphoid progenitors (CLPs), which then migrate to the thymus to develop into T cells [70, 71]. In the thymus, CLPs undergo several stages of T cell maturation. During this process, they undergo somatic recombination to generate a diverse repertoire of TCRs. As they develop, T cells commit to either the CD8+ cytotoxic or CD4+ helper lineage, depending on the binding specificity of their TCR. After completing maturation, T cells emerge from the thymus to populate different peripheral tissues where they carry out their immune functions.

1.3.1 TCR recombination

TCRs are generated by the recombination of different germline encoded gene segments during T cell development in the thymus. The TCRα locus contains V and J segments, followed by the constant region. In addition to the V and J segments, the TCRβ locus also contains D segments [72]. There are two TCRβ constant region isoforms, but they only differ by a few amino acids in sequence and are expected to function similarly [73, 74]. In total, the TCRα locus is composed of $\sim$70 V_α and 61 J_α segments, whilst the TCRβ locus contains 52 V_β, 13 J_β and 2 D_β segments [75].

TCR diversity is generated by combining one V segment, one J segment, and in the case of TCRβ, one D segment to form a unique polypeptide chain. V(D)J recombination is mediated by the recombination-activating genes (RAG) recombinases, RAG1 and RAG2. RAG proteins bind recombination signal sequences (RSS) that flank each of the V(D)J segments and induce a DNA double-strand break. The V(D)J segments are joined by non-homologous end joining (NHEJ) of the DNA breaks, and the intermediate DNA sequences are excised [76].

In addition to this V(D)J combinatorial diversity, the imprecise repair of the double-strand breaks further increases the diversity at the junctions between each of the gene segments. NHEJ of the DNA breaks can introduce small insertions or deletions, due to the absence of a DNA repair template. Furthermore, the terminal deoxynucleotidyl transferase (TdT) nuclease randomly adds nucleotides at the DNA ends which further increases junctional diversity [77].

In its 3D folded structure, TCRs bind their ligands via six complementarity determining region (CDR) loops, three in each TCR chain. CDR1 and CDR2 are encoded in the inherited V segments, whilst CDR3 is the most variable as it includes the junctions between the V(D)J segments [72]. Therefore, the combinatorial and junctional diversity generated by V(D)J recombination generates a huge diversity of CDR loops and therefore a huge diversity of TCRs that bind different ligands.

1.3.2 Thymic selection

Since T cells generate novel TCRs with many specificities, a selection process is necessary to eliminate T cells that do not recognise peptides presented on MHC molecules. T cells that recognise self-peptides presented on MHC could cause autoimmune damage and must also be removed [78].

Before they undergo TCR recombination, developing thymocytes are co-receptor double negative (DN), as they do not express neither the CD8 nor the CD4 co-receptors. Recombination of the TCRβ locus happens first, and DN T cells express a pre-TCR composed of the re-arranged β chain and an invariant α chain. If TCRβ recombination has produced a functional TCRβ chain, the pre-TCR induces signalling either by binding ligands in the thymus or by ligand-independent tonic signalling, which enables T cell proliferation and maturation to proceed [79, 80]. Next, T cells upregulate the expression of both the CD8 and CD4 co-receptors. These double positive (DP) T cells undergo recombination at the TCRα locus to replace the invariant pre-TCRα chain for a newly re-arranged one [81].

Positive selection of DP T cells takes place in the thymic cortex, and its aim is to ensure that the newly generated TCR is MHC-restricted, specifically to peptides presented on the host's MHC alleles. For instance, experiments in mice demonstrated that T cells expressing a transgenic H2-D^b-restricted TCR could only mature if H2-D^b was expressed in the thymus [82, 83]. Cortical thymic epithelial cells (cTECs) present self pMHCs to developing T cells. If T cells do not interact with the self peptides presented, they undergo apoptosis or death by neglect. Interestingly, if T cells do not recognise any of the self pMHCs, the TCRα locus can undergo additional re-arrangements to increase the likelihood of producing a TCR that can survive positive selection [84, 85].

Since TCR recombination occurs randomly, only a very small proportion of T cells would be expected to have TCRs that bind pMHC ligands, as opposed to a much larger number of TCRs that bind folded proteins or other ligands. However, it has been suggested that the TCR repertoire has an intrinsic bias for MHC. Supporting this hypothesis, it was demonstrated that mice expressing a single type of pMHC ligand in the thymus can positively select a diverse TCR repertoire that binds peptides presented on different MHC alleles [86]. More recently, it has been shown that germline encoded TCR V segments have specificity to motifs shared by different MHC alleles [87, 88].

Once T cells that bind peptides presented on MHC are positively selected, an additional step is required to eliminate any T cells that could pose an autoimmune risk. This process is known as negative selection or central tolerance. T cells that react too strongly against self pMHCs presented in the thymus undergo apoptosis. It is important to ensure that T cells are not reactive to any self-antigens, even if those proteins are normally expressed outside the thymus. Therefore, medullary thymic epithelial cells (mTECs) deploy different mechanisms to increase the diversity of peptides that they present. For example, the autoimmune regulator (AIRE) is a key transcription factors that induces the ectopic expression of antigens from peripheral tissues in the thymus [89]. Additionally, mTECs activate the expression

of transcription factors that are normally restricted to other tissues, such as the lung or liver [90].

Alternatively, some CD4+ T cells that undergo strong signalling in the thymus can differentiate into regulatory T cells (Tregs). Development into Tregs is regulated by the expression of the FOXP3 transcription factor and epigenetic changes such as hypomethylation of specific DNA loci [91]. In the periphery, Tregs are activated in a pMHC dependent manner, but they have an immunosuppressive role, by producing cytokines such as IL-10 and TGF-β [92].

In conclusion, thymic selection ensures that T cells that do not bind pMHCs at all or T cells that bind self pMHCs too strongly are eliminated. As a result, only T cells that bind self pMHCs with intermediate affinity can survive. Therefore, the set of peptides used to positively and negatively select developing T cells shapes the specificites of the TCR repertoire in the periphery. cTECs express β5t, a proteasomal subunit only expressed in the thymus with unique protein cleaving specificites. Consequently, cTECs present a set of peptides that is different from the self-peptides presented by cells in the periphery [93, 94]. Therefore, even though peripheral T cells have intermediate affinity to the self-selecting peptide repertoire, they might have lower affinity to actual self peptides in the periphery.

Additionally, it has been suggested that this testing-set of self pMHCs could introduce blindspots in the T cell repertoire, where foreign peptides with motifs that are very different from these positively-selecting peptides being less likely to be recognised [95]. Similarly, individuals with heterozygous MHC alleles can present a greater diversity of peptides for negative selection, which results in a smaller diversity in the TCR repertoire [96, 97].

1.3.3 Commitment to the CD8 or CD4 lineages

Following the random process of TCR gene rearrangement, T cells must commit to either the CD8+ or CD4+ lineages, depending on whether their TCR binds pMHC-I or pMHC-II antigens, respectively. During positive selection, DP T cells become single positive (SP) for CD8 or CD4. They do not only repress expression of one of the co-receptors, but they also undergo extensive transcriptional reprogramming to differentiate into T cells with helper or cytotoxic effector functions. The transcription factors RUNX3 and ThPOK regulate the maturation into CD8+ cytotoxic T cells or CD4+ helper T cells, respectively [78].

The kinetic-signalling model postulates that the duration of TCR signalling at this stage determines the lineage choice. During positive selection, developing DP T cells transiently lose expression of CD8 [98]. As a result, T cells with an MHC-I restricted TCR, will lose signalling and will differentiate into CD8+ cytotoxic T cells. Alternatively, T cells that have an MHC-II restricted TCR will continue to signal after CD8 downregulation, as they express CD4, and they will differentiate into CD4+ helper T cells.

More recently, this model has been confirmed using FlipFlop mice, where the CD4 protein is encoded at the *Cd8* locus and the CD8 protein is encoded at the *Cd4* locus [99]. During positive selection, the *Cd8* locus encoding the CD4 protein is transiently silenced. Therefore, FlipFlop mice T cells that are MHC-II restricted stop signalling, whilst T cells that are MHC-I restricted continue signalling, as they express CD8. Consequently, MHC-I restricted T cells become CD8+ helper T cells and MHC-II restricted T cells become CD4+ cytotoxic T cells. This reversal of the T cell phenotypes led to ineffective immunity, since professional APCs presenting foreign pMHC-II antigens were eliminated by CD4+ T cell cytotoxic activity [99].

1.4 T cell ligand discrimination

T cells display intermediate affinity to self-peptides as a result of thymic positive selection. Even though they also undergo negative selection in the thymus, there remains a risk that T cells could become activated against self-peptides in the periphery and lead to autoimmune damage. To prevent this, the immune system has evolved several mechanisms to ensure that T cells that have evaded central tolerance do not become activated against self-peptides in other tissues.

Peripheral tolerance provides contextual information to T cells, ensuring that they are only activated in the presence of other inflammatory signals. In addition to these peripheral tolerance mechanisms, which involve other immune cells, it has been proposed that T cells are able to intrinsically discriminate between self and non-self peptides. However, the precise molecular mechanisms underlying this discrimination are still not fully understood. The kinetic proofreading model proposes that ligand discrimination is an emergent property of the T cell signal transduction pathway. In contrast, the mechanosensing model proposes that discrimination is an intrinsic feature of the physical properties of the TCR.

1.4.1 Peripheral tolerance

Following development in the thymus, naïve T cells that have not yet encountered their cognate antigen require additional co-stimulatory signals from a professional antigen presenting cell to become activated. If a naïve T cell is stimulated with pMHC alone (Signal 1) without receiving additional co-stimulation (Signal 2), it enters a state of unresponsiveness known as anergy [100–102]. Anergic T cells are unable to proliferate or trigger effector functions in response to subsequent pMHC stimulations. It has been proposed that TCR signalling in the absence of co-stimulation results in preferential activation of NFAT, with reduced activity of the AP-1 and NF-κB transcription factors. This imbalance results in the upregulation of anergy-associated genes and the repression of effector genes, such as cytokines [103, 104].

Stimulation of naïve T cells with both pMHC and co-stimulatory ligands enables their differentiation into effector and memory T cells. One of the most important T cell co-stimulatory molecules is CD28, which engages CD80 or CD86 ligands presented by professional antigen presenting cells. Simultaneous T cell activation via TCR and CD28 signalling promotes T cell proliferation and enhances the stabilisation of cytokine mRNAs such as IL-2 [105]. Upon recognition of pathogenic (PAMPs) or danger signals (DAMPs) via their innate immune receptors, dendritic cells mature and upregulate the expression of MHC and co-stimulatory molecules [106, 107]. Therefore, mature dendritic cells have the ability to prime naïve T cells and initiate immune responses. Once a T cell has been primed, future exposures to pMHC antigen (Signal 1) can result in activation even in the absence of co-stimulatory ligands (Signal 2) [106].

T cells that are repeatedly activated by self-peptides in the periphery can undergo apoptosis or clonal deletion. In a study with mice expressing a transgenic TCR with low affinity against a self-peptide from myelin basic protein (MBP), mature T cells with the MBP-specific TCR could develop [108]. These T cells did not induce autoimmunity due to the low affinity of their transgenic TCR against the MBP peptide. However, injecting mice with a mutant peptide with much higher affinity to the TCR resulted in the deletion of the MBP-reactive T cell clones [108]. One of the mechanisms that mediate peripheral clonal deletion is activation-induced cell death (AICD). Upon repeated activation, T cells upregulate expression of Fas and its ligand FasL, leading to apoptosis [109].

Alternatively, T cells that become activated against self-peptides in the periphery can also be induced to differentiate into Tregs to establish peripheral tolerance to these antigens [110, 111]. In addition to producing inhibitory cytokines, Tregs can also induce tolerance by preventing the co-stimulation of effector T cells. Tregs constitutively express CTLA-4, which binds the same ligands as the co-stimulatory receptor CD28 (CD80 and CD86), but with higher binding affinity. CTLA-4 removes

these co-stimulatory ligands from antigen presenting cells by transendocytosis, which prevents the activation of effector T cells [112, 113].

1.4.2 Kinetic proofreading

Many cell surface receptors, such as G protein-coupled receptors (GPCRs), induce signalling proportionally to the amount of ligand that they bind [114, 115]. In these cases, receptor occupancy directly determines the strength of signalling. For example, a 10 fold reduction in ligand affinity can be compensated by increasing the ligand concentration by 10 fold. However, TCRs are unique in that they have to discriminate between a huge number of structurally similar pMHC ligands. The differences in TCR binding affinity between self and foreign-pMHCs are often small [116, 117]. Moreover, self-peptides are presented at much higher densities than foreign peptides. Multiple studies have found that small differences in pMHC affinity leads to disproportionately larger reductions in T cell activation, and this cannot be compensated by simply increasing the concentration of the pMHC ligand [118–120]. Therefore, TCRs display enhanced ligand discrimination compared to other cell surface receptors, and the relationship between ligand affinity and activation is non-linear. One study calculated that T cell discrimination follows a power-law relationship with an exponent of 2 [114]. This means that a 10 fold reduction in ligand affinity requires a 100 fold (10^2) increase in ligand concentration to achieve the same level of activation.

The kinetic proofreading model was proposed to explain the observed enhanced ligand discrimination of T cells [121]. This model postulates that T cells amplify small differences in binding affinity into larger differences in functional responses by introducing a time-delay between ligand binding and signal transduction. Upon TCR/pMHC binding, a number of biochemical steps must take place before the signal is transmitted. If the TCR unbinds before these proofreading steps are completed, the system quickly resets to its initial state. Therefore, only ligands that remain bound for a sufficient duration can initiate signalling. The strength

of ligand discrimination is therefore determined by the length of the proofreading time.

TCR/pMHC binding kinetics have traditionally been studied using Surface Plasmon Resonance (SPR). In these experiments, pMHC is immobilised on a surface, and purified TCR is flowed over it to measure the binding kinetics in solution. These 3D affinity measurements generally correlate well with T cell activation [114, 116, 117]. However, it has been argued that the 3D binding parameters do not accurately reflect the 2D interactions between a TCR and pMHC anchored at opposing cell membranes. At the cellular interface, TCRs and pMHCs can only diffuse laterally. One study found that 2D binding affinity correlated better with T cell activation than 3D measurements [122]. However, a more recent study found that both 2D and 3D binding measurements correlated well with T cell activation [123].

It has also been proposed that in the native 2D environment, the orientation of the TCR and pMHC molecules at the cell surface can increase the likelihood of pMHC rebinding immediately after dissociation. Additionally, TCR clustering would further increase the probability of re-binding the same pMHC molecule. Therefore, pMHC ligands with short binding life-times can generate a longer aggregate half-life or confinement time by re-binding the same TCR multiple times [124–126]. As a result, pMHC ligands with fast on-rates are more likely to re-bind and increase T cell activation. These predictions are consistent with observations that TCRs with extremely slow dissociation rates have impaired T cell activation, as they do not benefit from serial re-binding [127].

The exact biochemical steps involved in kinetic proofreading are not known. It is unclear if these proofreading steps occur exclusively at the TCR, such as phosphorylation of ITAMs, or if they extend to further downstream signalling molecules, such as ZAP-70 recruitment to phosphorylated ITAMs or the formation of LAT microclusters by ZAP-70 phosphorylation. These TCR proximal phosphorylation events can be quickly reversed by abundant phosphatases, such as CD45. The kinetic segregation model proposes that exclusion of CD45 is required for receptor

triggering [63]. Following TCR unbinding, the receptor can diffuse away from CD45 depleted regions and reset to the unphosphorylated state.

The first step in TCR signal transduction is the phosphorylation of CD3 ITAMs by Lck. It has been demonstrated that chemical inhibition of Lck increases T cell ligand discrimination by selectively reducing activation to low affinity ligands [128]. This suggests, that Lck-mediated ITAM phosphorylation is a kinetic proofreading step. Lck activity is regulated by two phosphorylation sites [128]. In its inactive closed state, Lck enzymatic activity is inhibited. Dephosphorylation of the inhibitory tyrosine (Y505) on its C-terminal tail allows Lck to adopt an open conformation. Phosphorylation of the activatory tyrosine (Y394) stabilises this active open conformation and further increases enzymatic activity [129].

Therefore, the regulation of Lck phosphorylation plays a key role in modulating kinetic proofreading. For example, the Csk kinase phosphorylates Lck at the inhibitory tyrosine (Y505) [130]. Consequently, chemical inhibition of Csk has been shown to reduce ligand discrimination by enhancing activation against low affinity ligands [128]. Moreover, negative feedback loops may further enhance ligand discrimination. Upon activation, Lck can phosphorylate the SHP-1 phosphatase, which in turn dephosphorylates Lck, providing a regulatory mechanism that reduces activation to lower affinity ligands [131].

Recruitment of ZAP-70 to the phosphorylated CD3 ITAMs is the second step in TCR signal transduction. The R360P mutation in ZAP-70 is associated with an autoimmune syndrome. A study found that this mutation reduces ZAP-70 autoinhibition and results in decreased T cell ligand discrimination [132]. T cells expressing this hyperactive ZAP-70 mutant responded to the high-affinity OVA peptide to the same extent as wild-type T cells. However, their activation against weaker affinity peptides was selectively increased. Therefore, these results strongly suggest that the phosphorylation of downstream signalling molecules by ZAP-70 acts as another proofreading step.

Phosphorylation of multiple tyrosine residues in LAT by ZAP-70 leads to the formation of signalling condensates. One study showed that the phosphorylation at the Y132 residue occurs more slowly than at the other LAT tyrosines, due to the preceding glycine (G131) [133]. Mutating this glycine to aspartate or glutamate increased the phosphorylation rate of Y132, as well as the recruitment rate of PLCγ1. Consequently, T cells displayed enhanced activation against low affinity peptides. The authors of the study suggested that the slow phosphorylation of LAT Y132 could be a rate-limiting step in kinetic proofreading, therefore enhancing ligand discrimination by elongating the proofreading time [133]. However, increasing the phosphorylation rate of Y132 also increased T cell activation against high affinity peptides. Therefore, LAT phosphorylation and recruitment of PLCγ1 might not constitute a proofreading step.

Optogenetic receptors can be used to precisely control ligand binding life-time. One study found that production of DAG by PLCγ1 correlated with ligand binding half-life [134]. However, this study did not directly measure T cell activation. Therefore, it remains unclear whether any signalling steps downstream of ZAP-70 or LAT are involved in kinetic proofreading.

1.4.3 Mechanosensing

T cells dynamically scan the surface of antigen presenting cells whilst they migrate. It has been observed that T cells generate pushing and pulling forces against their target cells [135]. These forces are generated by T cell cystoskeletal re-arrangements and displacements between the T cell and its target cell [135, 136]. Additionally, the segregation of large molecules such as CD45 and CD43 from the immune synapse induces a deformation of the T cell membrane, which increases the tension experienced by the TCR/pMHC bond [137].

Some studies have suggested that force can amplify TCR antigen discrimination [138–141]. Applying force increases the dissociation rate of TCR/pMHC bonds, which reduces the variability in bond life-times and amplifies the discrimination between ligands with small differences in their off-rates [140]. Furthermore, a study using Biomembrane Force Probe (BFP) experiments found that strong agonist peptides exhibit catch-bond behaviour, whilst non-agonist peptides display slip bonds [139].

In slip bonds, the dissociation rate increases proportionally with force, like most protein bonds. In contrast, applying force to a catch-bond leads to a counter-intuitive increase in bond life-time. However, if the force exceeds an optimal threshold, it ultimately leads to increased dissociation of catch-bonds as well. Therefore, force can amplify the differences in bond life-times between agonists and non-agonists. The optimal force to elongate the life-time of catch-bonds was measured at approximately 10 pN [139]. This is consistent with another study using DNA-based nanosensors, which measured the forces experienced by the TCR/pMHC bond to be between 12-19 pN [142].

In BFP experiments, bond lifetime is measured between a live T cell and pMHC presented on a probe bead attached to a target cell. These interactions typically last several hundred milliseconds [139]. During this time, initial T cell activation events could lead to different cellular responses that affect T cell adhesion. Furthermore, BFP experiments measure up to 50 repeated contacts between a single T cell and the pMHC surface, potentially resulting in the accumulation of T cell activation signals from independent contacts. Therefore, it remains unclear whether the observed catch-bond behaviour is a cause or a consequence of T cell activation. In contrast, cell-free experiments using purified TCR and pMHC proteins found no catch-bond behaviour when measuring the TCR/pMHC interaction under force [143, 144]. These findings suggest that catch-bonds may not be an intrinsic feature of the TCR itself but rather a cellular phenomenon.

1.5 T cell co-signalling receptors

T cells scan target cells for activating ligands while migrating through tissues at a speed of approximately 5 μm per minute [145–147]. They use membrane protrusions, called microvilli, to examine the surface of antigen presenting cells within one minute [145]. When an agonist pMHC binds, the microvilli are selectively stabilised, prolonging the contact between the T cell and the APC [145]. Even though T cells recognize target pMHCs via the TCR, other surface molecules can also have an impact on activation. T cell co-signalling receptors engage different ligands on the target cell, which can influence both extracellular TCR/pMHC binding and intracellular signal transduction.

Co-signalling receptors can be broadly categorised as either co-stimulatory, promoting T cell activation, or co-inhibitory, reducing it. Unlike TCRs, co-signalling receptors are invariant, but different T cell subsets express various combinations of these accessory receptors and target cells express different combinations of their ligands. A single co-signalling receptor, such as PD-1, can bind multiple ligands on target cells, such as PD-L1 and PD-L2 [148, 149]. Similarly, some ligands, like CD80 and CD86, can bind to multiple co-signalling receptors on T cells, such as CD28 (co-stimulatory) and CTLA-4 (co-inhibitory) [150]. Co-signalling receptors modulate diverse signalling pathways through their intracellular tails [151]. Ultimately, T cells decide whether to activate or not by integrating a large number of signals from both their TCR and accessory receptors.

1.5.1 The CD8 and CD4 co-receptors

The CD8 and CD4 co-receptors are unique co-signalling molecules because they engage the same ligand as the TCR, namely MHC-I and MHC-II, respectively (Figure 1.5). They both also bind Lck intracellularly. CD8 is a heterodimer of an α and β polypeptide chains, whilst CD4 is a monomeric protein. The CD8 α and β chains are encoded in two closely associated loci on chromosome 2 with the same transcriptional orientation [152], whereas CD4 is encoded in chromosome 12 [153].

A CD8$\alpha\alpha$ homodimer can also be expressed by intraepithelial lymphocytes, mucosal associated invariant T (MAIT) cells and some memory T cells [154, 155]. However, CD8$\alpha\alpha$ is less effective in potentiating signalling than the CD8$\alpha\beta$ heterodimer [156].

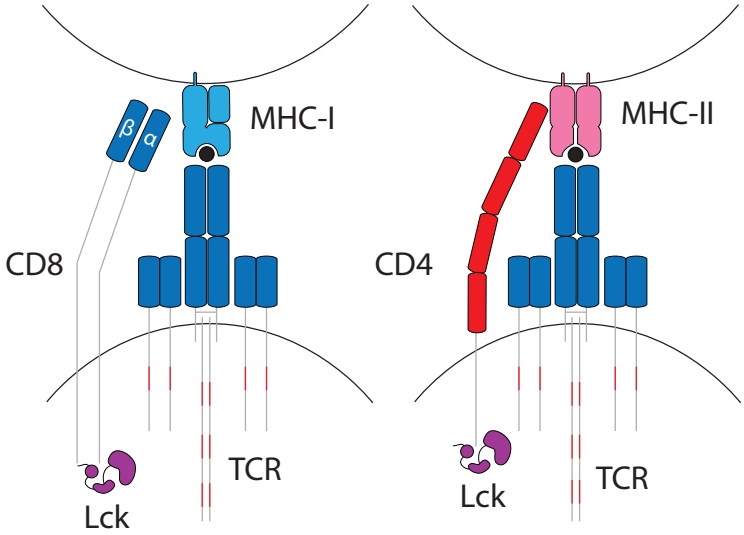


Figure 1.5: Schematic of the CD8 and CD4 co-receptors.
(Left) The CD8 co-receptor is an $\alpha\beta$ heterodimer. It binds MHC-I molecules extracellularly and Lck intracellularly. (Right) The CD4 co-receptor is a monomer. It binds MHC-II molecules extracellularly and Lck intracellularly.

CD8α binds MHC-I molecules at conserved residues on the α3 domain [157]. CD8 interacts with most MHC-I allotypes with a similar affinity, typically in the range of 100-200 μM [158, 159]. However, there are some exceptions, such as HLA-A*6801 and HLA-A*4801, which contain mutations in the α3 domain, and bind CD8 with a much lower affinity, exceeding >1 mM [159]. Both alleles are polymorphic at position 245, and replacing this residue with the consensus alanine restored binding to CD8, resulting in an affinity of ~100 μM. In contrast, the interaction between CD4 and MHC-II is much weaker. CD4 binds to MHC-II molecules at conserved residues in the α2 and β2 domains [160, 161]. The 3D binding affinity in solution was too weak to be measured. A 2D affinity of ~5000 molecules/μm^2 was calculated using B cells expressing MHC-II and a supported lipid bilayer containing CD4 [162]. However, the addition of CD2 at the bilayer was required to increase adhesion to the B cells and enable detection of CD4 binding.

Despite the relatively low binding affinity of co-receptors to MHC, it has been suggested that they help stabilise the TCR/pMHC interaction. It was found that the binding of CD8 to MHC-I slowed the dissociation rate of the TCR/pMHC interaction by approximately 2 fold [163, 164]. Similarly, despite the extremely weak initial binding affinity of CD4 to MHC-II, its affinity increased by 3-6 log when binding to a pMHC-TCR complex, resulting in a slower dissociation rate for the tri-molecular complex [165].

Increasing the binding affinity of CD8 to MHC-I to ∼30 μM was shown to increase T cell sensitivity to pMHC [166]. However, increasing the affinity further to ∼10 μM resulted in the activation of T cells even in the absence of the cognate antigen of the TCR [167, 168]. Therefore, these results suggest that the binding affinity of CD8 to MHC-I tightly regulates T cell activation and cross-reactivity. For instance, mouse CD8 binds MHC with a 3 fold higher affinity (K_D = ∼49 μM) than human CD8. This could enhance mouse T cell cross-reactivity, enabling their smaller T cell repertoire to provide full immune coverage against the large array of pathogenic peptides [168].

In addition to stabilising the TCR/pMHC interaction, it has been proposed that CD8 and CD4 primarily increase T cell sensitivity to antigen by recruiting Lck to the TCR. The intracellular tails of CD8α and CD4 bind Lck via a zinc clasp. The association with Lck is mediated by two cysteines in a CxCP motif in the co-receptors and two cysteines in the CxxC motif in Lck, which co-ordinate a Zn^{2+} ion [169]. Furthermore, one study showed that the CD8-mediated stabilisation of the TCR/pMHC interaction depends on signalling. If Lck was inhibited, this stabilisation effect was lost [170]. Therefore, this data supports the idea that Lck serves as an adaptor that indirectly associates the co-receptor and the TCR [171].

It has been shown that TCRs typically bind pMHCs with a canonical docking polarity. TCRs that bind pMHC with high affinity but in a reversed-polarity lead to poor T cell activation [172]. Although CD8 can still co-engage the same pMHC

molecules as reversed-polarity TCRs, CD8-bound Lck is mislocalised relative to the TCR-CD3 chains [172]. As a result, reversed-polarity TCRs cannot benefit from CD8-mediated Lck recruitment. These findings emphasize that while co-receptor binding may slow TCR/pMHC dissociation, Lck recruitment is the key factor in promoting T cell activation.

One study found that only ∼7% of CD4 molecules and ∼1% of CD8 molecules in thymocytes were bound to Lck [173]. Therefore, the authors proposed that upon binding a pMHC molecule, the TCR must scan multiple co-receptors until one that is bound to Lck is found. This 'co-receptor scanning' was proposed to constitute a rate-limiting kinetic proofreading step. However, recent studies found higher levels of co-receptor/Lck occupancy [129, 174]. Moreover, co-receptor unbound or 'free' Lck has been shown to trigger TCR signalling and activate T cells [175].

In summary, the co-receptor scanning model proposes that CD8 increases T cell ligand discrimination. This is further supported by studies that suggest that CD8 selectively stabilises high-affinity pMHC/TCR interactions [170, 176]. In contrast, other studies have proposed that CD8 decreases ligand discrimination, by selectively increasing activation to low affinity pMHCs [164, 177]. Therefore, the precise impact of co-receptors on ligand discrimination remains unclear.

It has also been proposed that regulating the expression level of CD8 could be a mechanism to control T cell reactivity. IFN-γ has been shown to upregulate CD8 expression, while IL-4 downregulates it [178]. In one study, male mice were injected with T cells expressing a TCR that recognises HY, a male tissue specific antigen. The adoptively transferred T cells lost reactivity to the HY antigen in the male mice, with CD8 downregulation being proposed as one of the tolerising mechanisms [179]. A different study also found that self-reactive T cells can lose CD8 expression and become double negative (CD4-CD8-) [180]. Collectively, this data suggests that CD8 downregulation may act as an alternative peripheral tolerance mechanism to clonal deletion.

1.5.2 Adhesion receptors

Cells are covered by a dense, negatively charged glycocalyx composed of abundant glycoproteins and carbohydrates, which extends for several hundred nanometers from the cell membrane [181, 182]. The TCR/pMHC bond spans approximately 15 nanometers, requiring a close interaction between the two cell membranes. Therefore, the glycocalyx hinders adhesion between T cells and their target cells and the formation of close contacts. T cell microvilli must penetrate the glycocalyx of target cells to scan their surface. On the T cell membrane, CD43 and CD45 are the key contributors to the glycocalyx, and they must be excluded, or heavily compressed, from the close contact sites [69]. Moreover, T cells express different adhesion receptors, such as CD2 and LFA-1, that strengthen the interactions with target cells.

CD2 is expressed by all T cells and its ligand, CD58, is broadly expressed in cells across different tissues [183]. CD2 binds CD58 with an affinity of 9-22 μM [184]. Despite this low binding affinity, the high density of CD2 and CD58 enables stable adhesion to the target cell by multiple binding interactions [185]. Moreover, since contacts with individual target cells are transient events, this low binding affinity enables adhesion to be quickly reversed [186]. CD2 has been shown to stabilise microvillar contacts, which increases antigen sensitivity [69]. Increasing CD2 expression levels to supra-physiological densities has been shown to enlarge the size of close contacts by 10 fold, which resulted in TCR triggering even in the absence of pMHC antigens [69]. Since the CD2/CD58 interaction spans a similar distance to the TCR/pMHC interaction ($\sim$15 nm), it has been proposed that CD2 aligns the T cell and target cell membranes at close contacts that promote TCR/pMHC binding [187]. Consequently, elongating the size of the interaction between CD2 and its ligand results in T cell inhibition, by preventing the formation of close contacts of the appropriate dimensions [188].

In addition to the effect of CD2 on adhesion, it has been proposed that its unstructured proline-rich intracellular tail can mediate signalling to promote T cell activation. The intracellular tail of CD2 contains five potential SH3 binding sites, two of which can also act as GYF binding sites [189]. Different effector and adaptor molecules such as Fyn [190], Lck [191], CD2AP [192] and CD2BP2 [193] have been shown to bind the intracellular tail of CD2. Even though no direct interaction between CD2 and the TCR-CD3 complex has been observed, they are indirectly associated and co-precipitate [194].

Another important adhesion receptor is LFA-1, which is a heterodimer of two protein chains, CD11a and CD18. The most studied LFA-1 ligand is ICAM-1 [195], but it can also bind other ligands such as ICAM-2, ICAM-3, ICAM-4, ICAM-5 and JAM-A [196–200]. LFA-1 can adopt different conformations, which modulate its activity. In its closed conformation, its affinity to ICAM-1 is very low ($\sim$1.7 mM). Upon T cell activation via other receptors, such as the TCR, LFA-1 undergoes a conformational change to adopt an open extended conformation with high affinity to ICAM-1 ($\sim$0.2 μM), in a process referred to as inside-out-signalling [201]. An intermediate affinity conformation ($\sim$5 μM) has also been identified [202, 203]. Therefore, T cell activation increases adhesion by inducing LFA-1 to adopt its open conformation.

Upon binding to its ligands, LFA-1 also undergoes outside-in-signalling. The intracellular tail of LFA-1 is bound by several adaptors, such as talins and kindlins, which connect it to the cytoskeleton [204]. Therefore, LFA-1 serves as a mechanical link between extracellular ligands and the cytoskeleton and regulates processes such as cell polarity and migration [205]. The intracellular tail of LFA-1 also associates with signalling molecules, such as Lck and ZAP-70, to provide T cell co-stimulation upon ligand binding, synergising with TCR signalling [206] .

1.6 TCR-engineered T cells in immunotherapies

T cells play an essential role in detecting and eliminating tumour cells. However, tumours frequently develop an immunosuppresive microenvironment to escape immune recognition [207]. One of the early historical indications that the immune system could be harnessed to treat cancer was the discovery that injecting patients with heat-inactivated bacteria led to tumour remission [208]. Many different strategies have been explored to induce the expansion and activation of T cells to treat cancer, including the administration of IL-2 [209] or the use of blocking antibodies against T cell inhibitory receptors, also known as immune checkpoint blockade [210].

One promising immunotherapy approach, known as adoptive cell therapy (ACT), is to isolate T cells from a patient, expand them *ex vivo* and re-infuse them into the patient to fight the tumour [211] (Fig. 1.6). The adoptively transferred cells can either be endogenous T cells expressing a diverse repertoire of TCRs or T cells engineered with a novel tumour-specific TCR.

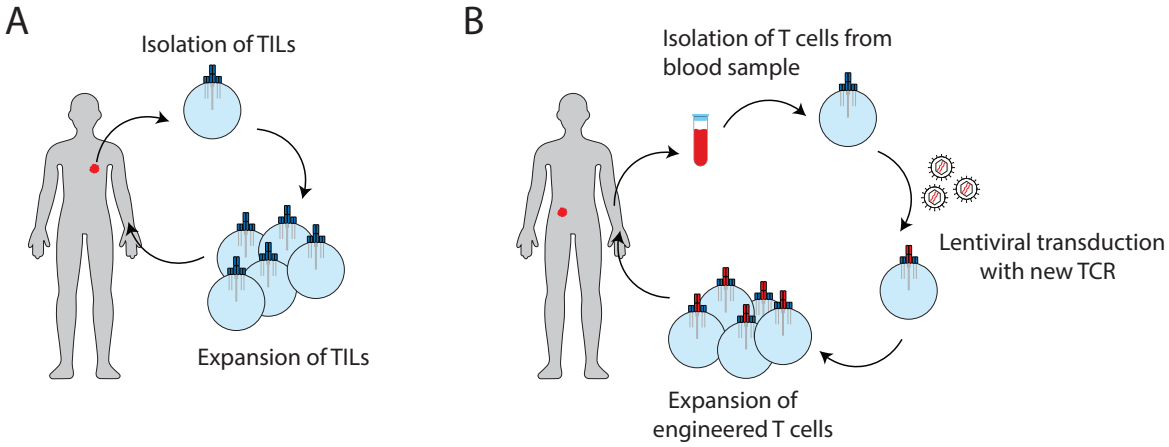


Figure 1.6: Adoptive T cell therapies.
(A) Tumour-infiltrating lymphocyte therapy. TILs are isolated from a patient's tumour (shown in red), expanded *ex vivo* and re-infused into the patient. (B) TCR-engineered T cell therapy. T cells are isolated from the patient's blood and engineered to express a new cancer-specific TCR. The engineered cells are expanded *ex vivo* and re-infused into the patient.

Tumour-reactive T cell clones have been shown to accumulate within tumours [212]. Therefore, tumour-infiltrating lymphocytes (TILs) were the first source of T cells used for ACT. TILs can be isolated from tumour resections, then expanded in the presence of IL-2 and administered to patients [213]. However, the antigen specificities of these expanded TILs are unknown, meaning that they could include many irrelevant T cell clones that do not recognise cancer peptides. Alternatively, cancer-reactive T cell clones can be enriched by co-culture with APCs pulsed with tumour lysates [214] or pulsed with specific tumour peptides [215]. This results in an ACT product containing a population of T cells with polyclonal TCRs that recognize the selected cancer peptides. Overall, the adoptive transfer of patient-derived TILs has shown promising clinical responses [216] and has now been approved by the FDA for the treatment of melanoma [217].

Despite these promising outcomes, TIL-based ACT has limited clinical efficacy. One potential reason is that some patients might not have any endogenous potent tumour-reactive T cell clones available for expansion. Therefore, engineering T cells with a novel TCR has been proposed to overcome these challenges. The key advantage of TCR gene transfer is that a TCR specific to a tumour epitope can be selected and then be broadly applied to any patient whose tumour expresses that antigen and HLA allele. This approach enables more tailored targeting and potentially a wider applicability across different cancer patients. Dozens of companies are running over a hundred clinical trials using TCR-engineered T cells against different tumour antigens (Fig. 1.7). The first of these therapies to receive FDA approval is afami-cel, which uses a MAGE-A4 specific TCR to treat synovial sarcoma [218, 219].

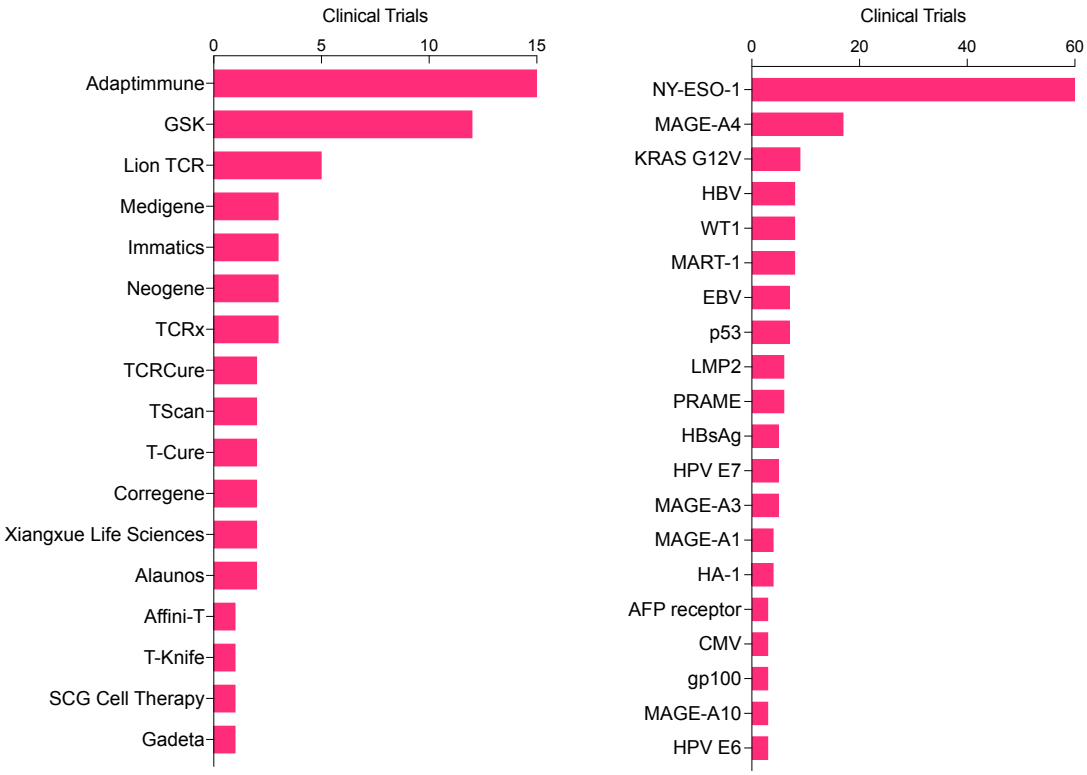


Figure 1.7: Landscape analysis of adoptive T cell therapies.
(Left) Number of clinical trials registered per company. (Right) Number of clinical trials registered per target antigen. Source data for the analysis was obtained from the Beacon database (Hanson Wade).

The first reported TCR-T clinical study targeted MART-1, a lineage-specific antigen in melanocytes, which is overexpressed in melanoma [220]. However, in a clinical trial using a high affinity MART-1 TCR, healthy melanocytes in the eye and ear were also targeted by the engineered T cells, resulting in severe side effects such as hearing loss [221]. A different clinical trial targeting CEA, a gastrointestinal differentiation marker, showed promising anti-tumour responses, but all patients developed severe inflammatory colitis [222]. Therefore, these trials demonstrate the risk of on-target off-tumour toxicity when targeting tissue-specific antigens, whose expression is not restricted to tumours.

Oncoviral infections can increase the probability of developing cancer. For example, chronic Hepatitis B virus (HBV) infection is associated with hepatocellular carcinoma [223]. TCRs have been developed against HBV antigens, such as HBsAg,

which are expressed in cancer cells but not in healthy liver tissue [224]. Similarly, human papillomavirus (HPV) infection is also associated with multiple cancer types. The HPV oncogenes E6 and E7 drive malignant transformation [225]. TCRs specific to E6 and E7 have been developed and tested in clinical trials [226, 227]. Therefore, targeting oncoviral proteins by ACT is an alternative strategy without the risk of on-target off-tumour toxicities.

Cancer-testis antigens are also an attractive target for ACT because they are normally only expressed in germline cells but they become re-activated in many different cancer types [228]. Moreover, germline cells do not express MHC-I molecules [229]. Therefore, targeting cancer-testis antigens reduces the risk of on-target off-tumour toxicities. TCRs specific to NY-ESO-1 [230], PRAME [231], MAGE-A1 [232], MAGE-A3 [233], MAGE-A4 [218] and MAGE-A10 [234] have been developed.

Tumours frequently accumulate mutations, leading to the presentation of neoantigens on MHC molecules. These neoantigens are not presented in healthy cells, making them appealing targets for ACT. Some neoantigens may arise from single-point mutations in self peptides, which makes them hard targets to be discriminated by T cells. More interestingly, some single point mutations in the MHC anchor residues of the peptide can result in the presentation of completely new epitopes, unique to cancer cells [235]. Many of these neoantigens are specific to individual cancer patients, arising either from passenger or driver mutations. However, some driver-mutations, which promote transformation or tumour growth, are recurrent across multiple patients, giving rise to public neoantigens [236]. For example, some driver-mutations in the GTPase KRAS have been identified as public neoantigens. TCRs specific to the KRAS-G12D [237] and KRAS-G12V mutations [238] have been developed and tested for therapy.

TCRs often display low binding affinity to tumour peptides due to thymic negative selection [116]. Therefore, once a suitable TCR has been identified, its affinity can be artificially increased by introducing mutations to their CDR loops [239–242], which improves anti-tumour responses [230, 243]. An alternative strategy is to isolate TCRs from humanised mice. These TCRs have higher affinity to the target cancer antigens because they did not undergo negative thymic selection against human proteins in the mouse [244].

However, even if the target cancer antigen is not expressed in healthy tissues, there is a risk that introducing a novel TCR that has not undergone thymic selection in the patient could result in cross-reactivities to off-target peptides [233, 245, 246]. For instance, in one clinical trial, the affinity-matured a3a TCR targetting MAGE-A3, a cancer-testis antigen, displayed cross-reactivity to a lower affinity off-target peptide from the cardiac muscle protein Titin, causing lethal autoimmune toxicities [233, 245]. In contrast, other affinity-matured TCRs targeting NY-ESO-1 or MAGE-A4 have been safely tested in clinical trials without off-target cross-reactivities [218, 230]. Therefore, it remains unclear whether the risk of cross-reactivity is primarily influenced by the affinity-maturation process or by the selection of the target tumour peptide.

Predicting potential off-target cross-reactivities of a TCR before first-in-human clinical trials is challenging [247–249] due to the lack of animal models or cell lines that represent the entire human proteome and HLA allele diversity. Therefore, there is an urgent need to identify methods to reduce the cross-reactivity of T cells to enhance the safety of TCR-T cell therapies.

Chapter 2

The role of T cell surface proteins in ligand discrimination

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2.1 Introduction

T cells must discriminate between foreign and self-peptides to mediate effective immunity whilst minimising the risk of autoimmunity. In the thymus, T cell clones that bind strongly to self-peptides are eliminated by negative selection [250, 251]. Therefore, T cells in the periphery typically bind to non-self peptides with higher affinity than to self-peptides. This enables T cells to discriminate between self and non-self peptides based on their affinity [114, 125, 133, 144, 252]. In addition to discrimination based on TCR/pMHC binding, the kinetic proofreading model provides a signalling-based mechanism for discrimination [253]. This model postulates that a time delay between pMHC binding and TCR signalling amplifies differences in the TCR/pMHC binding lifetime, with longer lifetimes more likely to transduce TCR signalling. A prediction of the model is that the TCR/pMHC lifetime (or off-rate) will correlate with T cell activation, which is supported by a large number of studies [114, 128, 164, 254].

T cells recognize their target antigens via their TCR, but other co-signalling receptors at the T cell membrane can influence the outcome of this recognition event [151]. These co-signalling receptors have largely been classified into co-stimulatory or co-inhibitory receptors, depending on whether they increase or decrease activation [151, 255]. However, there is limited data on their ability to selectively impact high or low affinity pMHC antigens and therefore, it is unclear whether they impact ligand discrimination.

One molecule that has previously been suggested to influence discrimination is the CD8 co-receptor. CD8 plays an essential role in thymic selection and T cell development. However, it can be dispensable for activation in mature peripheral T cells [240, 256]. CD8 improves T cell activation by both stabilising the TCR-pMHC interaction [170] and recruiting Lck to signalling TCRs [257]. However, the exact contribution of CD8 to ligand discrimination has been debated. On the one hand, it has been proposed that CD8 can selectively stabilise high-affinity TCR/pMHC interactions. Therefore, this positive feedback loop amplifies differences in binding

affinity and enhances ligand discrimination [170, 176]. On the other hand, it has also been proposed that CD8 slows the dissociation rate of TCR/pMHC interactions, which preferentially increases T cell sensitivity to low affinity peptides and decreases ligand discrimination [163, 164, 177, 258].

Adhesion receptors such as CD2 and LFA-1 have been shown to increase T cell sensitivity to pMHC antigens by binding to their ligands on target cells, CD58 and ICAM-1, respectively [186, 259–261]. However, their impact on ligand discrimination is not fully understood. It has been proposed that the engagement of CD2 and LFA-1 could increase T cell ligand discrimination by shielding the TCR/pMHC interaction from mechanical forces, which preferentially reduce the lifetime of high affinity interactions [114, 144]. However, the effect of CD2 and LFA-1 on discrimination has been studied individually, and not in combination. Moreover, the evidence that they impact ligand discrimination was obtained by stimulating T cells with plate immobilised pMHC with or without CD58 and ICAM-1. Therefore, there is no information on whether they impact ligand discrimination at cellular interfaces.

CD5 is a negative regulator of T cell activation, but the precise mechanism of inhibition is unclear [262–264]. CD5 associates with inhibitory molecules such as Ubash3a and Cbl-b and represses TCR signalling [265]. Additionally, CD5 expression levels are associated with the affinity of the TCR to self peptides during positive thymic selection [266, 267]. This fine-tuning of TCR signalling has been proposed to reduce the risk of autoimmune T cell activation against self-peptides in the periphery [264]. Antibody blockade or the knock-out of CD5 have been shown to enhance anti-tumour activity in TCR and CAR-T cells [263, 268, 269]. However, it is unknown whether the inhibitory effect of CD5 is dependent on TCR/pMHC affinity and its role on ligand discrimination remains unclear.

CD43 is a large glycosylated protein abundantly expressed at the surface of T cells, and excluded from the immune synapse [270]. It has been proposed to be a negative regulator of T cell activation by reducing adhesion to target cells by steric hindrance. The knock-out of CD43 led to enhanced cell adhesion and proliferation both in mice [271–274] and in the human CEM T cell line [275]. Additionally, it has been found that the intracellular tail of CD43 can negatively regulate T cell responses, independently of its ectodomain [272, 273]. Furthermore, it has been predicted that the exclusion of large surface molecules such as CD43 from close contacts could increase ligand discrimination by inducing a deformation of the membrane that preferentially increases the off-rates of low-affinity peptides [137]. However, the impact of CD43 on ligand discrimination has not been previously experimentally measured.

In this chapter, we quantified the impact of CD8, CD2, LFA-1, CD5 and CD43 on T cell ligand discrimination using the NY-ESO-1 specific c259 TCR as a model system. We knocked-out these molecules on human primary T cells transduced with the c259 TCR and activated them with peptides presented on U87 glioblastoma cancer cells. We find that CD43 or the adhesion receptors CD2 and LFA-1 do not impact antigen discrimination. The knock-out of CD8 selectively reduced T cell activation to lower affinity ligands, whilst the knock-out of CD5 improved activation to low affinity peptides. Therefore, CD8 reduces ligand discrimination whilst CD5 enhances it. Taken together, these results offer a direct quantitative comparison of the impact of different T cell surface molecules on ligand discrimination. We show that T cell ligand discrimination results from a combination of TCR binding discrimination and TCR signalling discrimination, which itself can be manipulated by co-signalling receptors.

2.2 Results

2.2.1 Establishing a platform to study ligand discrimination

We developed a pipeline to investigate the role of different T cell proteins in ligand discrimination. We isolated human primary CD8+ T cells from healthy donors, knocked-out a gene of interest by CRISPR/Cas9 electroporation and transduced them with a model TCR. Subsequently, we co-cultured these T cells with cancer cells pulsed with a titration of different peptides and measured T cell activation (Figure 2.1). The human U87 glioblastoma cell line has previously been used as an antigen presenting cell in T cell studies [261, 276, 277]. We examined three different measures of T cell activation: (I) flow cytometry analysis of cell surface expression of the 4-1BB activation marker, (II) ELISA analysis of the secretion of IFNγ and (III) cytotoxic activity of T cells with a luciferase-based assay. Target cell death can be quantified in a simple and sensitive way by monitoring the release of a cytosolic expressed luciferase using a microplate reader [278, 279]. We constructed a U87 glioblastoma reporter cell line expressing the thermostable marine luciferase Nluc without a signal peptide. Therefore, Nluc activity in the co-culture supernatant serves as a measure of target cell death.

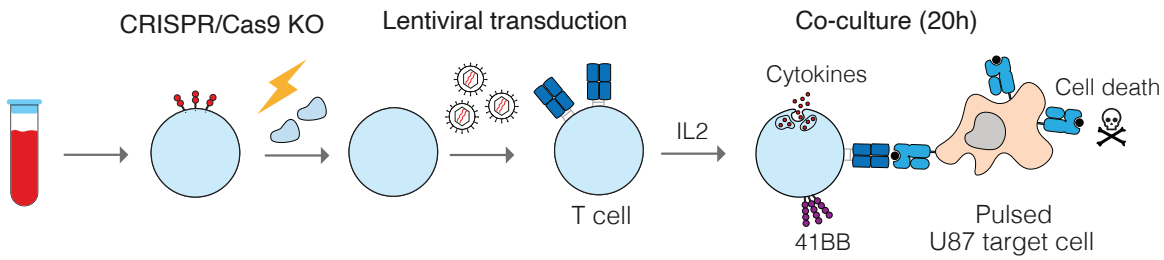


Figure 2.1: Experimental workflow to produce gene knockout primary human TCR-T cells.
T cells were isolated from the blood of healthy donors, electroporated with Cas9/sgRNA ribonucleoprotein complexes, transduced with lentivirus encoding a TCR and expanded with IL2. After expansion, the T cells were co-cultured with peptide-pulsed target cells for 20 hours and T cell activation was measured.

To quantify the contribution of a T cell protein to ligand discrimination, we compared the activation of WT and KO T cells to peptides with different affinities to the TCR (Figure 2.2). Firstly, we define peptide potency (P_{15}) as the concentration of peptide required to elicit 15% of the maximal T cell response in order to include the lowest affinity pMHCs. If a KO reduces activation to a particular peptide, the fold change in potency compared to WT cells (ΔP_{15}) is greater than 0. Alternatively, if a KO increases activation to a peptide, its ΔP_{15} is smaller than 0. Subsequently, we plot ΔP_{15} over peptide affinity. If a T cell protein affects discrimination, ΔP_{15} will vary for peptides of different affinities and the slope of this plot will be significantly different from zero. A positive slope indicates an increase in ligand discrimination, because the fold change in activation (ΔP_{15}) is larger for lower affinity peptides. Conversely, a negative slope indicates a decrease in ligand discrimination, because activation is selectively increased against lower affinity peptides, leading to smaller ΔP_{15} values.

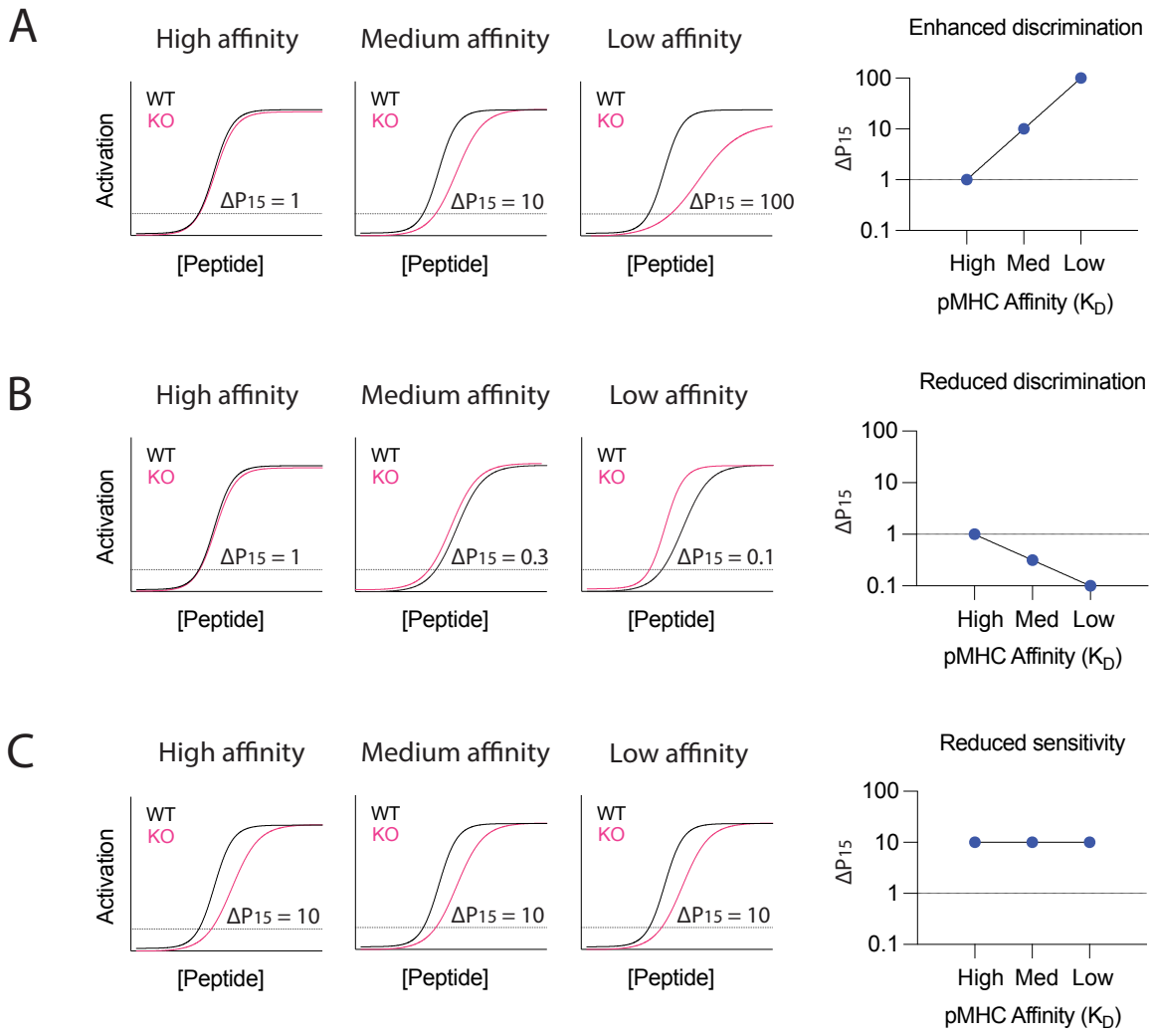


Figure 2.2: Method to analyse the impact of a gene knock-out on T cell ligand discrimination.
Ligand potency (P_{15}) is the ligand concentration required to activate 15% of maximum response. The fold change in potency between KO and WT T cells (ΔP_{15}) is plotted for different ligand affinities. **(A)** Example of a gene knock-out that enhances ligand discrimination. **(B)** Example of a gene knock-out that reduces ligand discrimination. **(C)** Example of a gene knock-out that reduces ligand sensitivity to all peptides, regardless of affinity. In the left panels, dashed line indicates P_{15}. In the right panels, dashed line indicates fold change of 1.

We selected the c259 TCR contained in the investigational TCR-T therapy lete-cel as our model receptor to study T cell ligand discrimination [280, 281]. The c259 TCR recognises a 9-mer NY-ESO-1 peptide (SLLMWITQC) presented on HLA-A*02:01. It has previously been shown that a mutation of the anchor residue at the 9th position from cysteine to valine increases peptide stability on the MHC [282, 283]. Therefore, we decided to use this peptide variant, to which we refer as 9V (SLLMWITQ**V**). First, we confirmed expression of the c259 TCR in transduced human primary T cells by 9V-pMHC tetramer staining (Figure 2.3A). Secondly, we loaded 9V and different single residue peptide mutants on U87 cells that endogenously express HLA-A*02:01 to stimulate c259 TCR-T cells (Figure 2.3B). We find that T cells can be activated by all 7 peptides, displaying a difference of ∼10,000 fold in potency between the strongest and weakest peptides.

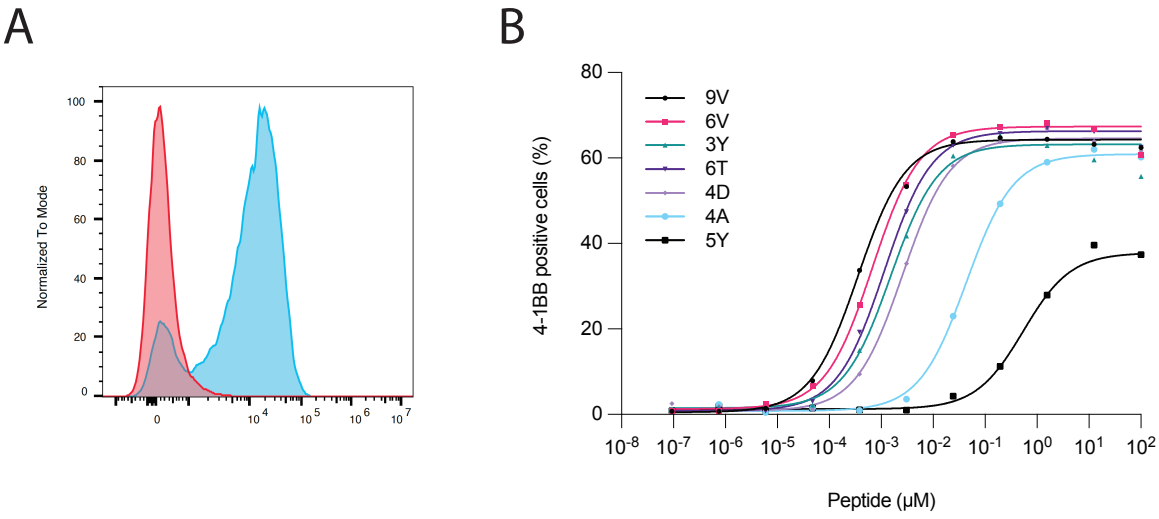


Figure 2.3: Characterisation of TCR expression and functional activity of c259 TCR-T cells.
(A) Flow cytometry analysis of TCR expression in c259 TCR-T cells (Blue) and untransduced T cells (Red). **(B)** c259 TCR-T cells were co-cultured with peptide-pulsed U87 cells. After 20 hours, expression of the activation marker 4-1BB was measured.

We then measured the binding affinity of the c259 TCR to this altered peptide ligands (APL) panel on HLA-A*02:01 by Surface Plasmon Resonance (SPR) using a previously published constrained B_{max} method [114] (Figure 2.4 A). The binding curve for low affinity pMHCs does not saturate even at the highest concentrations of TCR used. Therefore, the fitted TCR B_{max} is not accurate, which means that the fitted K_D (defined as the concentration of TCR that elicits 50% of B_{max}) is not accurate. BBM.1 is a conformation-specific antibody that only binds correctly folded pMHC on the chip surface. Therefore, there is a linear relationship between BBM.1 binding (RU) and the B_{max} of the TCR. An empirical standard curve was created using high affinity-peptides which saturate the binding curve and allow for accurate measurements of the TCR B_{max} (Figure 2.4 B). This calibration curve was then used to constrain the TCR B_{max} of other low-affinity peptides using the BBM.1 antibody binding. We find a broad range of K_D values ranging from ∼1-150 μM (Figure 2.4, Table 2.1).

Table 2.1: The c259 TCR affinities (K_D) to the NY-ESO-1 peptide variants.

Abbr	Sequence	Mean	SD	N
9V	SLLMWITQV	1.240	0.129	3
6V	SLLMWVTQV	3.538	0.283	2
3Y	SLYMWITQV	10.350	1.634	4
6T	SLLMWTTQV	27.628	4.598	11
4D	SLLDWITQV	41.777	4.703	7
4A	SLLAWITQV	62.319	10.313	8
5Y	SLLMYITQV	157.666	23.818	3

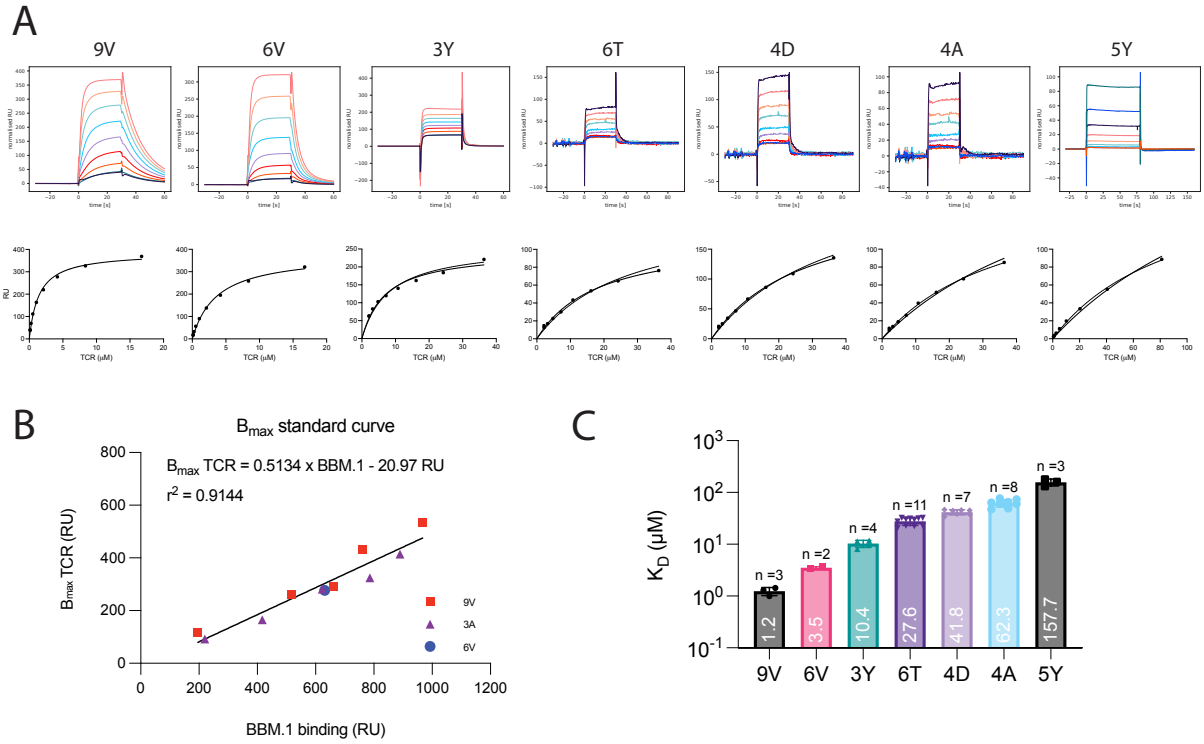


Figure 2.4: Establishing a panel of peptides that bind the c259 TCR with a range of affinities as measured by SPR at 37°C.
(A) (Top) Representative SPR sensograms depicting injections of increasing concentrations of the c259 TCR. (Bottom) Representative steady-state curves of c259 TCR binding to different pMHCs. 3D affinity (K_D) was calculated by constraining B_{max} (dashed line) or fitting B_{max} (solid line). **(B)** Empirical standard curve relating the binding of the BBM.1 antibody (x-axis) to the fitted TCR B_{max}. Only data for the higher-affinity pMHCs is used to generate the standard curve. **(C)** Steady-state binding affinity for the selected 7-peptide panel. Barplot represents mean $K_D \pm$ SDs. The affinities were calculated by constraining B_{max} to the value obtained from the standard curve in (B) based on the amount of BBM.1 antibody that bound the chip surface (see Materials and Methods). All data fitting was performed using a one site-specific binding model in GraphPad Prism.

We plot the potency (P_{15}) values from Figure 2.3B over the measured K_D values from Table 2.1 and find that T cell activation correlates non-linearly with pMHC affinity (Figure 2.5). Furthermore, we find that there is an affinity threshold of approximately $\sim$10 μM above which T cell activity cannot be enhanced, consistent with previous studies [127, 284].

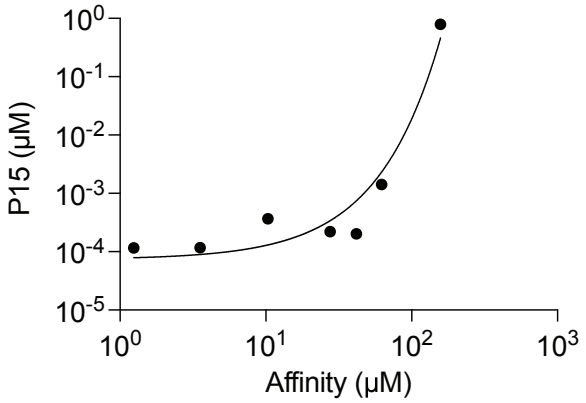


Figure 2.5: c259 TCR activation correlates with pMHC affinity.
Potency data (P_{15}) from Figure 2.3B is plotted over K_D values from Table 2.1.

Next, we used the c259 TCR and this APL panel to quantify the role of different T cell surface molecules in ligand discrimination. We focused on five molecules that had previously been suggested to impact ligand discrimination (CD43 [137], CD2, LFA-1 [114, 144], CD5 [267] and CD8 [285]). We optimised a protocol for the electroporation of human primary T cells with Cas9:sgRNA ribonucleoproteins (RNPs). Following several rounds of synthetic guide RNA (sgRNA) design and testing, we obtained a knock-out efficiency of up to 95% for the different target genes. The remaining positive T cells were stained with an anti PE-conjugated antibody and then pulled down with magnetic anti-PE beads (see Materials and Methods). We obtained pure populations of 100% negative primary T cells for the different targeted genes (Figure 2.6 A).

We stained the different T cell populations with pMHC tetramers to confirm that the knock-out process did not modify TCR expression level (Figure 2.6 B). We found that only CD8 knock-out T cells displayed lower pMHC tetramer staining, which was expected since CD8 binds MHC and has been shown to increase tetramer binding [286]. Therefore, we stained wild-type and CD8 knock-out T cells with a TCR Vβ13.1 antibody to confirm that expression of the c259 TCR was not altered (Figure 2.7).

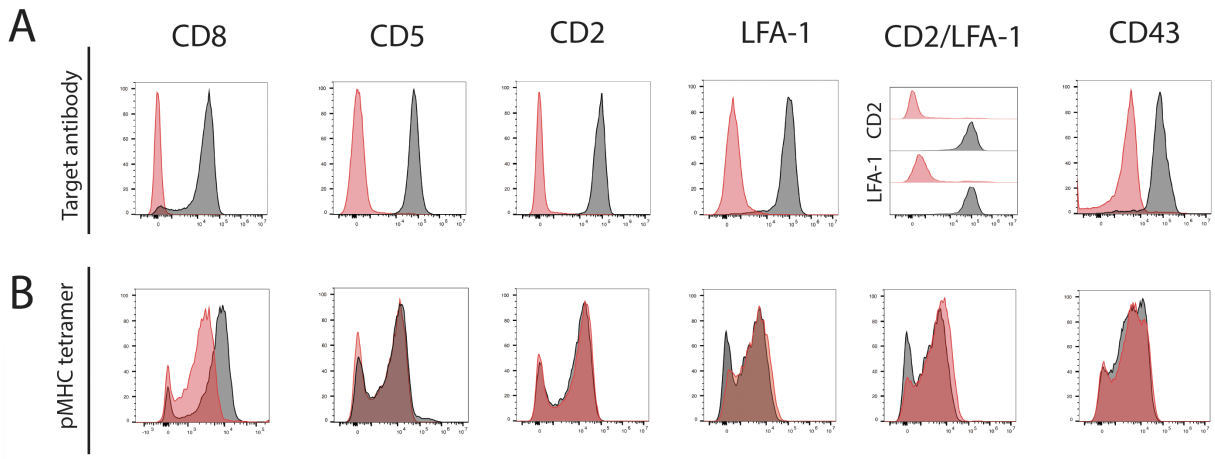


Figure 2.6: Knocking-out different T cell surface proteins by CRISPR/Cas9. **(A)** Flow cytometry analysis of KO efficiency for the different targets. **(B)** Flow cytometry analysis of c259 TCR expression using 9V-pMHC tetramers. KO T cells shown in red or WT T cells shown in black.

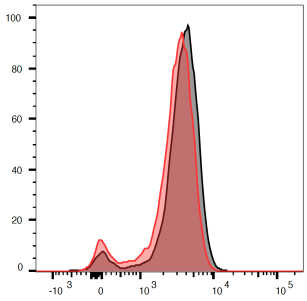


Figure 2.7: CD8 knock-out does not modify TCR expression.
Flow cytometry analysis of c259 TCR expression using a TCR Vβ13.1 antibody. KO T cells shown in red or WT T cells shown in black.

2.2.2 The role of CD43 in T cell ligand discrimination

The knock-out of CD43 did not have any impact on T cell sensitivity to the high affinity 9V NY-ESO-1 peptide (Figure 2.8). Interestingly, we observed a small increase in target cell killing and 4-1BB upregulation against the lowest affinity peptide (5Y). When combining the data from the whole peptide panel, no significant difference in ligand discrimination was measured for the target cell killing data (Figure 2.9). The slope of the ΔP_{15} over K_D plots was technically significant for the 4-1BB and IFNγ activation data (p = 0.0396 and p = 0.0469). However, ΔP_{15} was approximately 1 for all peptides except 5Y. Taken together, we conclude that CD43 does not impact T cell antigen recognition in this experimental system.

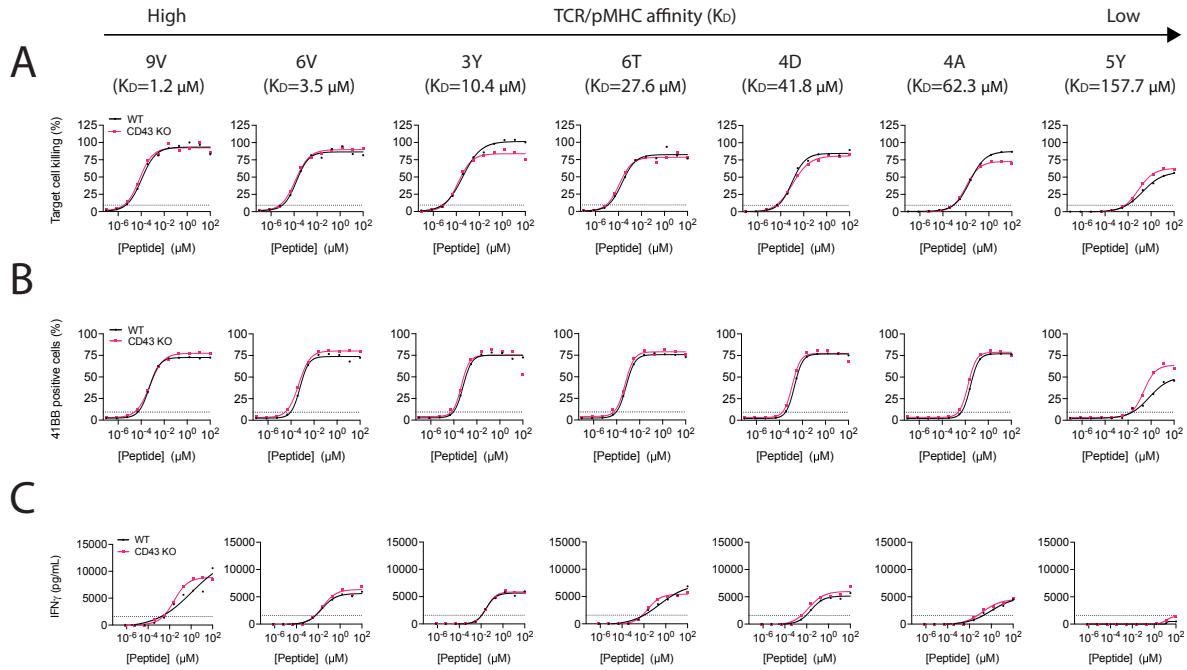


Figure 2.8: Measuring the impact of CD43 KO on T cell activation.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD43 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of N=3 independent experiments with different blood donors.

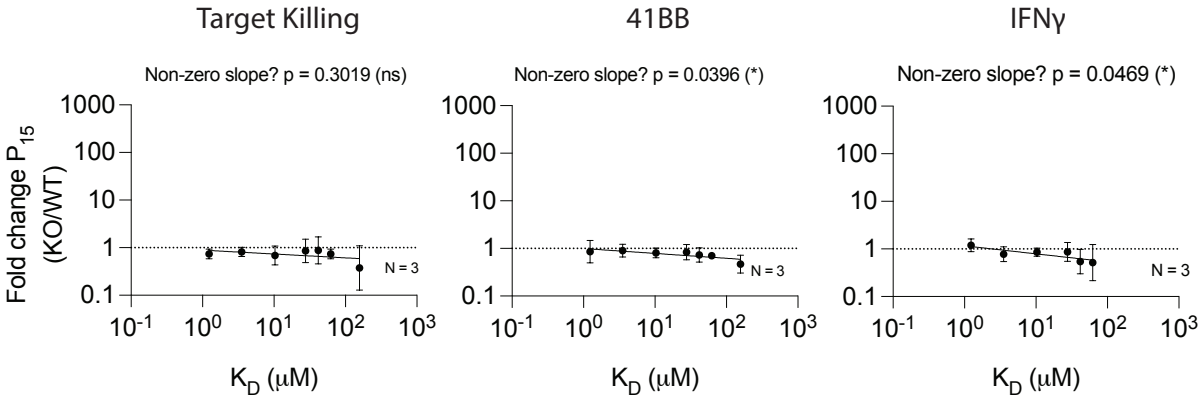


Figure 2.9: CD43 KO does not impact T cell activation.
Fold change in potency (P_{15}) between CD43 KO and WT c259 TCR-T cells from (Fig. 2.8) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of N=3 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

2.2.3 The role of the LFA-1 and CD2 adhesion receptors in T cell ligand discrimination

LFA-1 is a heterodimer composed of CD11a and CD18. The CD18 chain can also associate with CD11b, CD11c or CD11d to form integrin complexes with different roles in immunity [287, 288]. Therefore, we decided to knock-out the CD11a gene to specifically abolish LFA-1 expression. The binding of LFA-1 to its major ligand ICAM-1 has been previously demonstrated to increase T cell sensitivity [260, 261]. LFA-1 can also bind to other ligands with lower affinity, such as ICAM-3 [197], ICAM-5 [199] and JAM-A [200]. The U87 cell line that we used to activate T cells endogenously express ICAM-1, ICAM-3, ICAM-5 and JAM-A, as confirmed by mRNA sequencing studies [289].

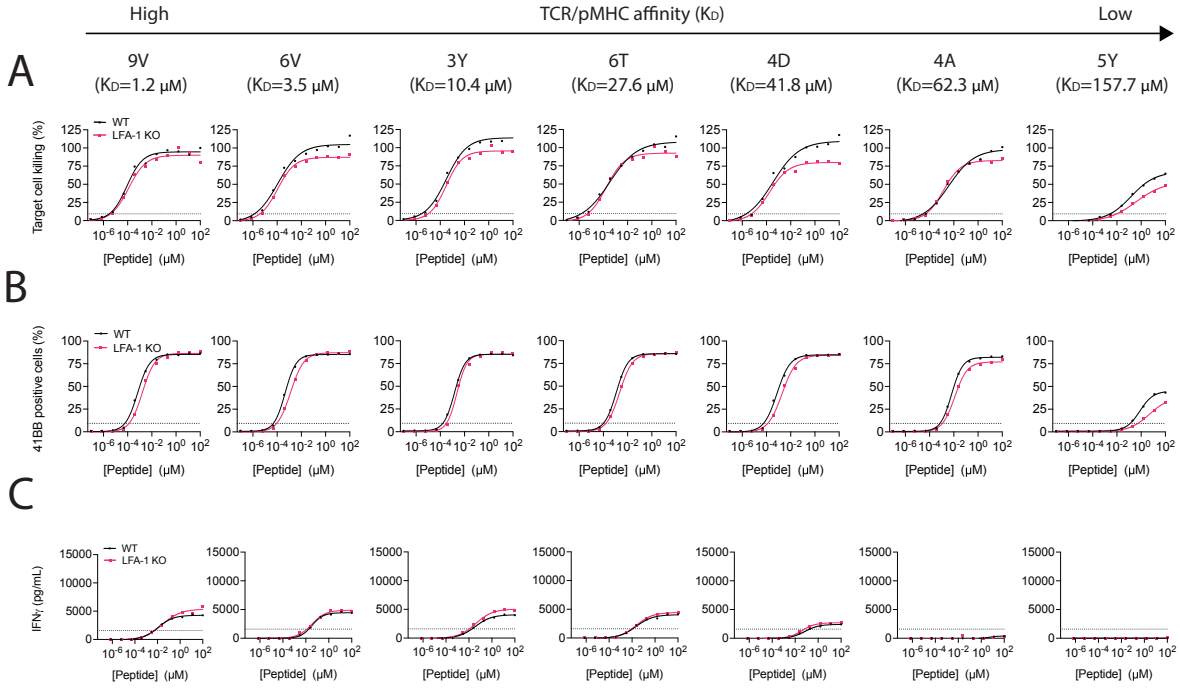


Figure 2.10: Measuring the impact of LFA-1 KO on T cell activation. U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or LFA-1 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of at least N=3 independent experiments with different blood donors.

LFA-1 knock-out had a very small effect on ligand sensitivity against all of the tested peptides (Figure 2.10). Across the peptide panel, the fold change in activation (ΔP_{15}) was between ∼1-5 for all peptides tested. There was no significant difference in ligand discrimination when analysing 4-1BB expression or IFNγ secretion (Figure 2.11). Even though the slope of the ΔP_{15} over K_D plot was significantly different from zero (p = 0.0234), taken together, these results suggest that LFA-1 does not impact ligand discrimination.

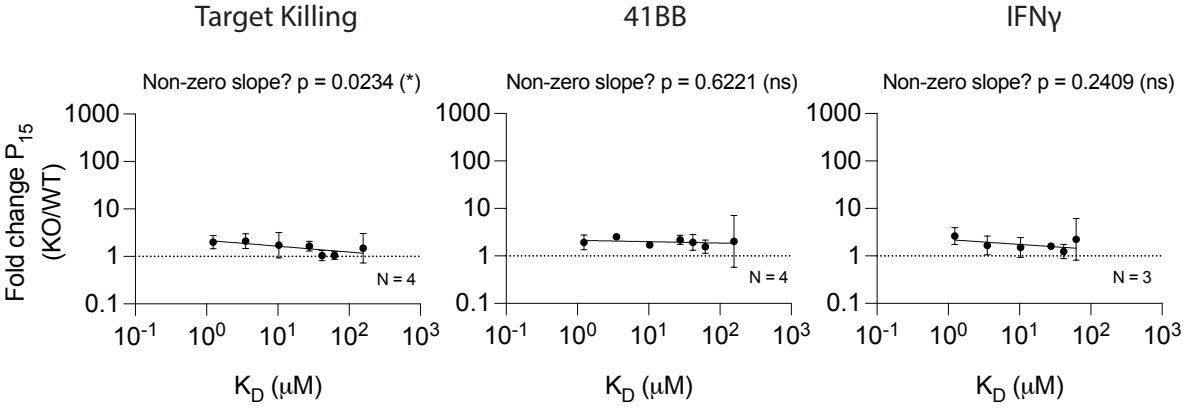


Figure 2.11: LFA-1 KO does not impact T cell activation.
Fold change in potency (P_{15}) between LFA-1 KO and WT c259 TCR-T cells from (Fig. 2.10) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of at least N=3 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

The knock-out of CD2 resulted in a stronger loss of sensitivity than LFA-1, of approximately ∼5-10 fold for all peptides tested. This effect was consistent across the different T cell activation metrics (killing, 4-1BB and IFNγ secretion) (Figure 2.12). There was no significant change in ligand discrimination compared with WT cells when analysing any of the T cell activation outputs (Figure 2.13).

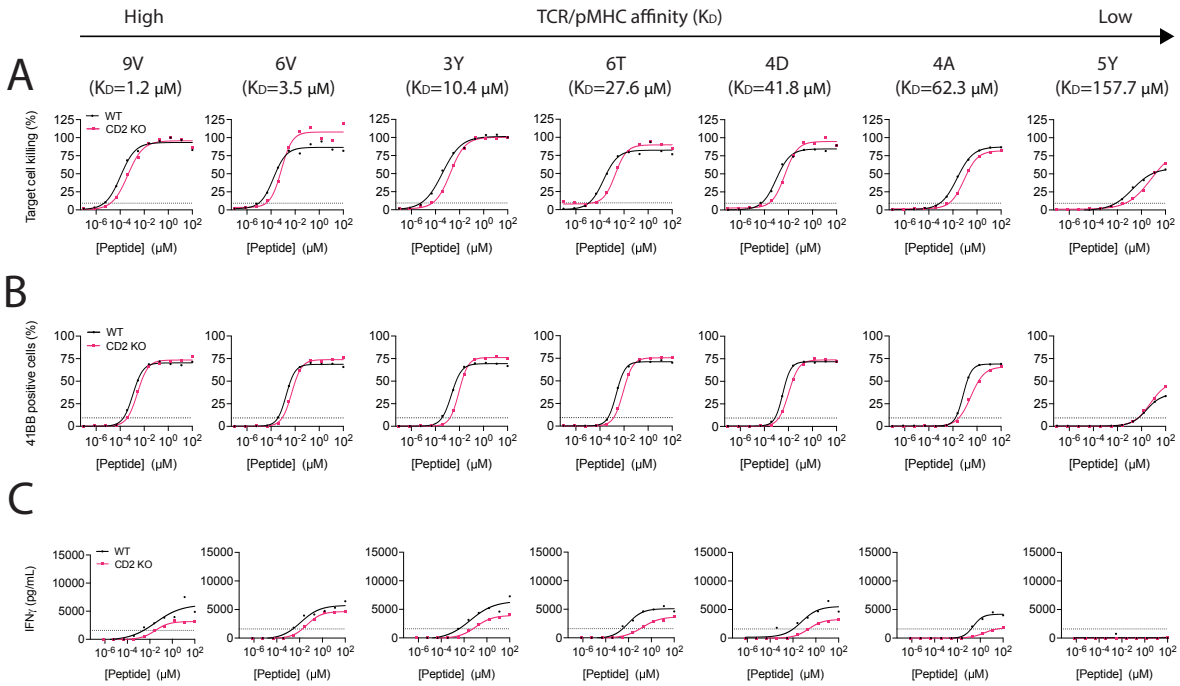


Figure 2.12: Measuring the impact of CD2 KO on T cell activation.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD2 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of at least N=2 independent experiments with different blood donors.

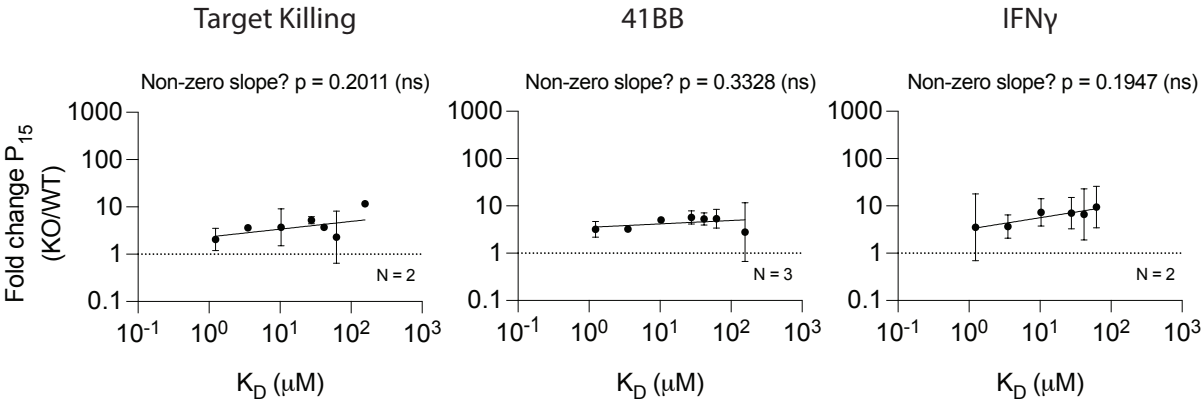


Figure 2.13: CD2 KO reduces T cell ligand sensitivity.
Fold change in potency (P_{15}) between CD2 KO and WT c259 TCR-T cells from (Fig. 2.12) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of at least N=2 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

Since the individual knock-out of either CD2 or LFA-1 did not result in large defects in T cell sensitivity, we decided to investigate the impact of simultaneously knocking-out both receptors in T cell activation. The double KO T cells displayed a stronger loss of sensitivity for all peptides tested of ∼10 fold (Figure 2.14). There were no differences in ligand discrimination when analysing the target killing or 4-1BB expression data (Figure 2.15). Interestingly, there was a significant increase in discrimination when analysing the IFNγ secretion data. This was surprising, because previous studies had predicted that the loss of adhesion receptors would lead to a decrease in ligand discrimination [114, 144]. However, for the IFNγ data, most peptides in the panel had a ΔP_{15} value of approximately 10, with 4A being one clear outlier. Since cytokine production in response to 4A was very low and almost at the detection limit, these results are inconclusive.

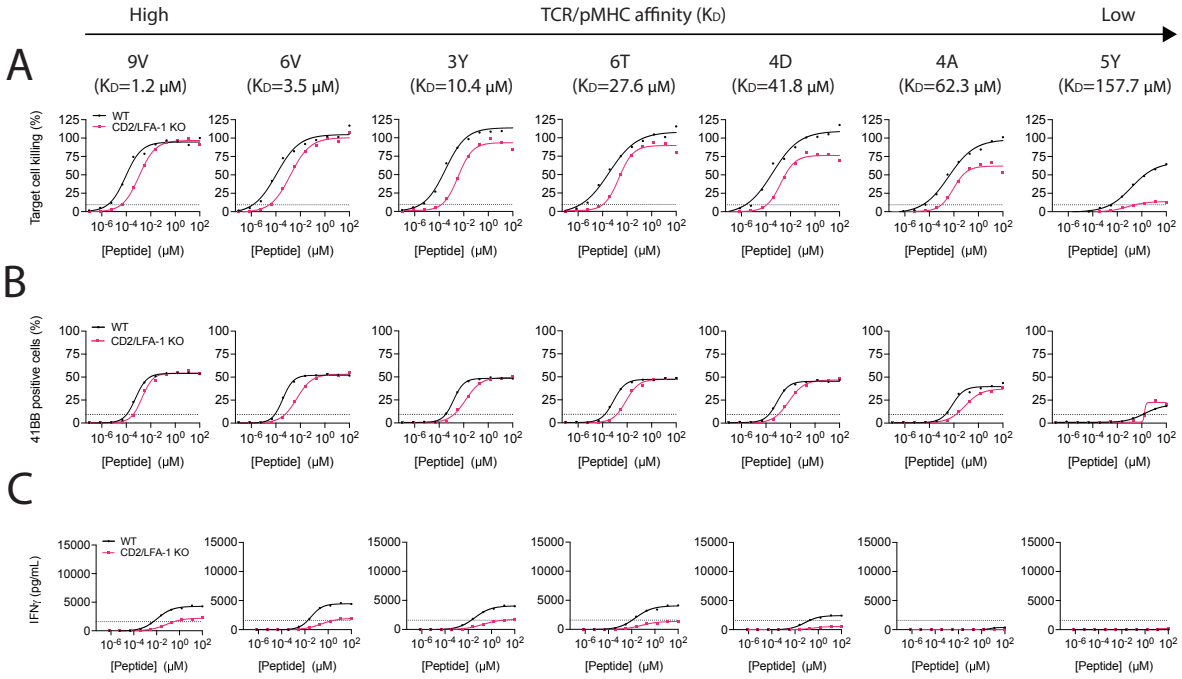


Figure 2.14: Measuring the impact of a double CD2 and LFA-1 KO on T cell activation.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD2/LFA-1 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of N=3 independent experiments with different blood donors.

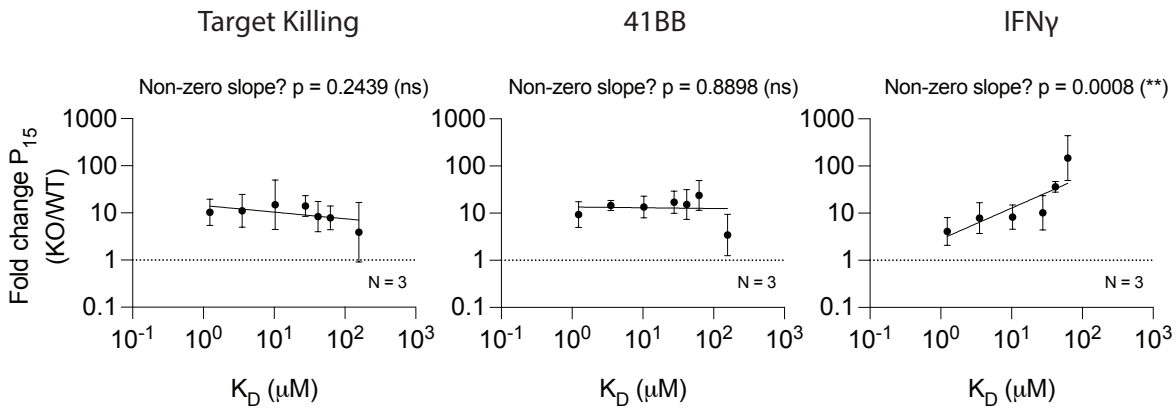


Figure 2.15: The double KO of CD2 and LFA-1 reduces T cell ligand sensitivity. Fold change in potency (P_{15}) between CD2/LFA-1 KO and WT c259 TCR-T cells from (Fig. 2.14) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of N=3 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

2.2.4 The role of CD5 in T cell ligand discrimination

The knock-out of CD5 did not impact activation against the high affinity 9V peptide, but it selectively increased activation against lower affinity peptides (Figure 2.16). The increase in killing of target cells pulsed with low affinity peptides was particularly striking, increasing the potency of these peptides by up to ∼100 fold. There was a significant decrease in ligand discrimination when analysing any metric of T cell activation, target killing, 4-1BB expression or IFNγ secretion (Figure 2.17). Collectively, these results suggest that CD5 increases ligand discrimination in T cells by preferentially inhibiting lower affinity peptides.

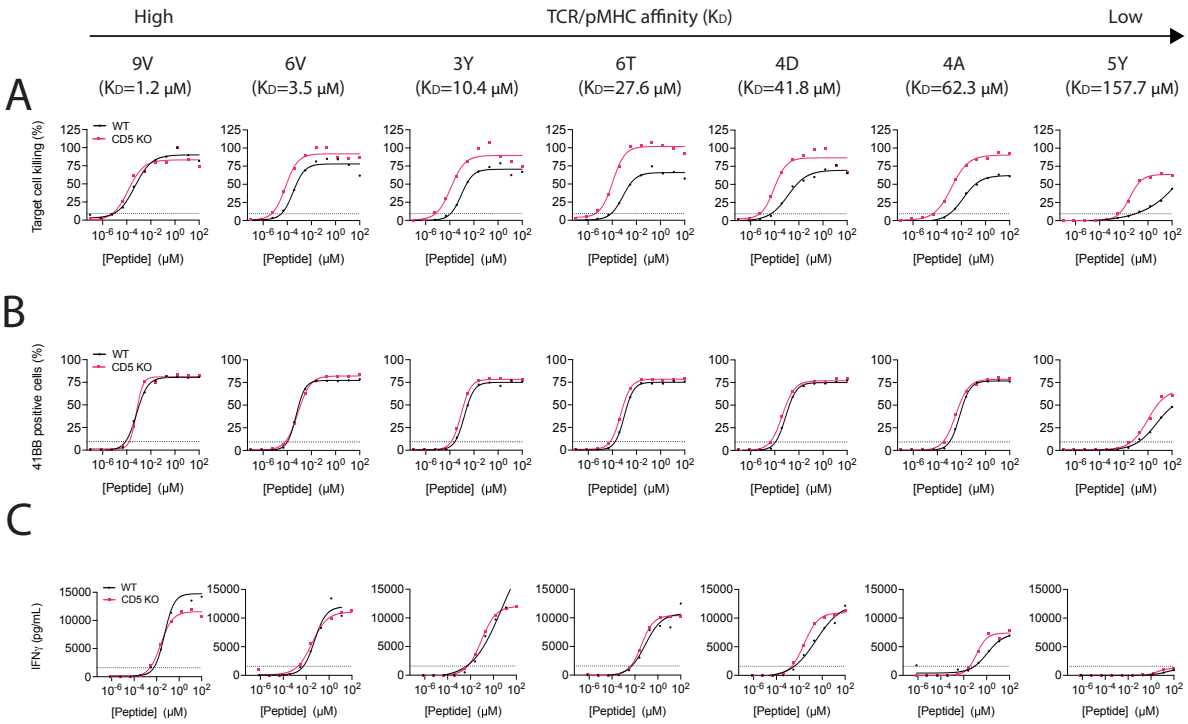


Figure 2.16: Measuring the impact of CD5 KO on T cell activation.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD5 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of at least N=3 independent experiments with different blood donors.

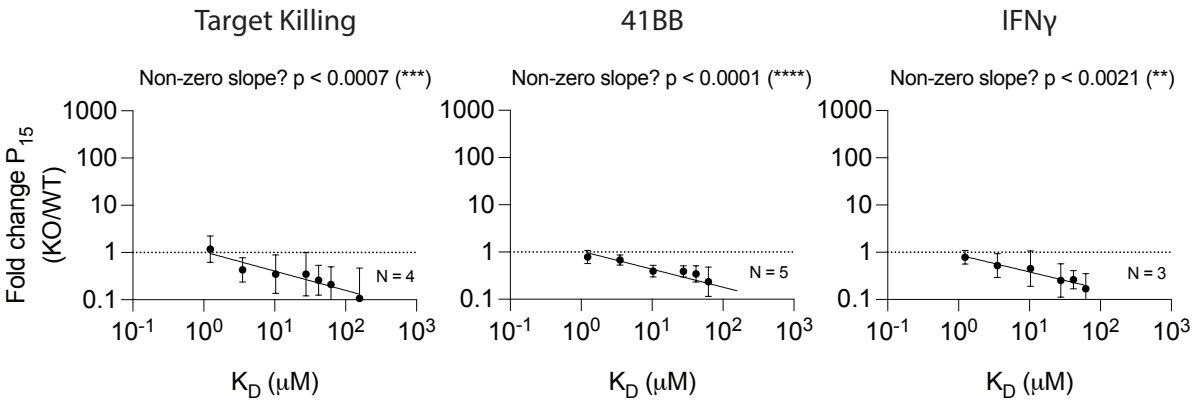


Figure 2.17: CD5 KO reduces T cell ligand discrimination.
Fold change in potency (P_{15}) between CD5 KO and WT c259 TCR-T cells from (Fig. 2.16) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of at least N=3 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

2.2.5 The role of the CD8 co-receptor in T cell ligand discrimination

The CD8 co-receptor is a heterodimer composed of an alpha and a beta chain. Whilst a CD8$\alpha\alpha$ homodimer can be expressed, CD8β cannot be expressed on the cell surface in the absence of CD8α [290]. Therefore, we knocked-out the CD8α chain to completely abolish CD8 co-receptor function and measure its contribution to ligand discrimination.

The knock-out of CD8 did not modify T cell activation against the high affinity 9V peptide, but it selectively reduced activation to low affinity peptides by up to ∼100-1,000 fold (Figure 2.18). There was a significant increase in ligand discriminaton for all T cell activation measures, target killing, 4-1BB expression or IFNγ secretion data (Figure 2.19). Therefore, these results suggest that CD8 decreases T cell ligand discrimination, by selectively increasing activation to lower affinity peptides.

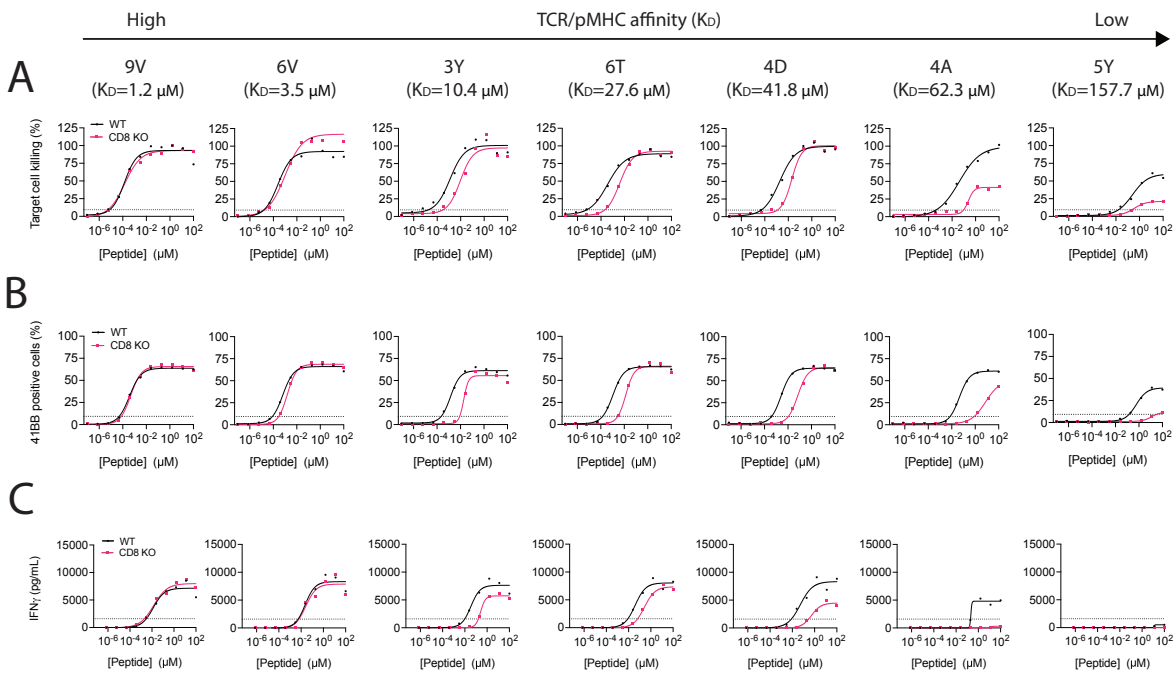


Figure 2.18: Measuring the impact of CD8 KO on T cell activation.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD8 KO c259 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of at least N=3 independent experiments with different blood donors.

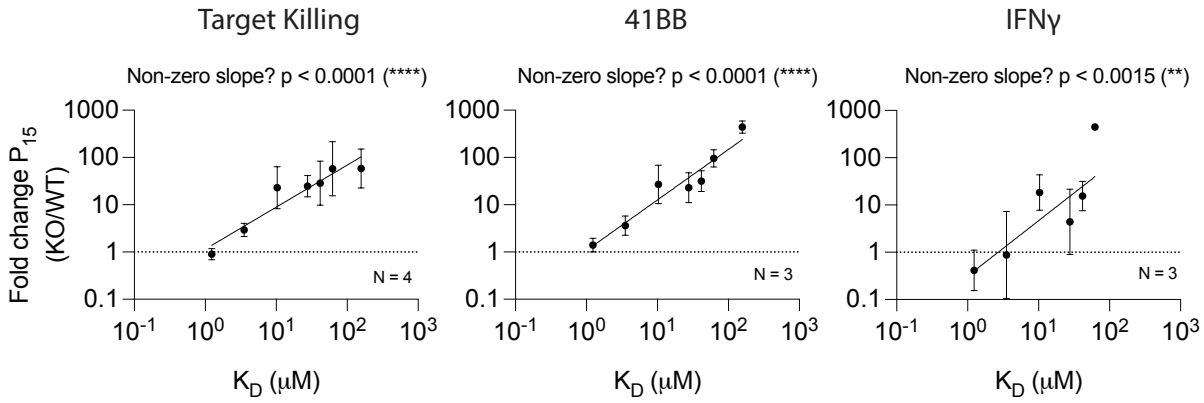


Figure 2.19: CD8 KO increases T cell ligand discrimination.
Fold change in potency (P_{15}) between CD8 KO and WT c259 TCR-T cells from (Fig. 2.18) is plotted over the TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Data is shown as means ± SDs of at least N=3 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

The c259 TCR is an affinity-matured receptor, therefore, we decided to investigate whether this increase in ligand discrimination would also be observed with a native TCR. The parental 1G4 TCR was isolated from a cancer patient [283] and two residues in its CDR3α loop were mutated to generate the c259 TCR, which has higher affinity to the NY-ESO-1 peptide [239, 243]. We transduced T cells with the 1G4 TCR and observed a striking ∼10,000-100,000 loss of sensitivity against the lowest affinity peptides when we knocked-out CD8. We also find a small loss of sensitivity against the high affinity 9V peptide of about ∼20 fold (Figure 2.20). This is consistent with our previous results using the c259 TCR, since the 1G4 TCR binds 9V with an affinity of 8μM. Overall, we confirmed that CD8 knock-out also increased the ligand discrimination of T cells with a native TCR (Figure 2.21).

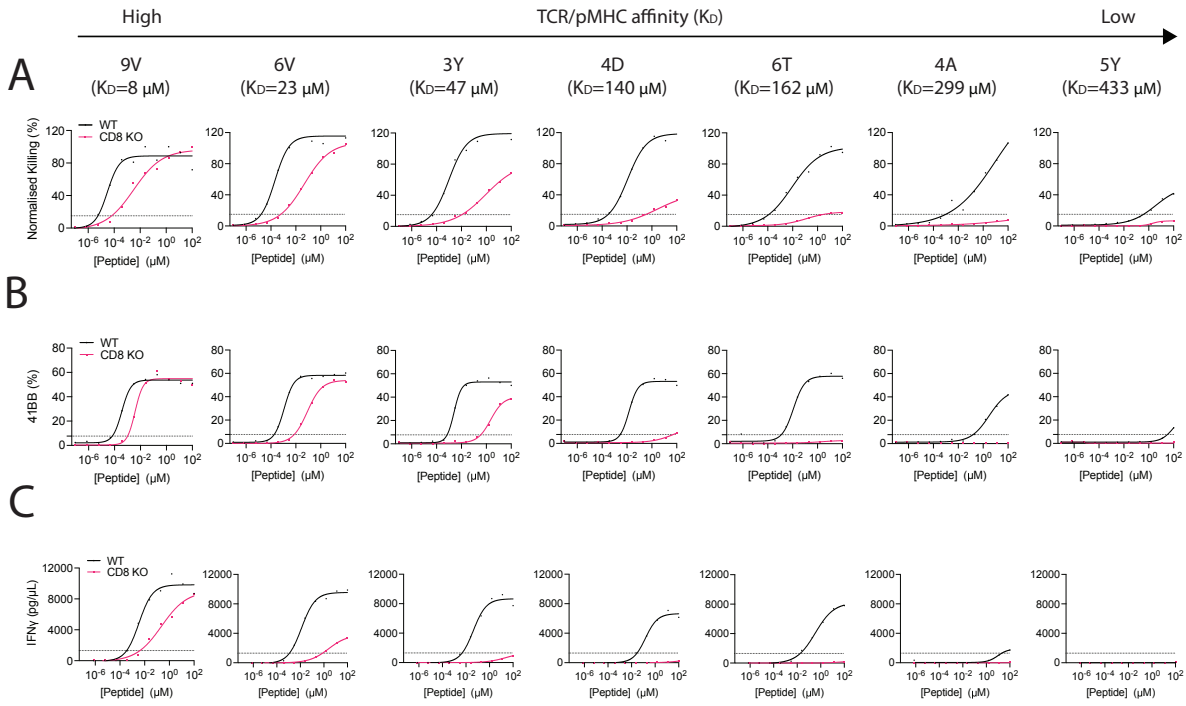


Figure 2.20: Measuring the impact of CD8 KO on T cell activation using the 1G4 TCR.
U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD8 KO 1G4 TCR-T cells. **(A)** Target killing, **(B)** expression of the 4-1BB activation marker and **(C)** IFNγ production were measured after 20 hours. Dashed line indicates potency (P_{15}). Data is representative of at least N=2 independent experiments with different blood donors.

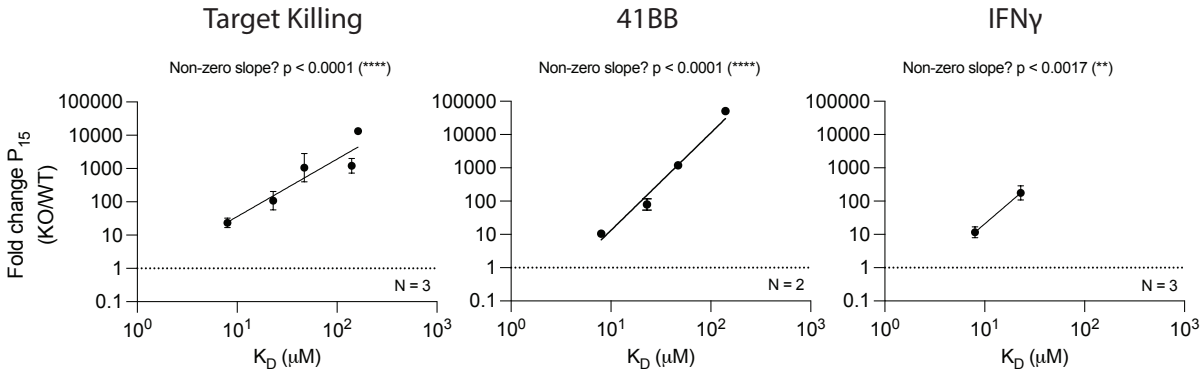


Figure 2.21: CD8 KO increases T cell ligand discrimination using the 1G4 TCR.
Fold change in potency (P_{15}) between CD8 KO and WT 1G4 TCR-T cells from (Fig. 2.20) is plotted over the TCR/pMHC affinity (K_D) from [114]. Dashed line indicates fold change of 1. Data is shown as means ± SDs of at least N=2 independent experiments with different blood donors. Significance of non-zero slope was assessed by an F-test.

The CD8 co-receptor recruits Lck to the TCR, which potentiates signalling, but our results show that this also reduces ligand discrimination. Previous studies have shown that Lck activity levels inversely correlate with T cell ligand discrimination [128]. Therefore, we validated that chemical inhibition of Lck led to a similar increase in ligand discrimination as observed when we knock-out CD8. We treated T cells with 100 nM of the Lck inhibitor A-770041 for one hour. We measured T cell activation at an earlier time-point, after 4 hours to ensure that the chemical inhibitor is not depleted during the co-culture. Due to the shorter activation time, we measure secretion of TNFα and expression of the activation marker CD69. We show that Lck inhibition preferentially reduced activation to low affinity peptides (Figure 2.22). Therefore, our results are consistent with the hypothesis that reducing Lck activity increases ligand discrimination.

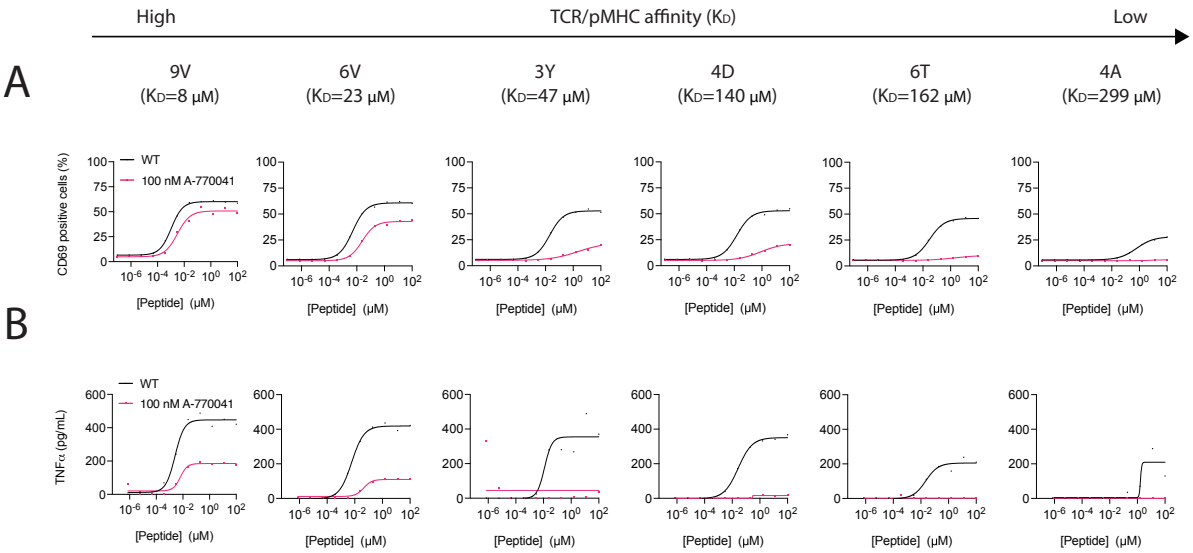


Figure 2.22: Chemically inhibiting Lck increases T cell ligand discrimination U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or A-770041 treated T cells. **(A)** CD69 expression and **(B)** TNFα production were measured after 4 hours. Dashed line indicates potency (P_{15}). Data is representative of N=3 independent experiments with different blood donors.

2.3 Discussion

Our understanding of how T cell co-signalling receptors impact ligand discrimination remains limited. Here, we have quantitatively assessed the contribution of various T cell co-signalling receptors to ligand discrimination in human primary T cells. We have shown that CD43 or the adhesion receptors CD2 and LFA-1 do not impact discrimination. Interestingly, we find that CD8 reduces ligand discrimination, whereas CD5 improves it.

We have established a platform to precisely study the impact of co-signalling receptors on T cell ligand discrimination, which has several advantages over existing approaches. Firstly, we use human primary T cells rather than immortalised T cell lines, which are known to contain mutations in key T cell receptor signalling genes [291]. Secondly, we knock-out the co-signalling receptors of interest rather than blocking them with antibodies [258]. Some blocking antibodies can actually trigger the targeted co-signalling receptors [292], and some co-signalling receptors can induce tonic signalling even if the binding to their ligand is blocked [293]. Thirdly, we selected a high affinity TCR (c259) to ensure that we can still measure activation to a wide-range of peptides even if T cell sensitivity is reduced. Finally, we use antigen presenting cells rather than immobilised pMHCs on plates, which is a more physiological system that captures all biophysical aspects, such as ligand mobility. Taken together, this platform is a useful tool to understand and exploit antigen discrimination.

We hypothesised that the negative effect of CD43 on adhesion could preferentially influence lower affinity pMHC interactions. However, we find no difference in T cell sensitivity or discrimination in CD43 KO human primary T cells. Other abundant proteins in the T cell glycocalyx such as CD45 could compensate for the absence of CD43. Indeed, previous studies have shown that the removal of CD43 or CD45 individually only had a small impact on synapse formation, whilst removal of both of them resulted in a significant increase in signalling and synapse formation [69]. However, removing CD45 is likely to impact not only the biophysics of the

glycocalyx but also TCR proximal signalling through the activity of its intracellular phosphatase domain, which regulates Lck, the TCR and other signalling molecules [294, 295].

We find that the knock-out of LFA-1 did not have any major impact on T cell activation. This was surprising, because previous experiments had found that the presence of its ligand, ICAM-1, increased T cell sensitivity in a 6 hour co-culture assay by ∼100 fold [296]. One possible explanation for this discrepancy is that LFA-1 accelerates the kinetics of T cell activation, but over a longer time-period, other adhesion interactions might compensate for the loss of LFA-1. Therefore, in our 20 hour co-culture assay we may not have captured these earlier differences in activation.

The knock-out of CD2 led to a decrease in T cell sensitivity, which is consistent with previous experiments using either CD58 on antigen presenting cells [296] or immobilised on plates [114, 297]. Moreover, the double knock-out of CD2 and LFA-1 led to an even larger reduction in T cell sensitivity. Pettmann et al. [114] found that immobilising CD58 or ICAM-1 in a plate system slightly increased ligand discrimination, though this increase was lower than the discrimination observed when activating T cells with antigen presenting cells. We find that the knock-out of LFA-1 or CD2 in primary T cells, either individually or in combination, does not impact ligand discrimination. While we cannot entirely rule out the role of adhesion interactions in modulating discrimination, our results do not support this hypothesis. One possibility is that when stimulating T cells in a cellular co-culture, other adhesion interactions compensate for the absence of CD2 and LFA-1, therefore maintaining the same discrimination. Additionally, the impact of adhesion receptors on ligand discrimination might be more pronounced for weaker affinity ligands than the range tested in this chapter (K_D = 1-150 μM). Indeed, Pettmann et al. [114] used the 1G4 TCR and found an impact on discrimination when using ligands with K_D = 7-1300 μM in the plate system.

We find that knocking-out CD5 selectively improves T cell activation only to lower affinity ligands. We did not observe any changes on T cell activation against high affinity peptides (∼1-10 μM). Therefore, our data suggest that CD5 increases T cell ligand discrimination. Since CD5 levels are higher in T cells that bind positively selecting peptides with higher affinity, this enhanced ligand discrimination would reduce the risk of autoimmune cross-reactivities. Although targeting CD5 function has been shown to enhance anti-tumour activity in T cell therapies [263, 268, 269], our findings suggest that this approach might be a double-edged sword due to the potential decrease in ligand discrimination. As a result, knocking-out CD5 could increase the risk of autoimmune toxicities against lower affinity self-peptides in adoptive cell therapies.

The role of CD8 in ligand discrimination has been subject to debate. Previous studies using mutant MHC molecules that cannot bind CD8, or CD8 blocking antibodies, have shown that CD8 selectively increases activation to low affinity ligands, thereby decreasing ligand discrimination [163, 164, 177, 258]. Alternatively, others have suggested that CD8 stabilises TCR/pMHC interactions that have already initiated signalling. This stabilisation is predicted to induce a positive feedback loop that selectively amplifies signalling from high affinity peptides and therefore increases ligand discrimination [170, 176]. We demonstrate that knocking-out CD8 in human primary T cell preferentially reduces responses to low affinity ligands. Therefore, our results are consistent with the former hypothesis, suggesting that CD8 decreases T cell ligand discrimination by increasing the potency of low affinity TCR/pMHC interactions. Knocking-out CD8 reduces the recruitment of Lck to the TCR [257], and it has been previously shown that reducing Lck activity with chemical inhibitors led to an increase in ligand discrimination [128]. Therefore, we propose that the reduction in Lck activity by knocking-out CD8 could explain the observed increase in ligand discrimination.

T cells must discriminate between self and non-self ligands. However, the number of non-self peptides that T cells must recognize far exceeds the number of T cell clones present in each individual. Therefore, T cells must be cross-reactive to achieve full immune coverage. It has been estimated that a single T cell can recognise over 10^6 different peptides [285, 298]. Our results suggest that CD8 increases the functional cross-reactivity of T cells by reducing their ligand discrimination. Interestingly, we find that CD8 knock-out did not affect the activation of human primary T cells against the high affinity 9V peptide ($K_D = 1.2\ \mu$M). This is consistent with previous studies that found the threshold for co-receptor independent TCR activation to be $K_D = \sim 1\ \mu$M [299]. Therefore, we propose that the knock-out of CD8 could be an interesting approach to increase the ligand discrimination of T cell therapies, which in turn would reduce the risk of off-target cross-reactivities without reducing potency to on-target peptides.

Chapter 3

Enhancing T cell ligand discrimination by modulating CD8 and CD4

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3.1 Introduction

The phosphorylation of ITAMs in the intracellular chains of the TCR by Lck is an essential step to initiate TCR signalling [300, 301]. The CD8 and CD4 co-receptors bind Lck via their intracellular domain [169] and interact with MHC molecules through their extracellular domain [157, 161, 302]. When a TCR binds a compatible pMHC ligand, class-I for CD8 and class-II for CD4, co-receptors actively recruit Lck to the signalling TCR [257]. While co-receptor mediated recruitment of Lck enhances TCR signalling, it is not essential for triggering. For example, we have shown in Chapter 2 that T cells lacking a co-receptor can still become activated. This is possible because Lck exists in the cell either in a co-receptor bound state or in a 'free' state, directly associated to the plasma membrane. Thus, ITAM phosphorylation by free Lck is sufficient to activate T cells [175].

Free Lck is more enzymatically active [129] and diffuses faster than [303] co-receptor bound Lck. As a result, co-receptors can actually inhibit signalling if they do not co-localise with the TCR, by reducing the pool of free Lck available [304, 305]. For instance, during thymic development, co-receptors sequester Lck from TCRs that do not bind pMHC antigens, preventing the positive selection of MHC-independent T cells. However, it is unclear if the sequestration of Lck by incompatible co-receptors affects ligand discrimination in mature T cells.

All nucleated cells in the body present peptides derived from their intracellular environment in MHC-I molecules. In contrast, MHC-II molecules are only expressed in professional antigen presenting cells, such as macrophages, dendritic cells or B cells, to display peptides derived from the extracellular environment. Accordingly, clinical efforts have typically focused on identifying TCRs that recognise tumour peptides presented on MHC-I molecules for their use in adoptive T cell therapies, since most tumour cells do not express MHC-II molecules. CD8+ cytotoxic T cells can directly recognise and kill cancer cells presenting the target pMHC-I antigens. However, it is also known that CD4+ helper T cells play an essential function

in natural immunity by supporting a broad and persistent anti-tumour response [306–309].

In adoptive T cell therapies, both CD8+ cytotoxic and CD4+ helper T cells are normally engineered with the same TCR that recognises a tumour pMHC-I antigen to elicit the strongest immune response [218, 220, 232, 245, 280, 310]. CD4+ helper cells recognising pMHC-I antigens produce cytokines such as IL-2, which might enhance the proliferation of CD8+ cytotoxic T cells in the tumour microenvironment [311]. However, it is unclear whether CD8+ cytotoxic or CD4+ helper T cells are responsible for the observed off-target autoimmune toxicities in multiple clinical trials [233, 245, 246].

Helper T cells re-directed to recognise pMHC-I antigens lack a functional co-receptor, since they do not express CD8. As discussed in Chapter 2, the absence of CD8 increases ligand discrimination. Moreover, helper T cells express an incompatible CD4 co-receptor that can bind Lck but cannot bind the pMHC-I antigen that their TCR recognises. Since Lck levels have been shown to regulate ligand discrimination [128], we decided to investigate whether these differences in co-receptor expression result in variations in ligand discrimination between helper and cytotoxic T cells expressing the same TCR.

In this chapter, we find that helper T cells exhibit higher discrimination than cytotoxic T cells when re-directed to recognise pMHC-I antigens. We demonstrate that their enhanced discrimination is not only because they do not express the compatible CD8 co-receptor, but also because they express the incompatible CD4 co-receptor. Next, we show that expressing CD4 in cytotoxic T cells can also increase their ligand discrimination. Finally, we apply the same strategy to investigate differences in ligand discrimination between cytotoxic T cells and helper T cells engineered with the therapeutic a3a TCR. We find that cytotoxic T cells, but not helper T cells, cross-react with the lower affinity Titin peptide that led to lethal autoimmune toxicities in a clinical trial. We also show that knocking-out the CD8 co-receptor abolishes this Titin cross-reactivity in cytotoxic T cells.

3.2 Results

3.2.1 MHC-I re-directed helper T cells have higher discrimination than cytotoxic T cells

We isolated CD8+ cytotoxic and CD4+ helper T cells from the same blood donors and transduced them with the MHC-I restricted c259 TCR (Figure 3.1 A). For simplicity, we will refer to these subsets as cytotoxic and helper T cells. Next, we activated them with U87 cells pulsed with a titration of the 7 peptide panel with different affinities that we measured in Chapter 2. Since cytotoxic and helper T cells have very different cytotoxic and cytokine secreting capacities, we decided to use the expression of the 4-1BB activation marker to directly compare the activation of the two T cell subsets. Moreover, in Chapter 2 we validated that expression of the 4-1BB activation marker correlated well with other effector functions, such as killing and cytokine production.

We find that helper and cytotoxic T cells display very different responses to the APL panel (Figure 3.1 B). For example, whilst all peptides activate cytotoxic cells, the lowest two affiny peptides (4A and 5Y) do not activate helper T cells. Furthermore, whilst cytotoxic T cells produce very similar responses against the top four affinity peptides (9V, 6V, 3Y and 6T), helper T cells show approximately a ∼1,000 fold difference in potency across these same four peptides. We quantify T cell ligand discrimination by plotting peptide potency (P_{15}) against peptide affinity (K_D) and find that helper T cells display higher ligand discrimination than cytotoxic T cells (Figure 3.2 A). We expected helper T cells to have higher discrimination because they lack a CD8 co-receptor, but surprisingly we find that they have even higher discrimination than cytotoxic T cells with a CD8 knock-out. Therefore, the enhanced discrimination of helper T cells cannot be explained by the absence of the CD8 co-receptor alone and there must be additional mechanisms that enhance their discrimination.

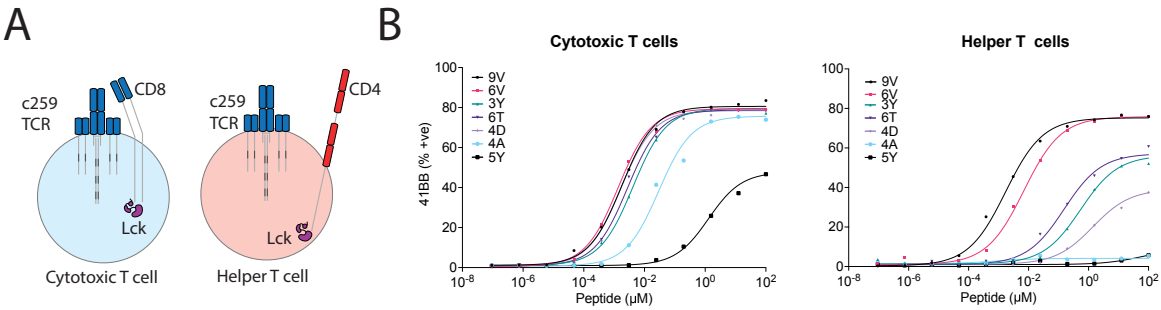


Figure 3.1: Comparing the ligand discrimination of cytotoxic and helper T cells.
(A) Schematic of cytotoxic and helper T cells expressing the c259 TCR. **(B)** U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate cytotoxic or helper c259 TCR-T cells. Expression of the 4-1BB activation marker was measured after a 20 hour co-culture. Data is representative of N=3 independent experiments with different blood donors.

We fit a simplified kinetic proofreading model to the potency over affinity data as previously described [114] (Figure 3.2 B):

$$log(P_{15}) = A + N \times log(1 + \frac{\tau_{KP} \times k_{on} \times K_D}{N})$$

The kinetic proofreading model uses three parameters: the number of proofreading steps (N), the proofreading time (τ_{KP}) and the sensitivity (A). Kinetic proofreading depends on the lifetime of the TCR/pMHC interaction. Since the off-rate of low affinity peptides cannot be accurately measured by SPR, we have used affinity (K_D) as a proxy for the off-rate (k_{off}) throughout this thesis. To fit the kinetic proofreading model, we estimated off-rates using the previously measured on-rate of the parental 1G4 TCR [125] ($k_{off} = K_D \times k_{on}$). After fitting the data, the slope of the curve is the number of proofreading steps (N), and the y-intercept is a measure of sensitivity (A). Since we do not expect differences in co-receptor expression to affect the number of proofreading steps, we constrain N to 2.7 as previously measured [114] and we fit proofreading time (τ_{KP}). We confirm that the kinetic proofreading time is significantly longer for helper cells than for either wild-type or CD8 knock-out cytotoxic T cells (Figure 3.2 C).

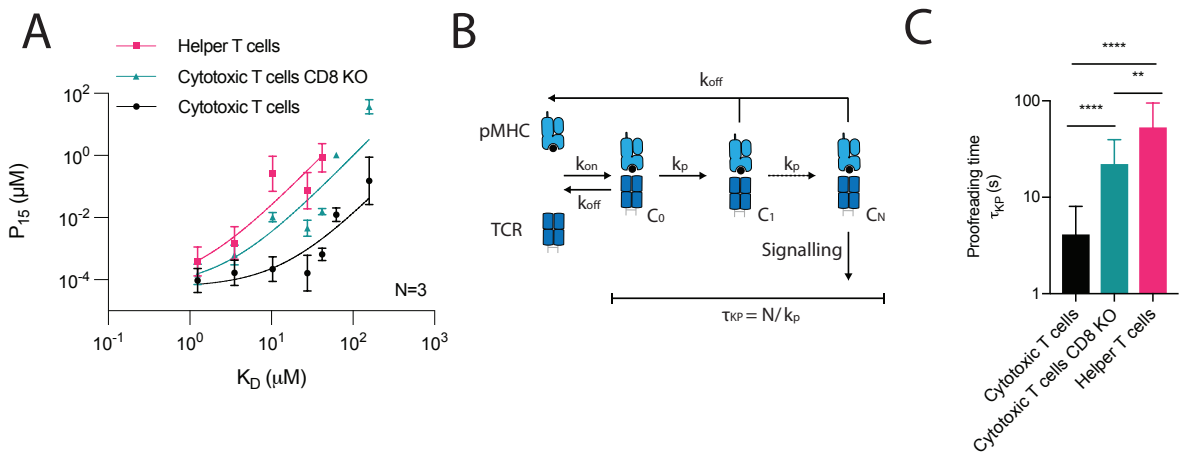


Figure 3.2: Helper T cells display higher discrimination than cytotoxic or CD8 KO cytotoxic T cells.
(A) Potency (P_{15}) from (Fig. 3.1 and 2.18) over TCR/pMHC affinity (K_D) is fitted to the kinetic proofreading model (solid line). **(B)** Kinetic proofreading introduces a time-delay (τ_{kp}) between pMHC binding (state C_0) and TCR signalling (state C_N) that selectively reduces signalling to low-affinity ligands. **(C)** Fitted time-delay from the data in (A). Data in (A) and (C) is displayed as means ± SDs of N=3 independent experiments with different blood donors. F-test compares the time-delay between conditions. **p<0.01, ****p<0.0001.

3.2.2 The CD4 co-receptor increases the ligand discrimination of MHC-I re-directed helper T cells

Helper T cells also express the CD4 co-receptor, which cannot bind the c259 TCR cognate class-I pMHC, but can bind Lck via its intracellular tail. We hypothesised that the presence of the incompatible CD4 co-receptor could be responsible for the enhanced discrimination of helper T cells. To test this hypothesis, we compared the discrimination of wild-type or CD4 knock-out helper T cells transduced with the c259 TCR (Figure 3.3 A-B). We find that CD4 KO did not change activation to the high affinity 9V peptide, but increased activation against low affinity peptides by up to ∼10 fold (Figure 3.3 C). Analysing the entire peptide panel, CD4 KO decreased ligand discrimination, as the slope of the ΔP_{15} over affinity (K_D) plot is negative and significantly different than zero (Figure 3.3 D). Overall, these results are consistent with our hypothesis that the expression of CD4 increases the ligand discrimination of helper T cells recognising peptides on class I MHC molecules.

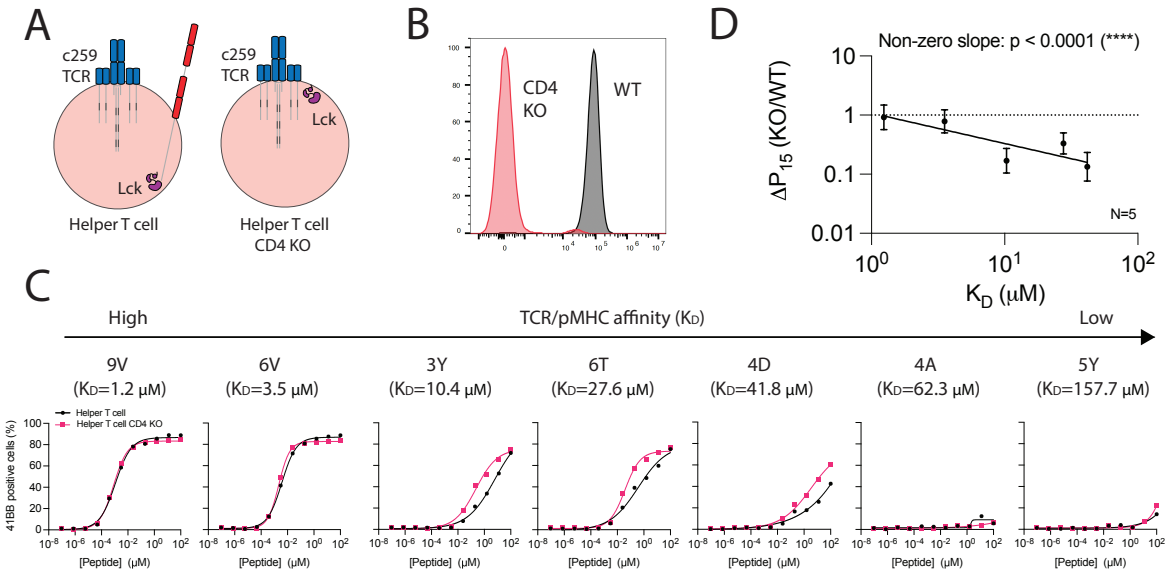


Figure 3.3: CD4 enhances ligand discrimination in c259-TCR helper T cells. **(A)** Schematic of helper WT and CD4 KO c259 TCR-T cells. **(B)** Flow cytometry staining of CD4 in WT and CD4 KO helper T cells. **(C)** U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD4 KO helper c259 TCR-T cells. Expression of the activation marker 4-1BB was measured after 20 hours. **(D)** Fold change in potency (P_{15}) between CD4 KO and WT helper T cells from (C) is plotted over TCR/pMHC affinity (K_D). Dashed line indicates fold change of 1. Significance of non-zero slope was assessed by an F-test. Data in (B) and (C) is representative of N=5 independent experiments from different blood donors. Data in (D) is displayed as means ± SDs.

3.2.3 Expression of the CD4 incompatible co-receptor in cytotoxic T cells increases ligand discrimination

Since we have shown that CD4 enhances the ligand discrimination of helper T cells, we decided to investigate whether it could also be used to increase the discrimination of cytotoxic T cells. We constructed a lentiviral vector to co-express the c259 TCR and CD4 co-receptor in human primary cells via a 2A peptide. We find that cytotoxic T cells can be engineered to express CD4 at high levels, without any impact on TCR expression (Figure 3.4).

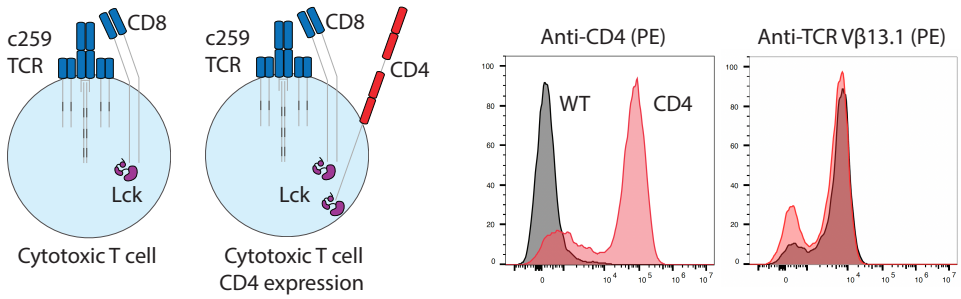


Figure 3.4: Engineering cytotoxic T cells to express the incompatible CD4 co-receptor. (Left) Schematic of CD4 expression in cytotoxic T cells. **(Right)** Flow cytometry staining of CD4 and TCR Vβ13.1 expression.

Expressing CD4 in cytotoxic T cells did not modify activation against the high affinity 9V peptide, but it gradually reduced activation against the lower affinity ligands. For the lowest affinity peptide (5Y), expressing CD4 resulted in a dramatic reduction in T cell activation (Figure 3.5). Overall, these results demonstrate that CD4 expression in cytotoxic T cells can increase their ligand discrimination, and were consistent for both target cell killing and expression of the 4-1BB activation marker.

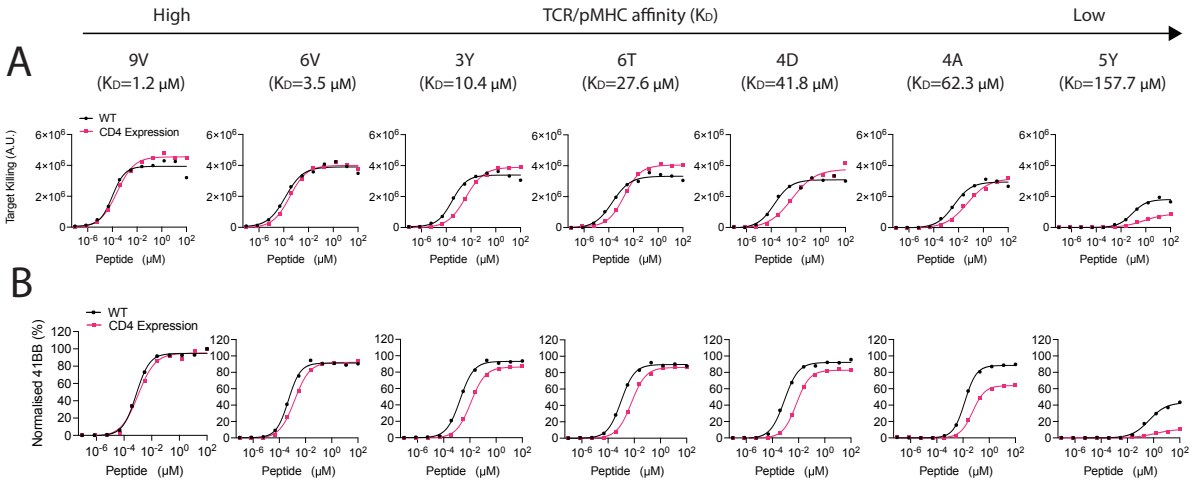


Figure 3.5: Measuring the ligand discrimination of cytotoxic T cells expressing the incompatible CD4 co-receptor. U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD4 expressing cytotoxic c259 TCR-T cells. Target cell killing **(A)** or expression of the activation marker 4-1BB **(B)** was measured after 20 hours. Data is representative of N=3 independent experiments using different blood donors.

We have shown in Chapter 2 that knocking-out the CD8 co-receptor can increase the ligand discrimination of cytotoxic T cells. Therefore, we hypothesised that combining CD8 knock-out and CD4 expression would synergise to produce T cells with an even higher degree of discrimination. We refer to these cells as CD8→CD4 co-receptor switch T cells (Figure 3.6).

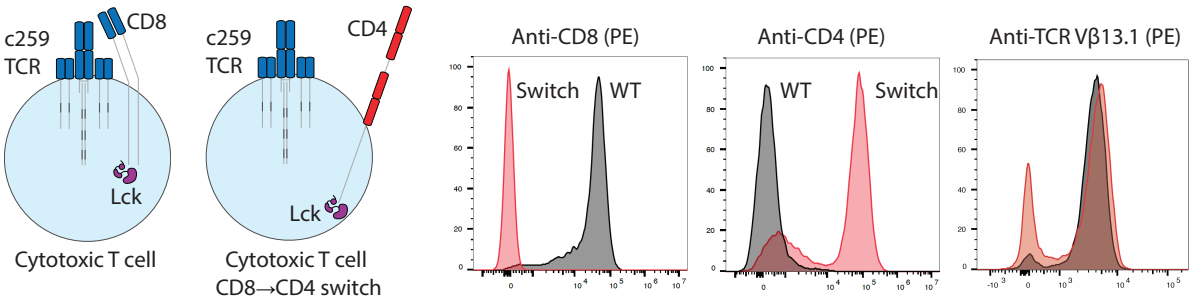


Figure 3.6: Engineering cytotoxic T cells with a CD8→CD4 co-receptor switch. **(Left)** Schematic of CD8→CD4 co-receptor switch in cytotoxic T cells. **(Right)** Flow cytometry staining of CD8, CD4 and TCR Vβ13.1 expression. CD8→CD4 co-receptor switch T cells shown in red, WT T cells shown in black.

CD8→CD4 co-receptor switch T cells displayed reduced responses against lower affinity peptides presented on U87 cells, whilst still eliciting a strong response against the high affinity 9V peptide (Figure 3.7). CD8→CD4 co-receptor switch T cells displayed higher ligand discrimination than T cells with either of the individual modifications, CD8 knock-out or CD4 expression (Figure 3.8). For the lowest affinity peptides, CD8→CD4 co-receptor switch T cells require approximately ∼10,000 fold higher concentration to activate than wild-type cells. Taken together, these results demonstrate that the combination of CD8 knock-out and CD4 expression can result in extremely high levels of ligand discrimination.

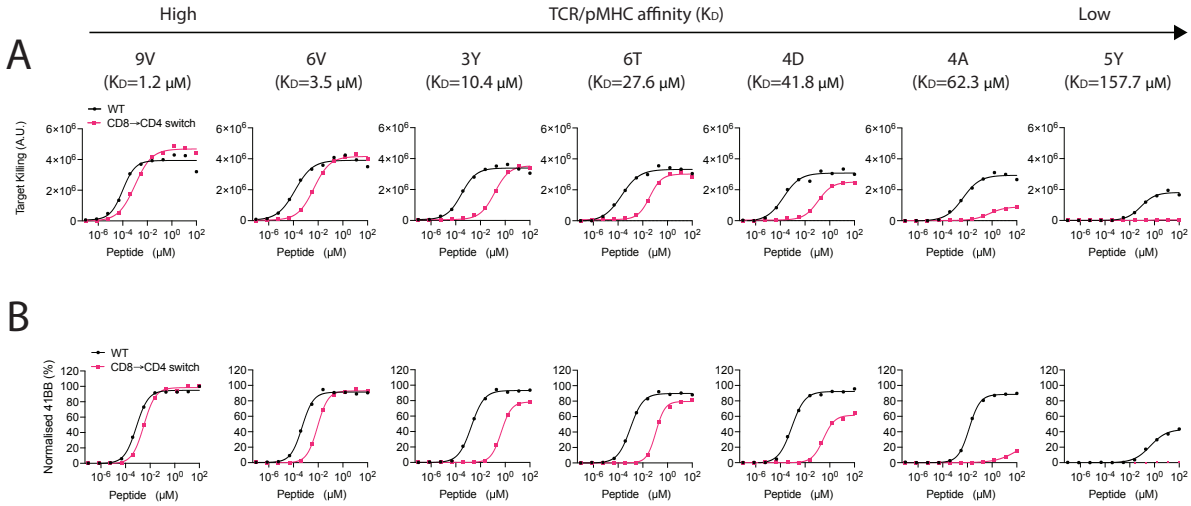


Figure 3.7: Measuring the ligand discrimination of CD8→CD4 co-receptor switch cytotoxic T cells. U87 cells were titrated with each of the 7 NY-ESO-1 peptides to stimulate WT or CD8→CD4 co-receptor switch cytotoxic c259 TCR-T cells. Target cell killing **(A)** or expression of the activation marker 4-1BB **(B)** was measured after 20 hours. Data is representative of N=3 independent experiments using different blood donors.

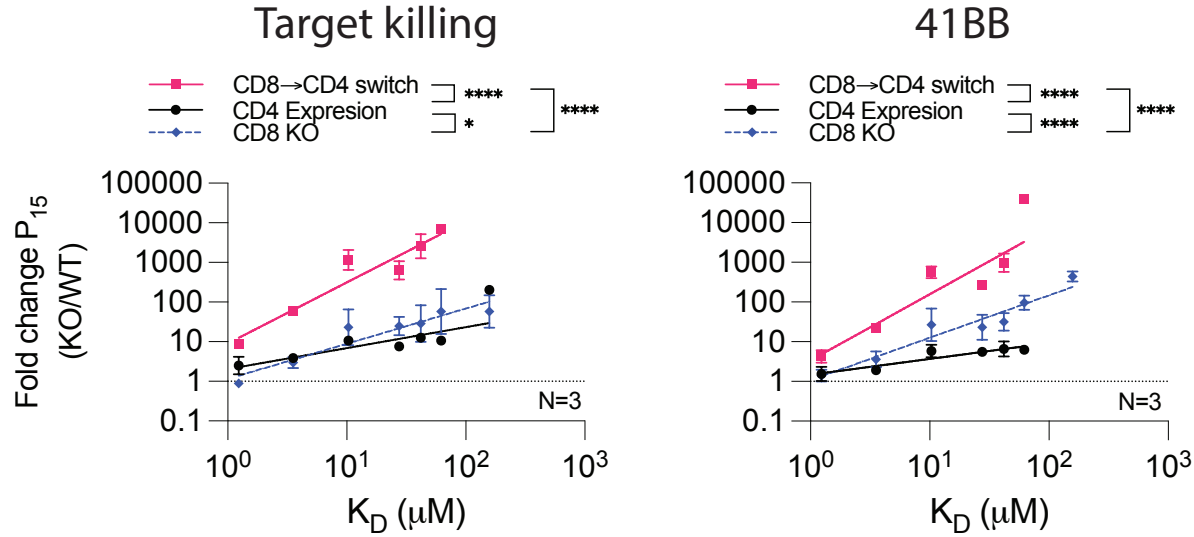


Figure 3.8: CD8→CD4 co-receptor switch cytotoxic T cells display higher ligand discrimination than CD8 KO or CD4 expressing cytotoxic T cells. The fold change in Potency (P_{15}) between the indicated modified and WT cytotoxic T cells over TCR/pMHC affinity (K_D) from (Fig. 3.5 and 3.7). Data for CD8 KO is shown from (Fig. 2.19). Data is shown as means ± SDs from N=3 independent experiments with different blood donors. P values were determined by F-test. *p<0.05, ****p<0.0001.

3.2.4 The cross-reactivity of the a3a TCR to Titin is dependent on CD8

We have demonstrated that differences in co-receptor expression between cytotoxic and helper T cells result in large differences in ligand discrimination when using the NY-ESO-1 specific c259 TCR. Next, we sought to investigate whether similar results would be observed with other clinically relevant TCRs. We selected the MAGE-A3 specific a3a TCR, since it caused lethal cardiac toxicities in a clinical trial due to cross-reactivity to a lower affinity peptide from the muscle protein Titin [233, 245].

The a3a TCR is restricted to HLA-A*01:01, which is not expressed in the U87 cell line we have used to study the c259 TCR. We constructed a HLA-A*01:01 positive T2 cell line by lentiviral transduction to use as an antigen presenting cell line to the a3a TCR. Peptide-pulsed T2 cells have been widely used previously to study T cell activation because they are TAP deficient and they do not process and present endogenous antigens [312–315].

We compared the activation of cytotoxic and helper T cells transduced with the a3a TCR against T2 cells pulsed with either MAGE-A3 or Titin. We find that while both cytotoxic and helper T cells responded to MAGE-A3, only cytotoxic T cells responded to Titin (Figure 3.9). This suggests that the autoimmune toxicities were likely driven by a3a TCR CD8+ cytotoxic T cells rather than CD4+ helper T cells.

We next investigated whether knocking-out CD8 would abolish cross-reactivity to Titin (Figure 3.10 A). We demonstrate that CD8 knock-out cytotoxic T cells do not respond at all against T2 cells pulsed with Titin (Figure 3.10 B). These results were consistent both by target cell killing and 4-1BB expression measurements. The knock-out of CD8 did not impact activation of cytotoxic T cells against the target MAGE-A3 peptide. Finally, we show that CD8 knock-out T cells also do not become activated when co-cultured with HLA-A*01:01 positive Nalm6 cells, which endogenously express Titin [233] (Figure 3.10 C).

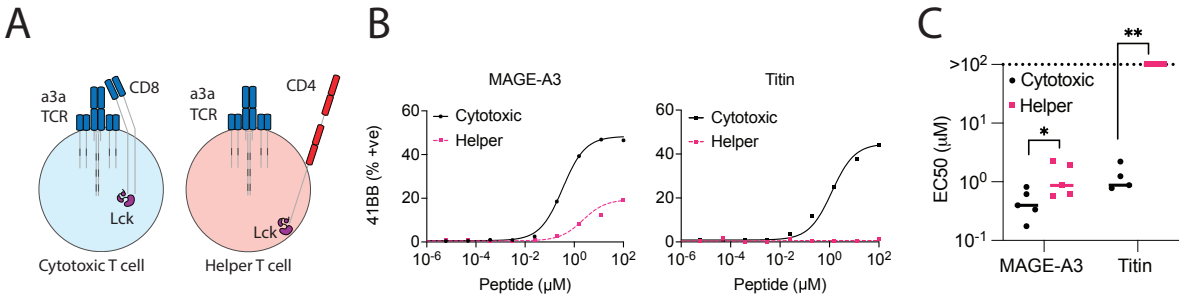


Figure 3.9: Cytotoxic, but not helper a3a TCR-T cells cross-react with Titin. **(A)** Schematic of helper and cytotoxic T cells transduced with the MAGE-A3 specific a3a TCR. **(B)** HLA-A1+ T2 cells were titrated with MAGE-A3 or Titin peptides to stimulate cytotoxic or helper a3a TCR-T cells. The activation marker 4-1BB was measured after 20 hours. **(C)** Mean sensitivity (EC50) from (B) is plotted. P values were determined by paired t-test; *p<0.05, **p<0.01.

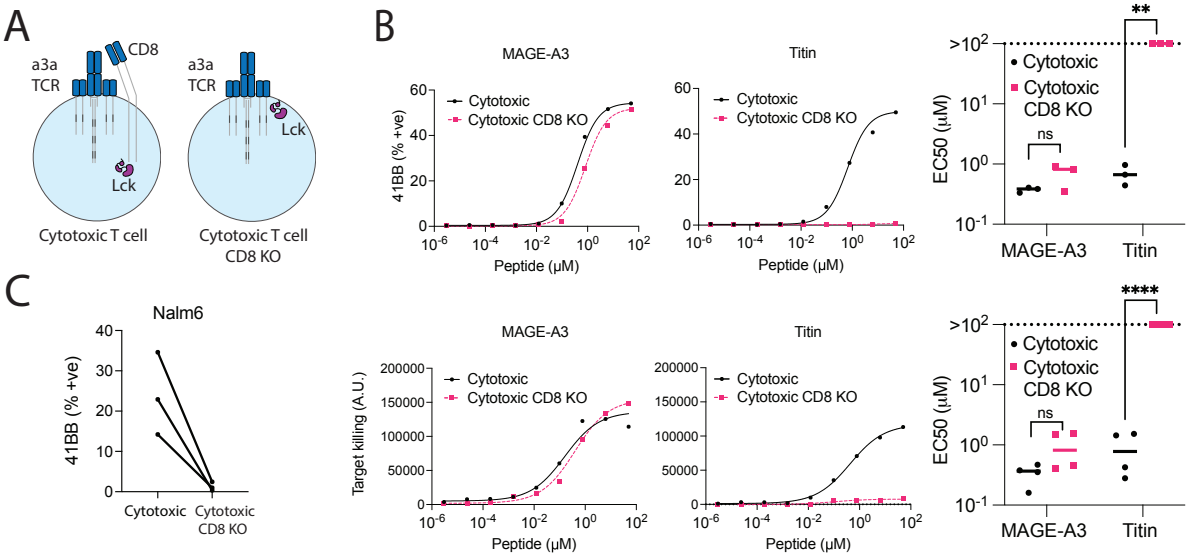


Figure 3.10: CD8 KO abolishes cytotoxic a3a TCR-T cell cross-reactivity to Titin. **(A)** Schematic of WT and CD8 KO cytotoxic a3a TCR-T cells. **(B)** HLA-A1+ T2 cells were titrated with MAGE-A3 or Titin peptides to stimulate WT or CD8 KO cytotoxic a3a TCR-T cells for 20 hours. Representative dose-responses (Left) and mean sensitivity as EC50 (Right). Data measuring 4-1BB surface activation marker (top) and target cell killing (bottom) are shown. Each EC50 point represents an independent experiment with different blood donors. **(C)** Nalm6 cells endogenously expressing the Titin protein were co-cultured with WT or CD8 KO cytotoxic a3a TCR-T cells for 20 hours. P values were determined by paired t-test; ns not significant, **p<0.01, ****p<0.0001

3.3 Discussion

Helper and cytotoxic T cells are routinely engineered with the same TCR in adoptive cell therapies. However, it remains unclear how their ability to discriminate between ligands differs. Here, we show that helper T cells display higher ligand discrimination than cytotoxic T cells when recognizing peptides on MHC-I molecules. We demonstrate that the incompatible CD4 co-receptor is responsible for the enhanced discrimination in helper T cells, and that the same effect can be achieved by engineering cytotoxic T cells to express CD4. Finally, we find that while cytotoxic T cells engineered with the therapeutic a3a TCR cross-react with a peptide from the cardiac protein Titin, helper T cells do not, and that knocking-out CD8 could abolish this cross-reactivity.

Using the c259 TCR and the NY-ESO-1 APL panel that we developed in Chapter 2, we find that helper T cells re-directed to recognise MHC-I peptides have a much higher ligand discrimination than cytotoxic T cells. Since helper T cells do not express the CD8 co-receptor, these results support our observations in Chapter 2 that the absence of a compatible co-receptor increases ligand discrimination. Our finding that helper T cells are even more discriminatory than CD8 knock-out cytotoxic T cells suggested that the absence of a compatible co-receptor was not the only factor that enhances their discrimination. We show that expression of the CD4 co-receptor is required to maintain the highest levels of ligand discrimination in helper T cells and hypothesize that this is due to the sequestration of Lck.

Previous studies have shown that antibodies against CD4 or CD8 inhibit T cell activation by preventing MHC binding and association with the TCR, and therefore sequestering co-receptor bound Lck away from the TCR [316, 317]. We show that this inhibition of Lck activity by CD4 elongates kinetic proofreading time and increases ligand discrimination. Our results are qualitatively consistent with a previous study that showed that CD4 inhibits lower affinity peptides presented on MHC-I but not higher affinity peptides [317]. However, while the previous study found that CD4 inhibited the 2C TCR (K_D=0.54μM) by ∼10,000 fold, we find

that CD4 achieves this level of inhibition only for much weaker affinity peptides (K_D>50μM). These quantitative differences in the range of peptide affinities that are susceptible to CD4 inhibition could be due to differences in signalling between the T cell hybridoma cell line used in the previous study and primary human T cells used in this thesis.

Since helper T cells were the most discriminatory, we explored whether cytotoxic T cells could be engineered to achieve similar levels of ligand discrimination. Firstly, we expressed CD4 in cytotoxic T cells and find that double positive (CD8+ CD4+) cytotoxic T cells had higher ligand discrimination than wild-type cytotoxic T cells. These results are consistent with previous reports that in double positive cells, CD4 outcompetes CD8 for Lck binding [303] due to the higher affinity of its intracellular tail for Lck [169]. The increase in discrimination that we observed when expressing CD4 was smaller than that achieved when knocking-out CD8 (Chapter 2). Therefore, we next combined both modifications to maximize ligand discrimination. We found that cytotoxic T cells with this CD8→CD4 switch required over a 10,000 fold increase in the concentration of the lowest affinity peptides to achieve the same response as wild-type T cells. Consequently, CD8→CD4 switch cytotoxic T cells demonstrated extremely high levels of ligand discrimination, comparable to that observed with helper T cells.

Collectively, these results show that T cell ligand discrimination can be titrated by the expression of different co-receptors. For an MHC-I restricted TCR: (I) CD8+ T cells display the lowest ligand discrimination, followed by (II) double positive (CD8+CD4+) T cells, then (III) double negative (CD4-CD8-) T cells and (IV) CD4+ T cells display the highest ligand discriminaton. We propose that the increasing levels of ligand discrimination can be explained by the decreasing levels of Lck activity across these T cell populations (Figure 3.11).

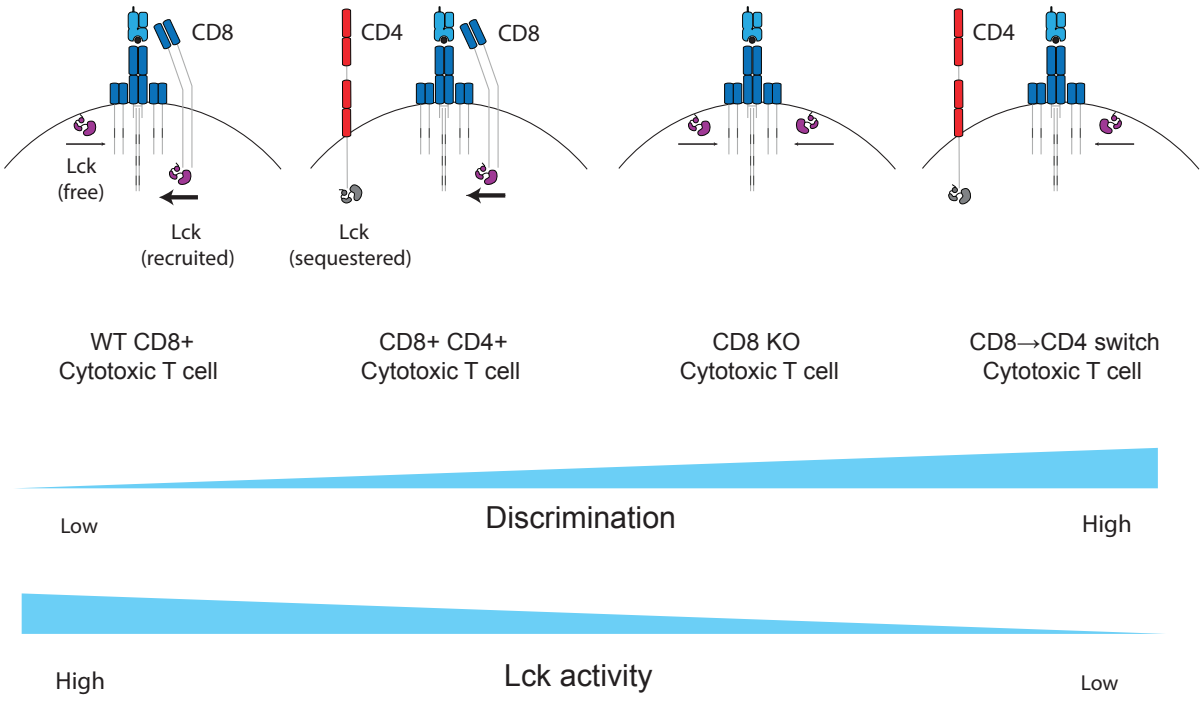


Figure 3.11: Co-receptor expression titrates ligand discrimination.
Free Lck can directly phosphorylate the TCR. If Lck is bound to the compatible CD8 co-receptor, which binds MHC-I, it is recruited to the TCR and its enzymatic activity is higher. Alternatively, if Lck is bound to the incompatible CD4 co-receptor, which cannot bind MHC-I, it is sequestered and its enzymatic activity is reduced. Decreasing amounts of Lck activity result in enhanced ligand discrimination.

We also investigated differences in ligand discrimination using T cells engineered with a different TCR. The therapeutic MAGE-A3 specific a3a TCR is a relevant case-study because it caused multiple lethal autoimmune toxicities against a peptide from the heart protein Titin in a clinical trial [233, 245]. We find that cytotoxic T cells, but not helper T cells, cross-react with the lower affinity Titin peptide. These results suggest that the fatalities in the clinical trial were driven by cytotoxic and not helper T cells. Histopathological analysis showed CD3+ T cells infiltrated the heart in both patients [245]. It would be interesting to investigate whether the proportion of a3a TCR cytotoxic T cells was higher than helper T cells in the heart infiltrate.

Since we demonstrated in Chapter 2 that the knock-out of CD8 could increase cytotoxic T cell ligand discrimination, we investigated whether the same strategy could reduce cross-reactivity to Titin. We show that CD8 knock-out cytotoxic T cells do not activate against Titin, whilst maintaining strong responses to the higher-affinity on-target MAGE-A3 tumour peptide.

In conclusion, we propose that a compatible co-receptor, CD8 for MHC-I restricted TCRs or CD4 for MHC-II restricted TCRs, functions to potentiate signalling and reduce ligand discrimination by actively recruiting Lck to the TCR. Conversly, an incompatible co-receptor increases ligand discrimination by sequestering Lck, thereby reducing its enzymatic activity. These findings have important implications for T cell therapies, where either helper or cytotoxic T cells are re-directed to recognise peptides presented on MHC-I or MHC-II molecules, respectively. Whilst we have focused on MHC-I restricted TCRs in this thesis, future experiments are needed to confirm that the same is true with MHC-II restricted TCRs. It would be interesting to test whether CD8 has the same inhibitory potency as CD4 when used as an incompatible co-receptor, since it has a lower affinity to Lck than CD4 [169].

Chapter 4

The cross-reactivity of enhanced discriminatory T cells

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4.1 Introduction

It has been estimated that each individual has $\sim 10^{12}$ T cells with $\sim 10^{6}$-10^{8} unique TCRs [318–320]. However, the total number of potential peptides that need to be recognized far exceeds the diversity of TCRs in any given individual. For example, there are 20^{10} (1.024×10^{13}) possible decamer peptides. This suggests that each T cell must recognize multiple peptides for an individual to achieve full immune coverage. It has been shown that the TCR CDR loops are flexible, and can accommodate structurally different pMHCs [321], which helps explain how a single single T cell can recognize over one million peptides [285, 298, 322, 323].

Different terms have been used in the literature to describe the ability of one T cell to recognize more than one peptide. For example, degeneracy and promiscuity have been used to refer to the recognition of different peptides with similar sequence or biochemical and structural properties [322, 324]. Alternatively, polyspecificity has been defined as the ability of T cells to recognize multiple unrelated ligands with high specificity for each of them, where for example single mutations could abolish activation [325]. Additionally, T cells can recognize peptides presented on different MHC molecules, known as alloreactivity [326–328] or even on MHC molecules from different species, termed xenoreactivity [329]. To avoid ambiguity, here we use the term cross-reactivity to broadly encompass the activation of T cells against multiple ligands.

Whilst T cell cross-reactivity is an essential feature of natural immunity, it is undesirable in adoptive T cell therapies. In these therapies, T cells are engineered with a novel TCR that recognises a target tumour pMHC. However, since the novel TCR has not undergone negative thymic selection in the patient, it can cross-react with self-peptides presented on healthy tissues. In multiple clinical trials, these cross-reactivities have caused lethal autoimmune toxicities [233, 245, 246]. Therefore, engineering T cells with reduced cross-reactivity could enhance the safety of T cell therapies.

Currently, efforts to reduce T cell cross-reactivity have focused on the binding properties of the therapeutic TCRs used. For example, the CDR loops of a TCR can be mutated to avoid binding to known cross-reactive self-peptides, whilst maintaining binding to the on-target tumour peptide [330, 331]. Additionally, it has been proposed that reducing CDR loop flexibility can reduce TCR binding cross-reactivity [332, 333]. However, all strategies that rely on mutating the TCR sequence to reduce binding cross-reactivity require optimisation of individual TCRs and are not generalizable.

Instead of modifying the TCR CDR loops to reduce binding cross-reactivity, we hypothesised that functional cross-reactivity can be reduced by manipulating T cell signalling without modifying the TCR. The mechanisms that control T cell cross-reactivity are not completely understood. Previous studies have proposed that the CD8 co-receptor increases cross-reactivity by increasing activation to low affinity pMHCs [177]. In chapter 3 we have shown that T cells engineered with a CD8→CD4 co-receptor switch displayed even greater ligand discrimination than CD8 knock-out T cells. In this chapter, we use different methods to measure the impact of the co-receptor switch on cross-reactivity. Using a positional scanning peptide library, we find that CD8→CD4 co-receptor switch T cells require a higher affinity threshold to activate than wild-type or CD8 KO T cells. Next, we show that CD8→CD4 co-receptor switch T cells display the lowest cross-reactivity to a pooled library of random 9-mer peptides. Finally, we demonstrate that knocking-out CD8 or especially, introducing a CD8→CD4 co-receptor switch reduces activation to self-peptides that are cross-recognized by the c259 TCR.

4.2 Results

4.2.1 Measuring T cell cross-reactivity to a positional scanning peptide library

In Chapters 2 and 3 we have used a panel of altered peptide ligands to measure T cell ligand discrimination. A key advantage of using APLs is that they exhibit similar loading and stability on MHC [114]. Additionally, APLs display small differences in their k_{on} rates to the TCR [125]. Therefore, differences in K_D can be used as a proxy for differences in k_{off}. Altogether, this approach enabled us to quantify differences in T cell responses to ligands with defined affinities to the TCR.

Next, we sought to confirm if these findings translate generally to larger sets of peptides. Since these 7 peptide variants only represent a small fraction of the total mutational landscape, we expanded our analysis by measuring T cell activation to a positional scanning peptide library. In this library, each position of the NY-ESO-1 peptide (SLLMWITQV) was mutated to any possible amino acid except cysteine, to prevent aggregation due to disulfide bridges. In total, the library is composed of 163 single amino acid mutant peptides.

We pulsed U87 cells with each of the 163 peptides to activate wild-type, CD8 KO or CD8→CD4 co-receptor switch T cells. We measured target cell killing following co-culture (Figure 4.1). We found that wild-type T cells are able to recognize a very large number of peptides from the positional scanning library. In particular, substitutions at positions 1 and 3 were the best tolerated and did not have a large impact on T cell activation. Surprisingly, T cells still became activated against most peptide mutants at position 9 and especially at position 2, despite being anchor residues on the MHC molecule [249, 282, 283]. On the other hand, mutations in position 5 led to the largest reduction in activation, indicating that the c259 TCR has a strong dependence on tryptophan at this central residue.

We observed a striking reduction in overall cross-reactivity for CD8 knock-out T cells and especially CD8→CD4 co-receptor switch T cells. Importantly, activation against the high affinity cognate peptide (SLLMWITQV) was not reduced (Black boxes). While the overall pattern of activation was similar to wild-type cells, the dependence of tryptophan at position 5 was even more pronounced. Any mutation at this position completely abolished activation of the enhanced discriminatory T cells. Positions 1 and 3 remained the most flexible, with mutations being somewhat tolerated, although to a lesser extent than in wild-type cells.

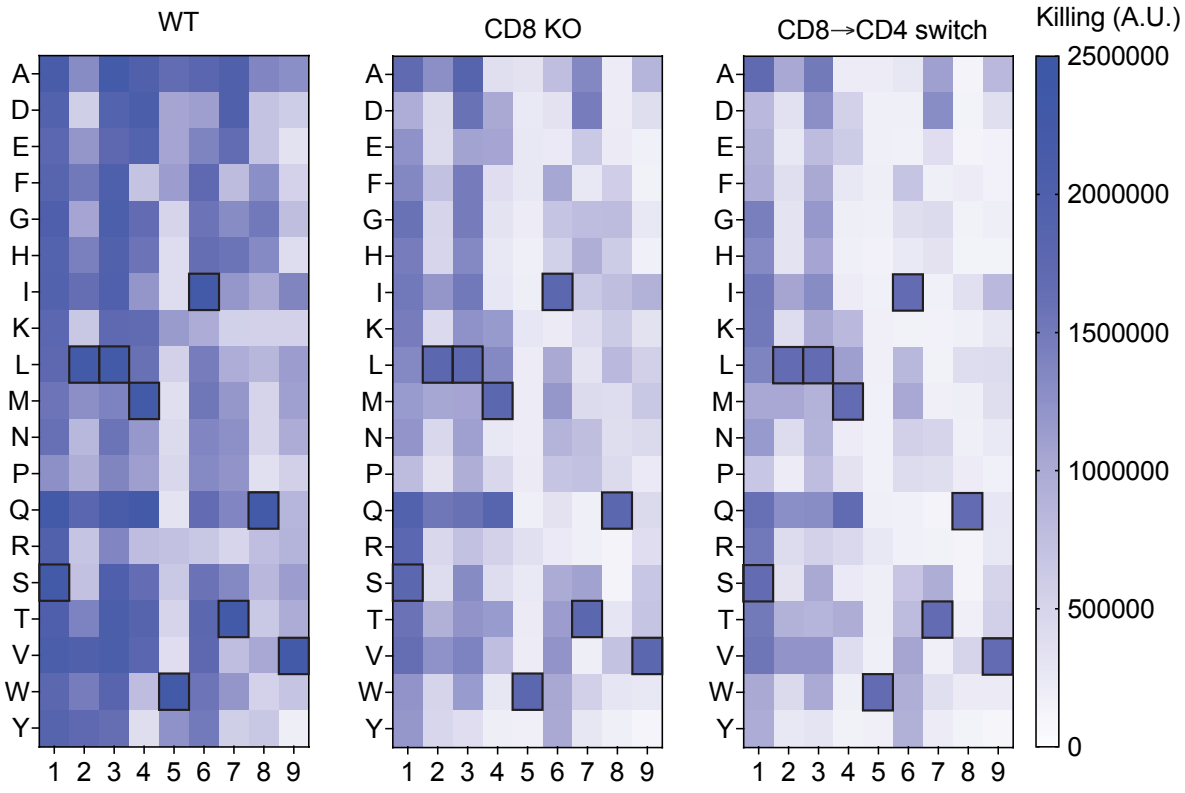


Figure 4.1: CD8→CD4 co-receptor switch T cells display lower cross-reactivity to a positional scanning peptide library than WT or CD8 KO T cells
U87 cells were individually pulsed with 0.1 µM of each of the 163 peptides in the positional library and co-cultured with T cells. Target killing was measured after 20 hours. Boxed amino acids represent the NY-ESO-1 peptide SLLMWITQV. Data is representative from N=4 independent experiments with different blood donors.

Next, we investigated whether this reduction in cross-reactivity to the positional scanning library was due to the increase in ligand discrimination based on affinity. Firstly, we developed a novel high-throughput SPR protocol to measure the affinity of the c259 TCR to the 163 pMHCs (See Materials and Methods). One limitation of the traditional SPR method that we used in Chapter 2 is that each pMHC must be individually re-folded and purified. Moreover, standard SPR instruments can only measure up to 3 pMHCs in parellel. Together, these limitations introduce a major bottleneck for determining TCR/pMHC affinities. For this high-throughput method, we decided to use a disulfide stabilised MHC [334] that can be individually loaded with each of the peptides, therefore enabling a much faster production of the 163 pMHC ligands. Additionally, the Carterra instrument used in the high throughput method enables the analysis of up to 396 pMHCs per run.

We find a broad range of affinities across the positional scanning library, spanning $\sim$1-10,000 μM (Figure 4.2 and Table 4.1). Some peptides could not be stably loaded on the MHC molecule, as determined by the lack of binding of an anti-β_2m antibody. Therefore, no affinity could be measured for these unstable pMHCs (white boxes), especially for peptides with mutations at the anchor positions 2 and 9.

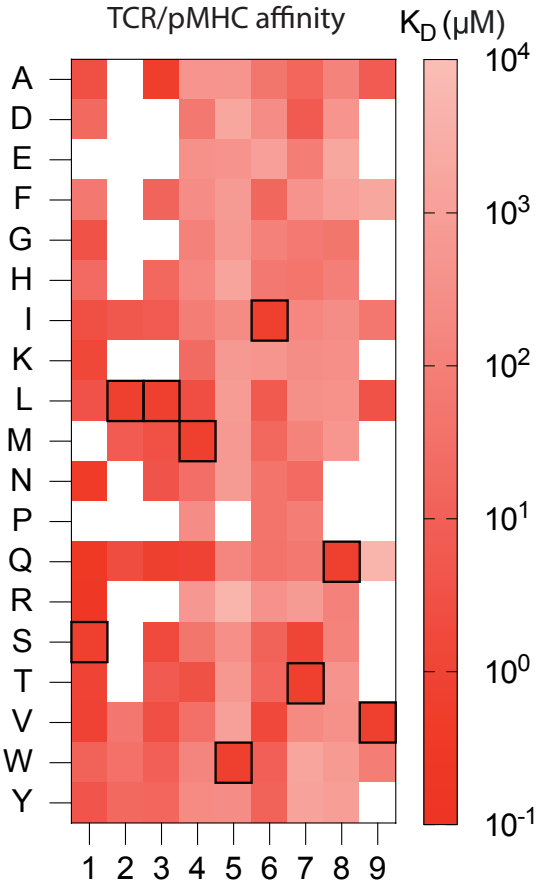


Figure 4.2: Measuring the binding affinity of the c259 TCR to a positional scanning peptide library.
Affinity between c259 TCR and each pMHC in the positional scanning peptide library was determined at 37 degrees by a high-throughput SPR method. Mean K_D values are shown from N=3 independent experiments. Boxed amino acids represent the NY-ESO-1 peptide SLLMWITQV. White boxes represent peptides without detectable MHC binding.

Table 4.1: The c259 TCR affinities to the positional scanning peptide library. Geometric mean, geometric standard deviation across N experiments is reported. K_D values have been excluded if pMHC was unstable (indicated as N/A) or no TCR binding response was observed (indicated as NB).

Position 1					Position 2					Position 3				
Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N
1A	ALLMWITQV	2.606	2.781	2	**2A**	SALMWITQV	N/A			**3A**	SLAMWITQV	0.596	1.057	2
1D	DLLMWITQV	18.846	1	1	**2D**	SDLMWITQV	N/A			**3D**	SLDMWITQV	N/A		
1E	ELLMWITQV	N/A			**2E**	SELMWITQV	N/A			**3E**	SLEMWITQV	N/A		
1F	FLLMWITQV	57.473	5.281	2	**2F**	SFLMWITQV	N/A			**3F**	SLFMWITQV	13.441	3.048	3
1G	GLLMWITQV	3.478	3.361	3	**2G**	SGLMWITQV	N/A			**3G**	SLGMWITQV	N/A		
1H	HLLMWITQV	21.339	10.308	3	**2H**	SHLMWITQV	N/A			**3H**	SLHMWITQV	17.216	18.519	2
1I	ILLMWITQV	2.787	6.294	3	**2I**	SILMWITQV	5.397	63.1	2	**3I**	SLIMWITQV	6.639	11.558	3
1K	KLLMWITQV	1.444	8.647	3	**2K**	SKLMWITQV	N/A			**3K**	SLKMWITQV	N/A		
1L	LLLMWITQV	3.7	9.695	3	**2L**	SLLMWITQV	0.701	1.966	3	**3L**	SLLMWITQV	0.701	1.966	3
1M	MLLMWITQV	N/A			**2M**	SMLMWITQV	6.684	23.015	2	**3M**	SLMMWITQV	2.885	3.632	3
1N	NLLMWITQV	0.409	1	1	**2N**	SNLMWITQV	N/A			**3N**	SLNMWITQV	3.951	19.691	2
1P	PLLMWITQV	N/A			**2P**	SPLMWITQV	N/A			**3P**	SLPMWITQV	N/A		
1Q	QLLMWITQV	0.309	1.403	2	**2Q**	SQLMWITQV	2.241	14.094	2	**3Q**	SLQMWITQV	0.688	6.074	2
1R	RLLMWITQV	0.226	1.082	2	**2R**	SRLMWITQV	N/A			**3R**	SLRMWITQV	N/A		
1S	SLLMWITQV	0.701	1.966	3	**2S**	SSLMWITQV	N/A			**3S**	SLSMWITQV	1.82	2.026	3
1T	TLLMWITQV	1.046	3.568	2	**2T**	STLMWITQV	N/A			**3T**	SLTMWITQV	6.228	1	1
1V	VLLMWITQV	0.91	1.746	3	**2V**	SVLMWITQV	50.287	1	1	**3V**	SLVMWITQV	2.657	1	1
1W	WLLMWITQV	11.631	1.329	3	**2W**	SWLMWITQV	33.447	3.481	3	**3W**	SLWMWITQV	9.861	1.691	3
1Y	YLLMWITQV	3.993	1.292	3	**2Y**	SYLMWITQV	18.095	1.288	3	**3Y**	SLYMWITQV	15.39	1.34	3
Position 4					**Position 5**					**Position 6**				
Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N
4A	SLLAWITQV	431.199	19.596	2	**5A**	SLLMAITQV	450.833	2.219	2	**6A**	SLLMWATQV	46.1	1.912	3
4D	SLLDWITQV	55.894	4.407	3	**5D**	SLLMDITQV	1779.52	1	1	**6D**	SLLMWDTQV	226.549	1.541	2
4E	SLLEWITQV	335.407	3.105	3	**5E**	SLLMEITQV	406.72	2.54	3	**6E**	SLLMWETQV	971.245	1.531	2
4F	SLLFWITQV	248.389	1.439	3	**5F**	SLLMFITQV	730.463	1.622	3	**6F**	SLLMWFTQV	16.258	4.03	3
4G	SLLGWITQV	111.398	2.492	3	**5G**	SLLMGITQV	658.784	1.293	2	**6G**	SLLMWGTQV	118.893	1	1
4H	SLLHWITQV	161.301	1.704	3	**5H**	SLLMHITQV	1436.123	1	1	**6H**	SLLMWHTQV	61.979	1.624	3
4I	SLLIWITQV	85.441	1.52	3	**5I**	SLLMIITQV	220.473	1	1	**6I**	SLLMWITQV	0.701	1.966	3
4K	SLLKWITQV	22.035	4.9	3	**5K**	SLLMKITQV	654.509	2.109	2	**6K**	SLLMWKTQV	476.51	1.479	3
4L	SLLLWITQV	2.392	17.993	3	**5L**	SLLMLITQV	815.925	1.446	3	**6L**	SLLMWLTQV	5.596	5.279	3
4M	SLLMWITQV	0.701	1.966	3	**5M**	SLLMMITQV	670.428	1.447	2	**6M**	SLLMWMTQV	16.572	16.638	3
4N	SLLNWITQV	26.905	2.998	3	**5N**	SLLMNITQV	758.195	1	1	**6N**	SLLMWNTQV	40.021	2.134	3
4P	SLLPWITQV	241.083	2.6	3	**5P**	SLLMPITQV	N/A			**6P**	SLLMWPTQV	41.366	1	1
4Q	SLLQWITQV	0.927	1.821	3	**5Q**	SLLMQITQV	154.879	1	1	**6Q**	SLLMWQTQV	38.707	1.705	3
4R	SLLRWITQV	604.902	1.555	2	**5R**	SLLMRITQV	4576.089	1	1	**6R**	SLLMWRTQV	389.248	1.019	2
4S	SLLSWITQV	48.243	1.186	3	**5S**	SLLMSITQV	289.89	1.941	3	**6S**	SLLMWSTQV	12.323	2.107	3
4T	SLLTWITQV	2.985	1.985	3	**5T**	SLLMTITQV	584.582	1.56	3	**6T**	SLLMWTTQV	15.427	1.101	3
4V	SLLVWITQV	30.833	1.08	2	**5V**	SLLMVITQV	1048.028	1.686	2	**6V**	SLLMWVTQV	1.796	1.671	3
4W	SLLWWITQV	146.053	1.285	3	**5W**	SLLMWITQV	0.701	1.966	3	**6W**	SLLMWWTQV	9.808	1.354	3
4Y	SLLYWITQV	208.894	1.518	3	**5Y**	SLLMYITQV	243.945	1.92	3	**6Y**	SLLMWYTQV	12.347	1.439	3
Position 7					**Position 8**					**Position 9**				
Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N	Abbr	Sequence	Geo. mean	Geo. SD	N
7A	SLLMWIAQV	15.201	3.121	3	**8A**	SLLMWITAV	126.651	3.221	3	**9A**	SLLMWITQA	6.31	18.975	3
7D	SLLMWIDQV	6.163	9.833	3	**8D**	SLLMWITDV	440.519	1.688	2	**9D**	SLLMWITQD	N/A		
7E	SLLMWIEQV	85.179	1.266	3	**8E**	SLLMWITEV	1758.587	2.616	3	**9E**	SLLMWITQE	N/A		
7F	SLLMWIFQV	408.909	1.802	3	**8F**	SLLMWITFV	961.039	1	1	**9F**	SLLMWITQF	1773.142	1	1
7G	SLLMWIGQV	65.685	9.19	2	**8G**	SLLMWITGV	45.631	3.139	3	**9G**	SLLMWITQG	N/A		
7H	SLLMWIHQV	45.044	4.503	3	**8H**	SLLMWITHV	99.112	1.372	3	**9H**	SLLMWITQH	N/A		
7I	SLLMWIIQV	168.64	2.155	3	**8I**	SLLMWITIV	267.092	1.481	2	**9I**	SLLMWITQI	51.817	1	1
7K	SLLMWIKQV	259.139	1	1	**8K**	SLLMWITKV	305.156	1.099	2	**9K**	SLLMWITQK	N/A		
7L	SLLMWILQV	321.614	1.129	2	**8L**	SLLMWITLV	357.078	2.024	2	**9L**	SLLMWITQL	3.39	1.913	2
7M	SLLMWIMQV	127.392	2.346	3	**8M**	SLLMWITMV	536.124	1.767	3	**9M**	SLLMWITQM	N/A		
7N	SLLMWINQV	20.053	7.134	3	**8N**	SLLMWITNV	N/A			**9N**	SLLMWITQN	N/A		
7P	SLLMWIPQV	83.799	4.303	3	**8P**	SLLMWITPV	N/A			**9P**	SLLMWITQP	N/A		
7Q	SLLMWIQQV	58.27	1.297	3	**8P**	SLLMWITQV	0.701	1.966	3	**9Q**	SLLMWITQQ	4607.455	1	1
7R	SLLMWIRQV	738.23	1.616	2	**8R**	SLLMWITRV	109.572	1.42	3	**9R**	SLLMWITQR	N/A		
7S	SLLMWISQV	1.199	1.839	3	**8S**	SLLMWITSV	122.849	1.544	3	**9S**	SLLMWITQS	N/A		
7T	SLLMWITQV	0.701	1.966	3	**8T**	SLLMWITTV	420.7	2.238	3	**9T**	SLLMWITQT	N/A		
7V	SLLMWIVQV	196.983	2.279	3	**8V**	SLLMWITVV	330.714	5.072	2	**9V**	SLLMWITQV	0.701	1.966	3
7W	SLLMWIWQV	1497.217	4.298	2	**8W**	SLLMWITWV	677.351	3.87	3	**9W**	SLLMWITQW	88.229	1	1
7Y	SLLMWIYQV	1190.897	2.148	3	**8Y**	SLLMWITYV	939.924	3.947	2	**9Y**	SLLMWITQY	N/A		

Finally, we plot the target killing results over their affinity (K_D) (Figure 4.3A). We find that affinity correlates with target cell killing across the positional scanning library. Wild-type T cells activate strongly against high affinity peptides, but some background killing is detected even for the lowest affinity peptides. Knocking-out CD8 or introducing a CD8→CD4 co-receptor switch did not reduce activation against the highest affinity peptides, but it selectively abolished activation against the lower affinity peptides. We quantify the affinity threshold for activation as the IC50 from this plot (Figure 4.3B). We find that wild-type cells have the lowest affinity threshold for activation. Knocking-out CD8 increases the affinity threshold for activation by approximately 10 fold compared to wild-type cells, while introducing a CD8→CD4 co-receptor switch increases the affinity threshold by approximately 2 fold further. Overall, these results demonstrate that the reduced cross-reactivity of CD8 KO and CD8→CD4 co-receptor switch T cells against the positional scanning library is a result of their enhanced ligand discrimination.

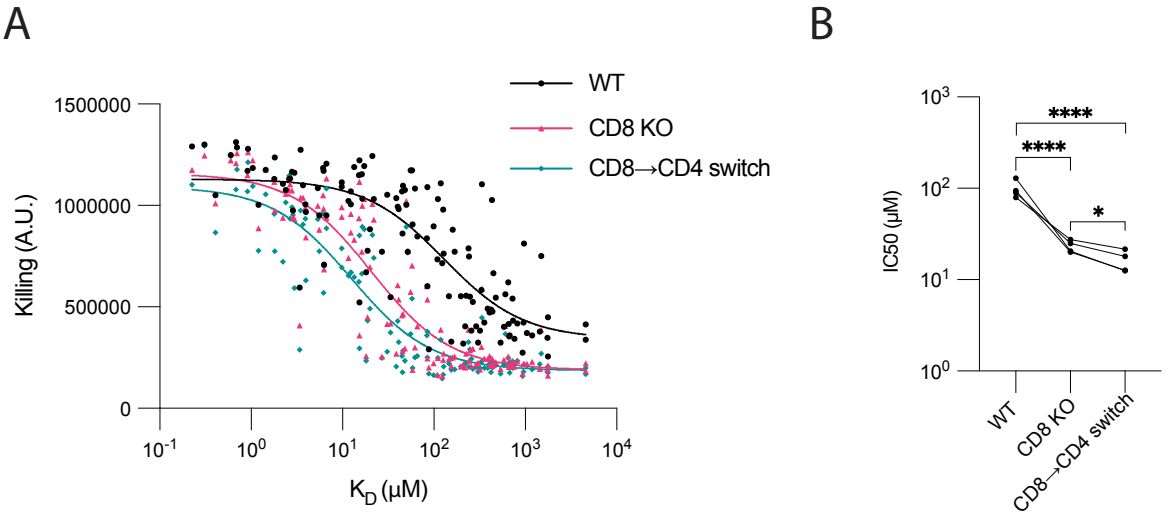


Figure 4.3: CD8→CD4 co-receptor switch T cells display lower cross-reactivity to a positional scanning peptide library due to their enhanced ligand discrimination.
(A) Representative target cell killing from (Fig. 4.1) plotted over the TCR/pMHC K_D from (Fig. 4.2). **(B)** IC50 from (A) is plotted with each data point representing an independent experiment with different blood donors. P values were determined by paired t-test. *p<0.05, ****p<0.0001.

4.2.2 Measuring T cell cross-reactivity to a pooled peptide library

We have demonstrated that enhanced discriminatory T cells display reduced cross-reactivity to selected NY-ESO-1 peptide mutants. However, all of the peptides that we have tested have high sequence similarity as they are only one-edit distance apart. Therefore, we sought to further validate our results using large, unbiased peptide libraries. In a completely random peptide mixture, we expect that a TCR will bind the large majority of peptides with low affinity, and only a small subset of peptides with high affinity. Therefore, we hypothesised that increasing the discrimination of T cells would reduce their cross-reactivity to a pooled peptide library. We measured the activation of c259 TCR expressing T cells to APCs loaded with random peptide mixtures of different lengths (Randomly synthesised peptides with all amino acids except cysteine) (Figure 4.4). We find that T cells only activate above background against cells loaded with the 9-mer peptide library. These results are consistent with previous findings that each TCR has a peptide length preference [335].

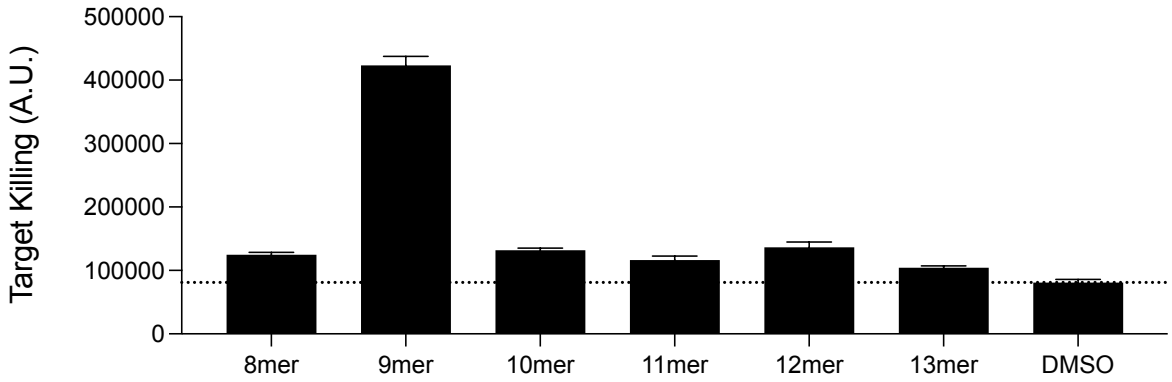


Figure 4.4: c259 TCR-T cells cross-react with a pooled library of 9-mer peptides. U87 cells were loaded with different pooled X-mer peptide libraries to stimulate c259 TCR-T cells. Target killing was measured after 20 hours. Data is displayed as mean of 3 technical replicates ± SDs. Data is representative of N=2 independent experiments with different blood donors.

Next, we compared the cross-reactivity of wild-type or enhanced discriminatory T cells against the pooled 9-mer library. This library has a theoretical diversity of 19^9 (3.2×10^{11}) peptides. We find that wild-type cells display the highest cross-reactivity. Knocking-out CD8 reduced cross-reactivity by approximately 40% and introducing a CD8→CD4 co-receptor switch reduced cross-reactivity by a further 30% (Figure 4.5). Overall, these results support our hypothesis that enhancing the ligand discrimination of T cells reduces their cross-reactivity to lower affinity peptides, regardless of their sequence identity.

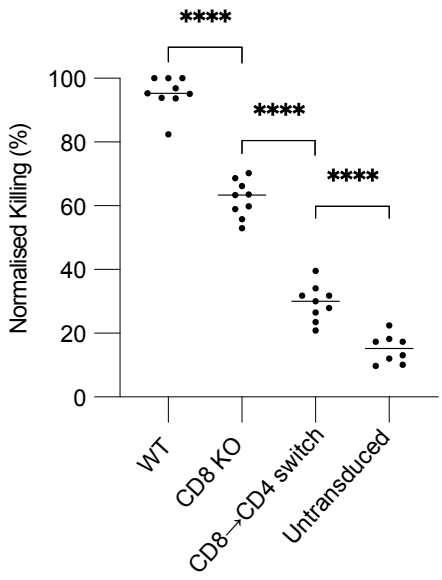


Figure 4.5: CD8→CD4 co-receptor switch T cells display lower cross-reactivity to a 9-mer pooled peptide library than WT or CD8 KO T cells
U87 cells were loaded with a pooled 9-mer peptide library to stimulate WT, CD8 KO or CD8→CD4 co-receptor switch cytotoxic c259 TCR-T cells. Target killing was measured after 20 hours. Each data point represents a technical replicate from N=3 independent experiments with different blood donors. P values were determined by paired t-test. ****$p<0.0001$.

4.2.3 Measuring T cell cross-reactivity to predicted self peptides

In T cell therapies, cross-reactivity can lead to lethal autoimmune toxicities if lower affinity self-peptides are recognized. Therefore, we sought to investigate if enhancing T cell ligand discrimination would reduce cross-reactivity to self-peptides. A previous study predicted peptides from the human proteome that can be presented by HLA-A*02:01 and cross-react with the c259 TCR [249]. Here, we selected the top predicted self-peptides and loaded them on U87 cells to activate c259 TCR-T cells (Figure 4.6). We find that 5 of these peptides can lead to detectable T cell activation, with a wide-range of potencies. These peptides have low sequence similarity to the cognate NY-ESO-1 peptide, with edit distances of 4-7. We subsequently measured the affinity of the c259 TCR to these self-pMHCs by Surface Plasmon Resonance (Figure 4.7 and Table 4.2). Most of these peptides have weak affinities to the TCR (K_D>100μM), except MKI67 (K_D=10μM).

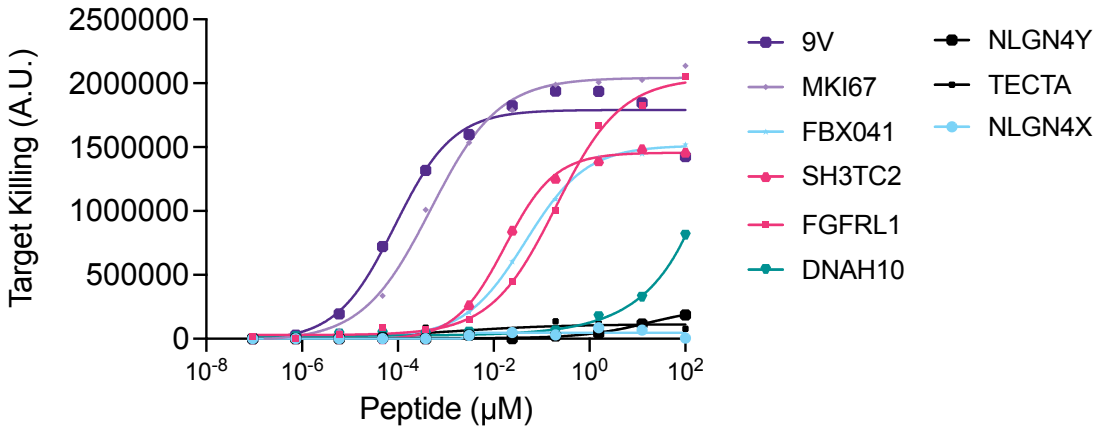


Figure 4.6: c259 TCR-T cells recognise predicted self-peptides.
U87 cells were pulsed with self-peptides predicted to cross-react with the c259 TCR [249]. Target cell killing was measured after 20 hours.

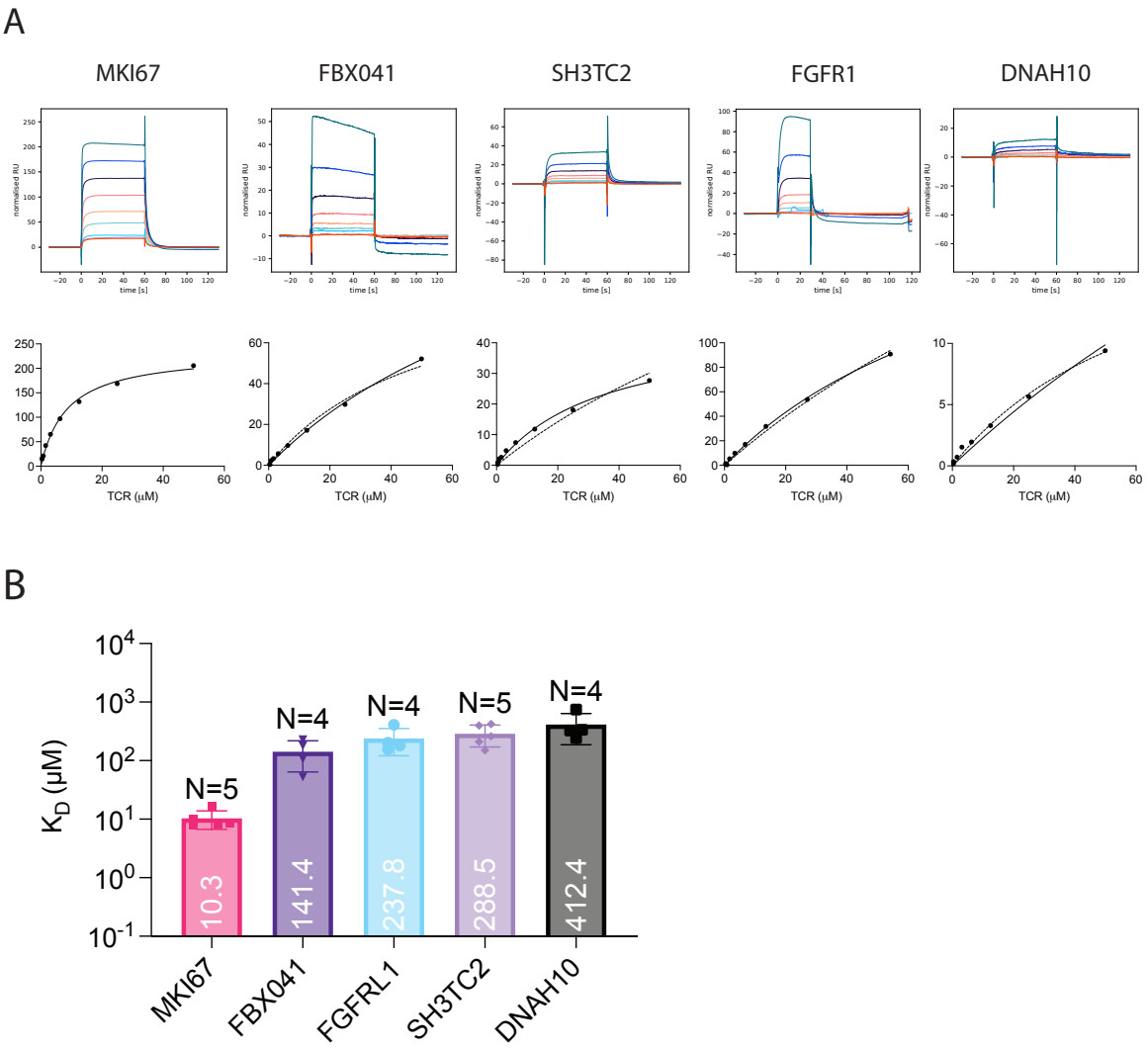


Figure 4.7: The c259 TCR affinity with a panel of self peptides measured by SPR at 37°C. (A) to stimulate WT c259 TCR-T cells. Target cell killing was measured after 20 hours. **(B)** The binding affinity of the c259 TCR to the peptides that induced T cell activation were measured. (Top) Representative SPR sensograms depicting injections of increasing concentrations of the c259 TCR. (Bottom) Representative equilibrium curves of c259 TCR binding to different self pMHCs. The TCR/pMHC affinity was calculated by constraining Bmax (dashed line) or fitting Bmax (solid line). **(C)** Steady-state binding affinity for the selected peptides. Barplot represents mean K_D ± SDs. The affinities were calculated by constraining Bmax to the value obtained from the standard curve in (B) based on the amount of BBM.1 antibody that bound the chip surface (see Methods for details). All data fitting was performed using a one site-specific binding model in GraphPad Prism.

Table 4.2: The c259 TCR affinities to predicted self-peptides.

Gene	Sequence	Mean	SD	N	Edit distance
NY-ESO-1 (9V)	SLLMWITQV	1.240	0.129	3	0
MKI67	FLTLWLTQV	10.314	3.612	5	4
FBX041	MLAQWCTQA	141.35	60.426	4	5
FGFRL1	TLLLWLCQA	237.775	116.323	4	5
SH3TC2	QVFLWLAQV	288.480	117.831	5	6
DNAH10	CVINWLNQI	412.425	225.050	4	7

We then investigated how CD8 KO impacted activation against the panel of self-peptides (Figure 4.8). We find that knocking-out CD8 reduces activation against all of the self-peptides, without reducing activation against the on-target NY-ESO-1 peptide (9V). These results were consistent for both target cell killing and the expression of the 4-1BB activation marker.

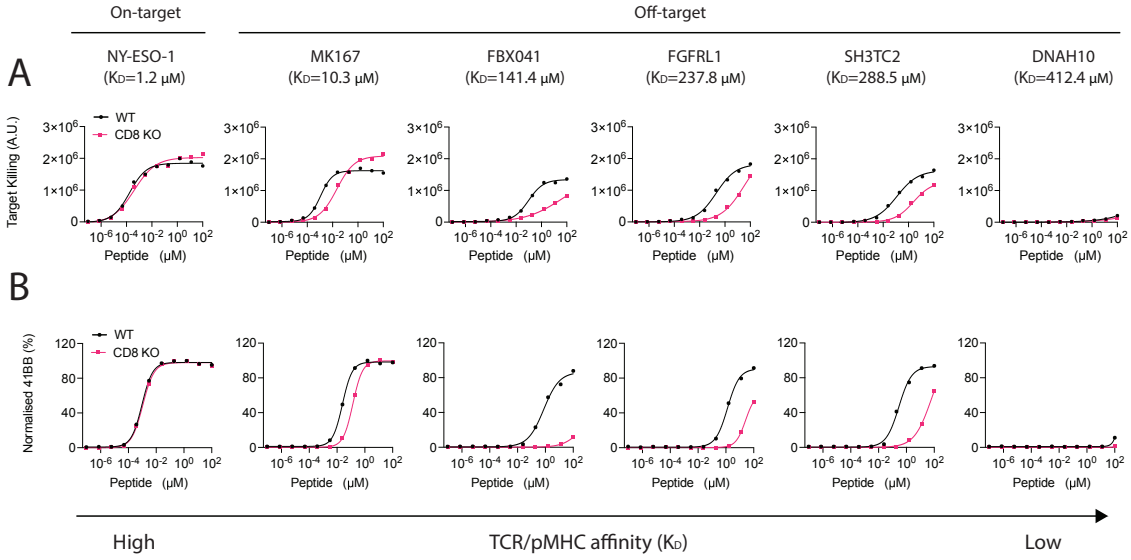


Figure 4.8: CD8 KO T cells display reduced cross-reactivity to a panel of self peptides.
U87 cells were pulsed with each of the self-peptides to stimulate WT or CD8 KO c259 TCR-T cells. Target cell killing **(A)** or expression of the activation marker 4-1BB **(B)** was measured after 20 hours. Data is representative of N=3 independent experiments with different blood donors.

Introducing a CD8→CD4 co-receptor switch led to an even greater reduction in activation against the predicted self-peptides (Figure 4.9). Activation was almost completely abolished for all self-peptides except the highest affinity one MKI67.

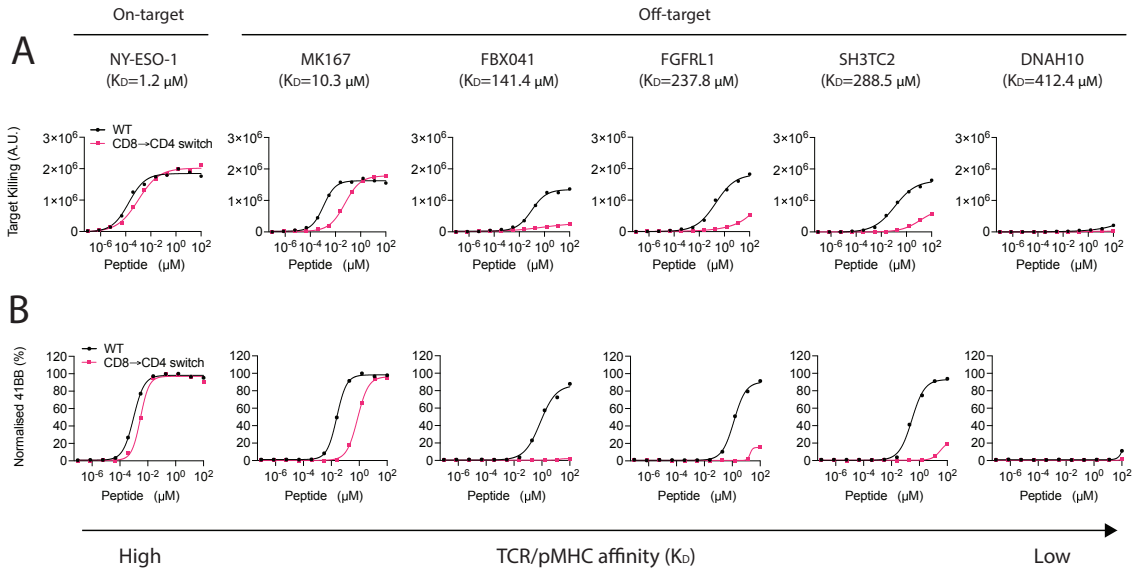


Figure 4.9: CD8→CD4 co-receptor switch T cells display reduced cross-reactivity to a panel of self peptides.
U87 cells were pulsed with each of the self-peptides to stimulate WT or CD8→CD4 co-receptor switch c259 TCR-T cells. Target cell killing **(A)** or expression of the activation marker 4-1BB **(B)** was measured after 20 hours. Data is representative of N=3 independent experiments with different blood donors.

We next plotted the fold changes in activation over peptide affinity (K_D) and we find that CD8→CD4 co-receptor switch T cells led to the largest increase in ligand discrimination, consistent with our results in Chapter 2 using the NY-ESO-1 APL panel (Figure 4.10). Overall, activation to the lowest affinity self-peptides was reduced by up to ∼1,000 fold, without reducing on-target potency to NY-ESO-1 (Figure 4.11).

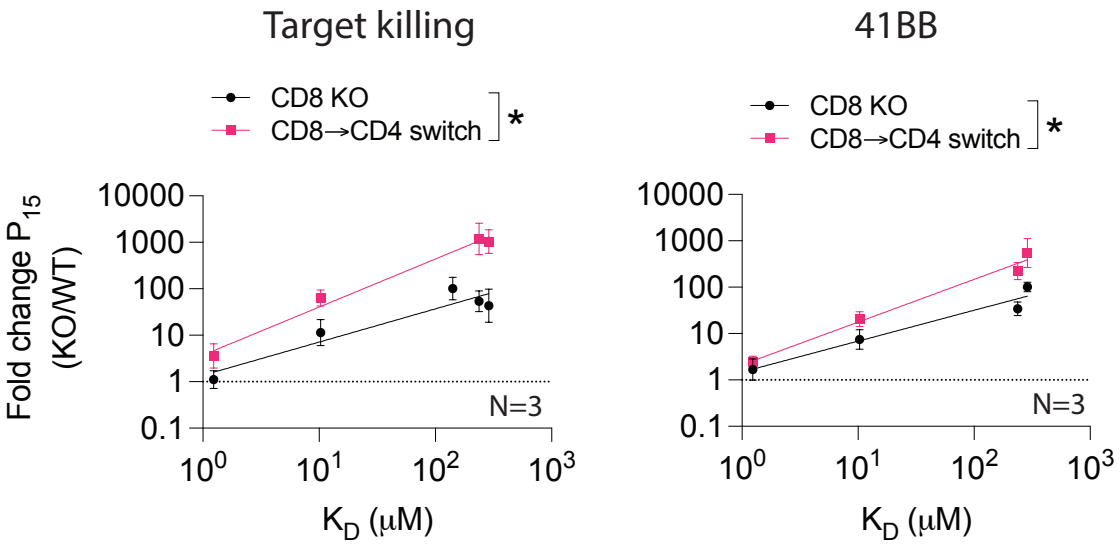


Figure 4.10: CD8→CD4 co-receptor switch T cells display lower cross-reactivity to a panel of self peptides due to their enhanced ligand discrimination.
Fold change in Potency (P_{15}) between modified and WT T cells from (Fig. 4.8 and 4.9) is plotted over TCR/pMHC affinity (K_D). Data is shown as means ± SDs from N=3 independent experiments with different blood donors. P values were determined by F-test. *p<0.05.

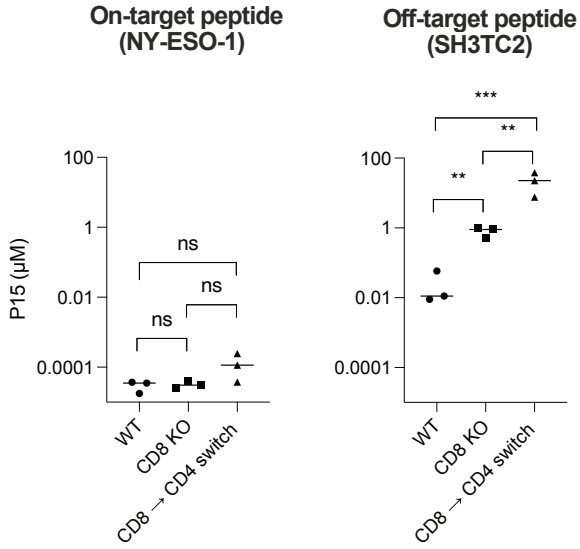


Figure 4.11: The CD8→CD4 co-receptor switch does not reduce T cells potency to their high-affinity on-target ligand.
Target cell killing potency (P_{15}) from (Fig. 4.8 and 4.9) is plotted for the indicated peptides. Each data point represents an independent experiment with different blood donors. P values were determined by paired t-test. ns not significant, **p<0.01, ***p<0.001.

4.3 Discussion

Identifying the potential off-target cross-reactivities of a TCR before first-in-human clinical trials is challenging [247–249]. For instance, the therapeutic a3a TCR did not show any cross-reactivity to different primary human cells, such as melanocytes, hepatocytes and epithelial cells in pre-clinical studies. However, it cross-reacted with an off-target Titin peptide in a clinical trial, causing multiple lethal autoimmune toxicities [233, 245].

Current efforts have focused on identifying TCRs that can recognise the target tumour pMHC but do not cross-react with healthy tissues. The CDR loops of a TCR can be rationally mutated to avoid known cross-reactivities, such as in the case of Titin [331]. However, this approach requires previous knowledge of a lethal cross-reactivity and changing the sequence of the TCR CDR loops could introduce yet new cross-reactivities. Additionally, it has been proposed that modifying the CDR loops to reduce their flexibility or introduce catch bonds may increase TCR specificity [330, 332, 333]. However, these strategies require challenging optimisation for each new therapeutic TCR and might not be generalizable. We hypothesized that enhancing T cell ligand discrimination could be an alternative strategy to reduce T cell cross-reactivity without modifying the TCR itself. In this chapter, we have shown that CD8→CD4 co-receptor switch T cells display reduced cross-reactivity using three different methods.

Firstly, we show that CD8 knock-out reduces T cell cross-reactivity to a positional scanning peptide library. Our results are consistent with a previous study that showed that the CD8 co-receptor increased cross-reactivity to a combinatorial peptide library (CPL) [177]. The CPL is composed of 180 mixtures of 9-mer peptides. In each pooled peptide mixture, one of the nine positions of the peptide sequence is fixed to a particular amino acid, whilst the remaining 8 positions are randomly synthesised. In contrast, our positional scanning library is composed of 163 defined peptides, where we individually mutate each position in a 9-mer cognate peptide to every possible amino acid. Whilst both of these methods provide information about the amino acid preference of a TCR at different peptide positions, our positional scanning library enables us to interrogate the relationship between TCR/pMHC affinity and T cell activation. We find that CD8 knock-out increases the affinity threshold required for T cell activation. Furthermore, introducing a CD8→CD4 co-receptor switch increased this affinity threshold even further. Overall, these results validate our findings from the APL panel from Chapters 2 and 3 using a much larger number of ligands.

Next, we investigated the cross-reactivity of T cells to a randomly synthesised peptide mixture. The total maximum diversity of this pooled peptide library is 19^9 (3.2×10^{11}) peptides and the concentration of each unique peptide is extremely low. We expect that the majority of these peptides will bind the c259 TCR with extremely weak affinities, and only a subset of these peptides will be strong agonists. Therefore, activation against this pooled library is likely the additive result of multiple interactions with different weak affinity peptides and serves as a metric of general T cell cross-reactivity to peptides of any sequence. We find that CD8→CD4 co-receptor switch T cells display the lowest cross-reactivity to the pooled peptide library, consistent with our findings that they have higher ligand discrimination than CD8 KO or wild-type T cells.

Finally, we investigate the cross-reactivity of c259 TCR-T cells to self-peptides. A previous study predicted off-target peptides from the human proteome that can be recognized by the c259 TCR [249]. We validate that c259 TCR-T cells can become activated against 5 of these self-peptides and we measured their binding affinity by SPR. We next show that knocking-out CD8 or especially introducing a CD8→CD4 co-receptor switch reduces activation to all of these self-peptides. One of these self-peptides (MKI67) bound the c259 TCR with a high affinity (K_D = 10 μM). Therefore, very high densities of this pMHC could activate even the enhanced discriminatory T cells. Interestingly, MKI67 has been described as a proliferation marker and its expression correlates with cancer progression [336–339]. Therefore, it would be interesting to test whether the MKI67 peptide is endogenously presented on HLA-A*02:01 molecules in healthy human tissues, and if so, at what density. This information would be required to understand whether CD8→CD4 co-receptor switching would be sufficient to de-risk the c259 TCR for therapeutic applications or if a stronger increase in ligand discrimination is required.

Whilst we have focused on the role of CD8 and CD4 in cross-reactivity in this chaper, we also identified that CD5 increases ligand discrimination in Chapter 2. Therefore, it would also be interesting to test the impact of CD5 on T cell cross-reactivity. For instance, we hypothesize that over-expressing CD5 could reduce cross-reactivity and knocking-it out would increase cross-reactivity. Whilst reducing T cell cross-reactivity can increase the safety of adoptive cell therapies, it has been proposed that increasing TCR cross-reactivity could be helpful to increase activation against lower affinity immune escape peptide variants in tumours with high genomic instability [340]. Therefore, CD5 knock-out could be exploited to achieve this effect without requiring modifications to the TCR.

Chapter 5

Discussion

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5.1 Summary

Engineering T cells with cancer-specific TCRs has shown promising clinical responses. However, these T cells can cross-react with off-target peptides in healthy tissues and cause lethal autoimmune toxicities. The aim of this thesis was to engineer T cells with enhanced ligand discriminatory powers to reduce the risk of off-target cross-reactivities in T cell therapies.

In Chapter 2, we investigated the role of different T cell co-signalling receptors in modulating ligand discrimination by knocking them out in human primary T cells. We show that the knock-out of the adhesion receptors CD2 or LFA-1, either individually or in combination, did not impact ligand discrimination. However, the knock-out of CD5 decreased ligand discrimination, while the knock-out of CD8 increased it. We demonstrate that CD8 knock-out increased the discrimination of T cells engineered with either a wild-type TCR (1G4) or an affinity-matured TCR (c259), both of which recognise an NY-ESO-1 peptide presented on HLA-A*02:01. Furthermore, CD8 knock-out also enhanced the discrimination of T cells engineered with the a3a TCR, which recognises a MAGE-A3 peptide presented on HLA-A*01:01 (Chapter 3). Therefore, this strategy could be used to improve the safety of TCRs recognising diverse peptides across different HLA alleles.

In Chapter 3, we compared the ligand discrimination of CD8+ cytotoxic and CD4+ helper T cells engineered with pMHC-I restricted TCRs. We found that CD4+ helper T cells displayed the highest ligand discrimination, which was expected since they do not express the CD8 co-receptor. Surprisingly, their ligand discrimination was even higher than that of CD8 knock-out cytotoxic T cells. We show that the CD4 incompatible co-receptor, which binds Lck but cannot bind the target pMHC-I, is responsible for this enhanced discrimination. Subsequently, we demonstrated that engineering cytotoxic T cells with a CD8-to-CD4 co-receptor switch further improved their ligand discrimination. We propose that the incompatible co-receptor enhances ligand discrimination by sequestering Lck from the TCR.

In Chapter 4, we measured the cross-reactivity of CD8-to-CD4 co-receptor switch T cells to large peptide libraries. CD8-to-CD4 co-receptor switch T cells display reduced cross-reactivity to a positional scanning library, where each position of the NY-ESO-1 peptide was mutated to every possible amino acid. Additionally, we developed a high-throughput SPR method and measured the binding affinity of the c259 TCR to these 163 single-mutant peptides. We demonstrate that the reduced cross-reactivity of CD8-to-CD4 co-receptor switch T cells to this positional scanning library is due to their enhanced ligand discrimination, since responses were selectively reduced to lower affinity peptides. Moreover, we show that CD8-to-CD4 co-receptor switch T cells display reduced cross-reactivity to a randomly synthesised pooled peptide library and to predicted self-peptides. Overall, these findings suggest that co-receptor switching is an attractive strategy to improve the safety of TCR-engineered T cell therapies without reducing activation against the high-affinity target tumour peptides.

5.2 Future implications

How do T cell co-signalling receptors influence ligand discrimination?

Our current understanding of the mechanisms that regulate T cell ligand discrimination is incomplete. In this thesis, we sought to investigate whether T cell discrimination can be modulated by its co-signalling receptors.

Previous work from our group demonstrated that T cells display very poor ligand discrimination when activated with plate-bound pMHC [114]. Therefore, this suggests that ligand discrimination is not an intrinsic feature of the TCR, but rather an emergent property of the interactions between a T cell and its target cell. Consequently, we hypothesised that the combination of co-signalling receptors expressed by a T cell, and the combination of ligands presented by the target cell, could both influence discrimination.

We did not find any changes in ligand discrimination when knocking-out the CD2 or LFA-1 adhesion receptors. This contradicts previous findings from our group, which suggested that adhesion interactions increase ligand discrimination [114, 144]. Our results in this thesis do not completely rule out a potential mechanism for adhesion interactions in modulating ligand discrimination. However, since there are many overlapping adhesion receptors, it would be hard to change the discrimination of T cells by deleting adhesion receptors in therapeutic applications. Interestingly, we found that knocking-out CD8 and CD5 could increase or decrease ligand discrimination, respectively.

These results demonstrate that T cell surface molecules can influence ligand discrimination. One limitation of our study is that we have only investigated primary T cell blasts. These are commonly used as a model for effector T cells, and are also directly relevant for therapeutic applications, which use similar blasting and expansion protocols. Therefore, future work will be required to investigate how different T cell subsets might fine-tune their discrimination by differential expression of T cell surface molecules. Previous work from our group did not find any differences in ligand discrimination between quiescent naïve or memory T

cells and T cell blasts [114]. However, we expect that differences in CD8 and CD5 expression levels across other T cell subsets may indeed impact discrimination.

It remains unclear whether target cells can modulate T cell ligand discrimination by the combination of co-stimulatory or co-inhibitory ligands that they express. CD8 is unique, because it binds the same ligand as the TCR and the identity of CD5 ligands remains controversial [341, 342]. Moreover, its inhibitory function may be independent of any putative ligand binding. One limitation of our study is that we have only investigated a limited number of co-signalling receptors. Future work that includes a larger variety of receptors will be required to answer this question. For example, some studies have suggested that the engagement of the co-inhibitory receptor PD-1 by its ligand, PD-L1, might influence T cell ligand discrimination [343, 344].

How does TCR affinity maturation impact cross-reactivity?

Increasing the binding affinity of TCRs to their target tumour-peptides is a common strategy to enhance T cell potency. However, while affinity maturation can improve activation against the target peptide, it may also affect TCR binding cross-reactivity. Antibodies undergo a natural affinity-maturation process *in vivo* that results in higher specificity to their target antigens [345]. However, unlike antibodies, TCRs do not bind folded protein antigens, but a complex surface composed by peptide and MHC. One would expect that if the mutations in the TCR primarily increase binding to MHC-residues, cross-reactivity would increase. On the other hand, if the mutations mainly affect interactions with the peptide residues, cross-reactivity would be unaffected or even reduced. Several affinity-matured TCRs have been developed and tested in clinical trials. While some have led to autoimmune toxicities due to off-target cross-reactivities [220, 233, 245], others have not [218, 230].

It is a common generalization that the germline encoded CDR1 and CDR2 loops primarily contact MHC residues, while the hypervariable CDR3 loops contact the peptide. However, this is not the case for every TCR [346]. In some cases, germline encoded CDR loops can interact with the peptide, [347] and CDR3 loops can contact conserved MHC residues [348] or influence how other CDR loops bind MHC [349].

For example, the MAGE-A3 specific a3a TCR, which caused two patient deaths due to off-target cross-reactivities in the heart, was generated by introducing 4 mutations in the CDR2α region of its parental TCR [245]. A different MAGE-A3 specific TCR, isolated from mice, was affinity-enhanced by introducing a single-point mutation in its CDR3α loop [350], which also led to two deaths due to off-target cross-reactivities in the brain. In contrast, the NY-ESO-1 specific c259 TCR, which carries two CDR3α mutations [243], did not cause any off-target toxicities in a clinical trial [230]. Similarly, a MAGE-A4 specific TCR with one CDR3α and one CDR3β single-point mutations [351] has shown a safe clinical profile [218]. Overall, regardless of which CDR region is selected for affinity-maturation, TCR cross-reactivity may be impacted, as evidenced by the clinical evidence. Therefore, structure-guided design is necessary when performing affinity-maturation to understand the potential impact of these mutations on TCR cross-reactivity.

We have shown that the affinity-matured c259 TCR has a higher binding affinity than its parental 1G4 TCR to both strong and weak agonist NY-ESO-1 single-mutant peptides (Fig. 5.1). Even though these are only a small number of ligands, these results suggest that the c259 affinity-enhancing mutations may increase binding to multiple ligands in addition to its NY-ESO-1 cognate peptide.

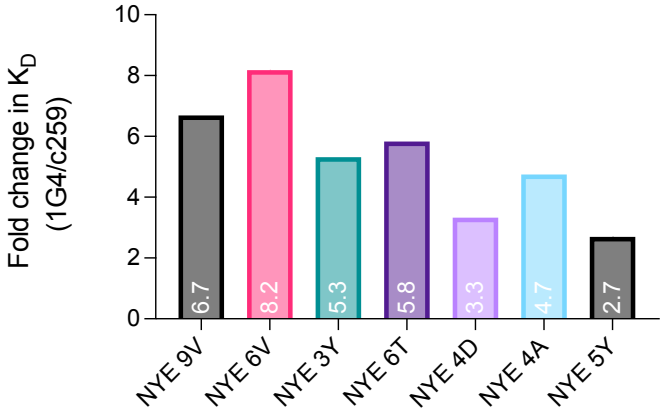


Figure 5.1: Fold change in binding affinity between the 1G4 and c259 TCR for a APL panel.
Steady-state binding affinity for the selected 7-peptide panel to the c259 TCR was measured in Figure 2.4, and to the 1G4 TCR was measured in [114]. Peptides are ordered from left to right according to decreasing potency.

It would also be interesting to compare the binding-affinity of the c259 and 1G4 TCRs to the full NY-ESO-1 positional scanning library. Nevertheless, studying TCR cross-reactivity to single-mutant peptides only provides information about specificity to peptides with high sequence similarity. TCRs can also cross-react with peptides with very divergent sequences, especially those exhibiting structural mimicry [315]. Therefore, alternative assays, such as the pooled peptide experiments used in this thesis or yeast-display libraries could provide additional information on TCR cross-reactivity to peptides with very diverse sequences.

Despite the **binding cross-reactivity** of TCRs to a large set of peptides, which may or may not be increased by affinity-maturation, T cells can modulate their **functional cross-reactivity** by signalling and kinetic proofreading. In this thesis, we have shown that CD4+ helper T cells display higher discrimination against pMHC-I antigens, and that the discrimination of CD8+ cytotoxic T cells can be increased with a CD8-to-CD4 co-receptor switch.

An alternative strategy that has previously been proposed to enhance the safety of T cell therapies is to engineer T cells with two different TCR variants [352]. On the one hand, a high affinity TCR would be used in CD4+ helper T cells to mediate potent responses, since they display lower cross-reactivity, as we show in this thesis. On the other hand, a lower affinity TCR would be transduced in CD8+ cytotoxic T cells, since they have higher cross-reactivity due to their CD8 co-receptor. However, this strategy would require additional optimisation and testing of the multiple TCR variants for each tumour target. Moreover, these variants would display different cross-reactivity profiles against diverse peptides.

Finally, one important consideration when engineering T cells with higher discrimination is to maintain sensitivity to the target tumour peptide. In the case of the c259 TCR, we have shown that activation against NY-ESO-1 ($K_D = \sim 1\ \mu M$) is not reduced by the CD8-to-CD4 co-receptor switch. A limitation of our method is that if a TCR does not bind its target cancer peptide with high affinity, its potency may be reduced by co-receptor switching. Therefore, affinity-maturation might be required for lower-affinity therapeutic TCRs to be used in combination with the co-receptor switch.

Chapter 6

Materials and Methods

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6.1 Reagents

Table 6.1: Antibodies for flow cytommetry.

Target	Clone	Conjugation	Supplier	RRID
CD45	HI30	BV421	Biolegend	RRID:AB_2561357
4-1BB	4B4-1	AF647	Biolegend	RRID:AB_2566258
CD69	FN50	AF647	Biolegend	RRID:AB_528871
CD8α	HIT8	PE	Biolegend	RRID:AB_314112
CD4	RPA-T4	PE	Biolegend	RRID:AB_314075
CD43	CD43-10G7	PE	Biolegend	RRID:AB_2255209
CD11a	TS2/4	PE	Biolegend	RRID:AB_10660819
CD5	UCHT2	PE	Biolegend	RRID:AB_314094
CD2	TS1/8	PE	Biolegend	RRID:AB_314758
TCR Vβ13.1	H131	APC	Biolegend	RRID:AB_2728348

Table 6.2: Cell lines.

Cell line	Source	Notes
U87	Dushek lab	HLA-A*02:01 positive.
T2	Dushek lab	HLA-A*02:01 positive.
T2-HLA-A1	This thesis	Transduced with HLA-A*01:01.
Nalm6	Crystal Mackall Lab	HLA-A*01:01 positive.
HEK-293T	Dushek lab	Used for lentivirus production.

Table 6.3: Cell culture media.

Name	Description
Complete RPMI	RPMI 10% FBS 1% Penicillin/Streptomycin
Complete DMEM	DMEM 10% FBS 1% Penicillin/Streptomycin

Table 6.4: General reagents.

Name	Supplier	Catalogue Number
RosetteSep Human CD8+ T Cell Enrichment Cocktail	STEMCELL technologies	15063
RosetteSep Human CD4+ T Cell Enrichment Cocktail	STEMCELL technologies	15062
Human recombinant IL2 (PeproTech)	Thermofisher Scientific	202-02-1MG
CD3/CD28 Human T-Activator Dynabeads	Thermofisher Scientific	11131D
X-tremeGENE HP	Merck	6366244001
Coelenterazine	Cambridge Bioscience	16123-1-mg-CAY
TrueCut Cas9 Protein v2	Thermofisher Scientific	A36499
Invitrogen™ IFN gamma Human Uncoated ELISA Kit	Fisher Scientific	88-7316-86
Cytiva Ficoll-Paque™ PLUS Media	Fisher Scientific	A36499
Opti-MEM (Gibco)	Thermofisher Scientific	31985062
BTX electroporation cuvette Plus	Fisher Scientific	15447270
MojoSort Human anti-PE nanobeads	Biolegend	480092

Table 6.5: Instruments and equipment.

Name	Company
Biacore T200 SPR system	Cytiva
Carterra LSA	Carterra
Carterra LSA-XT	Carterra
BD LRSFortessa X-20 cell analyzer	BD Biosciences
CytoFLEX flow cytometer	Beckman Coulter
BTX ECM 830 Square Wave Electroporation System	Harvard Bioscience
SpectraMax M3 microplate reader	Molecular Devides

Table 6.6: Software.

Name	Version	Company
FlowJo	10.10.0	BD Biosciences
GraphPad Prism	10.2.1	GraphPad Software

6.2 Sequences

Sequence 1: c259 TCR alpha chain (Insert in lentiviral transfer plasmid).

METLLGLLILWLQLQWVSSKQEVTQIPAALSVPEGENLVLNCSFTDSAIYNL
QWFRQDPGKGLTSLLLIQSSQREQTSGRLNASLDKSSGRSTLYIAASQPGDS
ATYLCAVRPLYGGSYIPTFGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKSVC
LFTDFDSQTNVSQSKDSDVYITDKTVLDMRSMDFKSNSAVAWSNKSDFAC
ANAFNNSIIPEDTFFPSPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILLLK
VAGFNLLMTLRLWSS*

Sequence 2: c259 TCR beta chain (Insert in lentiviral transfer plasmid).

MSIGLLCCAALSLLWAGPVNAGVTQTPKFQVLKTGQSMTLQCAQDMNHE
YMSWYRQDPGMGLRLIHYSVGAGITDQGEVPNGYNVSRSTTEDFPLRLLS
AAPSQTSVYFCASSYVGNTGELFFGEGSRLTVLEDLKNVFPPEVAVFEPSE
AEISHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVSTDPQPLKEQPA
LNDSRYCLSSRLRVSATFWQNPRNHFRCQVQFYGLSENDEWTQDRAKPV
TQIVSAEAWGRADCGFTSESYQQGVLSATILYEILLGKATLYAVLVSALVL
MAMVKRKDSRG*

Sequence 3: c259 TCR alpha chain with His Tag shown in bold (For soluble expression in *E. coli.*).

MQEVTQIPAALSVPEGENLVLNCSFTDSAIYNLQWFRQDPGKGLTSLLLIQ
SSQREQTSGRLNASLDKSSGRSTLYIAASQPGDSATYLCAVRPLYGGSYIPT
FGRGTSLIVHPYIQNPDPAVYQLRDSKSSDKSVCLFTDFDSQTNVSQSKDSD
VYITDKTVLDMRSMDFKSNSAVAWSNKSDFACANAFNNSIIPEDTFFPSPE
NDGGGCKLE**HHHHHH***

Sequence 4: c259 TCR beta chain (For soluble expression in *E. coli.*).

MGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYS
VGAGITDQGEVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASSYVGNTG
ELFFGEGSRLTVLEDLKNVFPPEVAVFEPSEAEISHTQKATLVCLATGFYP
DHVELSWWVNGKEVHSGVSTDPQPLKEQPALNDSRYCLSSRLRVSATFW
QNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQIVSAEAWGRAD*

Sequence 5: a3a TCR alpha chain (Insert in lentiviral transfer plasmid).

METLLGLLILWLQLQWVSSKQEVTQIPAALSVPEGENLVLNCSFTDSAIYNL
QWFRQDPGKGLTSLLLVRPYQREQTSGRLNASLDKSSGRSTLYIAASQPGD
SATYLCAVRPGGAGSYQLTFGKGTKLSVIPNIQNPDPAVYQLRDSKSSDKS
VCLFTDFDSQTNVSQSKDSDVYITDKTVLDMRSMDFKSNSAVAWSNKSDF
ACANAFNNSIIPEDTFFPSPESSCDVKLVEKSFETDTNLNFQNLSVIGFRILL
LKVAGFNLLMTLRLWSS*

Sequence 6: a3a TCR beta chain (Insert in lentiviral transfer plasmid).

MGSRLLCWVLLCLLGAGPVKAGVTQTPRYLIKTRGQQVTLSCSPISGHRSV
SWYQQTPGQGLQFLFEYFSETQRNKGNFPGRFSGRQFSNSRSEMNVSTLE
LGDSALYLCASSPNMADEQYFGPGTRLTVTEDLKNVFPPEVAVFEPSEAEI
SHTQKATLVCLATGFYPDHVELSWWVNGKEVHSGVSTDPQPLKEQPALN
DSRYCLSSRLRVSATFWQNPRNHFRCQVQFYGLSENDEWTQDRAKPVTQ
IVSAEAWGRADCGFTSESYQQGVLSATILYEILLGKATLYAVLVSALVLMA
MVKRKDSR*

Sequence 7: CD4 (Insert in lentiviral transfer plasmid).

MNRGVPFRHLLLVLQLALLPAATQGKKVVLGKKGDTVELTCTASQKKSIQ
FHWKNSNQIKILGNQGSFLTKGPSKLNDRADSRRSLWDQGNFPLIIKNLKIE
DSDTYICEVEDQKEEVQLLVFGLTANSDTHLLQGQSLTLTLESPPGSSPSVQ
CRSPRGKNIQGGKTLSVSQLELQDSGTWTCTVLQNQKKVEFKIDIVVLAFQ
KASSIVYKKEGEQVEFSFPLAFTVEKLTGSGELWWQAERASSSKSWITFDL
KNKEVSVKRVTQDPKLQMGKKLPLHLTLPQALPQYAGSGNLTLALEAKT
GKLHQEVNLVVMRATQLQKNLTCEVWGPTSPKLMLSLKLENKEAKVSKR
EKAVWVLNPEAGMWQCLLSDSGQVLLESNIKVLPTWSTPVQPMALIVLGG
VAGLLLFIGLGIFFCVRCRHRRRQAERMSQIKRLLSEKKTCQCPHRFQKTC
SPI*

Sequence 8: Nluc luciferase with AcV5 tag shown in bold (Insert in lentiviral transfer plasmid).

MVFTLEDFVGDWRQTAGYNLDQVLEQGGVSSLFQNLGVSVTPIQRIVLSG
ENGLKIDIHVIIPYEGLSGDQMGQIEKIFKVVYPVDDHHFKVILHYGTLVID
GVTPNMIDYFGRPYEGIAVFDGKKITVTGTLWNGNKIIDERLINPDGSLLF
RVTINGVTGWRLCERILA**SWKDASGWS***

Sequence 9: sgRNAs for CD8α knock-out.

Guide 1: ATACTGTTGTGCGCACATCG
Guide 2: GTTAGACGTATCTCGCCGAA
Guide 3: GCTGCTGTCCAACCCGACGT
Guide 4: GAGCAAGGCGGTCACTGGTA

Sequence 10: sgRNAs for CD5 knock-out.

Guide 1: GCAGACTTTTGACGCTTGAC

Guide 2: CCGTTCCAACTCGAAGTGCC

Guide 3: ATCATCTGCTACGGACAACT

Guide 4: AGGTCTACCTCAAGGACGGA

Sequence 11: sgRNAs for CD43 knock-out.

Guide 1: GGCTCGCTAGTAGAGACCAA

Guide 2: GCACCAATGGAAGTCCAAAG

Guide 3: AGGTTGTTGGCTCAGGTAAA

Sequence 12: sgRNAs for CD2 knock-out.

Guide 1: CAAGGCACCCCAGGTTTCCA

Guide 2: CAAAGAGATTACGAATGCCT

Guide 3: CTTGTAGATATCCTGATCAT

Guide 4: GCATCTGAAGACCGATGATC

Sequence 13: sgRNAs for CD11a knock-out.

Guide 1: CTTTGGATACCGCGTCCTGC

Guide 2: CAAGTACTTGGAGGTATAGT

Guide 3: GTAACACAGGCCACTCAGAT

Guide 4: GUAGCUCGAGGCCGGCGCUG

Sequence 14: sgRNAs for CD4 knock-out.

Guide 1: GTCAGCGCGATCATTCAGCT

Guide 2: GAGGTGCAATTGCTAGTGTT

Guide 3: AACTGTAAAGGCGAGTGGGA

Guide 4: CTGTTTTCGCTTCAAGGGCC

6.3 Experimental methods

Lentivirus production

0.8 million HEK 293T cells were seeded in a 6-well plate (Day 1) and incubated overnight. Cells in each well were co-transfected (Day 2) using X-tremeGENE™ HP with 0.8 μg of the appropriate lentiviral transfer plasmid encoding an antigen receptor (1G4 TCR or c259 TCR) and the lentiviral packaging plasmids: pRSV-Rev (0.25 μg), pMDLg/pRRE (0.53 μg), and pVSV-G (0.35 μg). The media was replaced 18 hours following transfection (Day 3). 24 hours after the media exchange, the supernatant from one well was harvested, filtered and used for the transduction of 1 million human T cells (Day 4).

Production of TCR transduced primary human T cells

T cells were isolated from anonymised leukocyte cones (Day 3) purchased from the NHS Blood Donor Centre at the John Radcliffe Hospital (Oxford University Hospitals). RosetteSep™ Human CD8+ Enrichment Cocktail was used for cytotoxic T cells or CD4+ T Cell Enrichment Cocktail for helper T cells. The enrichment cocktail was added to the blood at 150 μL/mL of sample and incubated at RT for 20 minutes. The sample was diluted with an equal volume of PBS and layered on Ficoll® Paque Plus (Cytiva) density gradient medium at a 0.8:1 ratio (Ficoll®:Sample).

The sample was centrifuged at 1200 g for 30 minutes (brake off). Cells at the interface of the Ficoll® media and plasma were collected (Buffy coat) and washed twice (Centrifuged at 500 g for 5 minutes). Cells were resuspended in complete RPMI media supplemented with IL2 (50 U/mL) at a density of 1 million cells per mL. Dynabeads® Human T-Activator CD3/CD28 were added (1 million beads per mL) and cells were incubated overnight.

1 million cells were transduced with the filtered lentiviral supernatant (Day 4). On Day 6 and on Day 8, 1 mL of media was removed and replaced with 1 mL of fresh medium. On Day 9, Dynabeads® were removed using a magnetic stand (6 days following isolation). Cells were resuspended in fresh media every other day at a

density of 1 million per mL and used for co-culture experiments. 17 days following isolation T cells were discarded.

CRISPR/Cas9 knock-out of T cell proteins

Cas9 ribonucleoproteins (RNPs) were prepared by mixing 8.5 μg of TruCut Cas9 protein v2 with 150 pmol of sgRNA mix (Truguide synthetic grna, Thermofisher) and Opti-MEM to a final volume of 5 μL. The RNPs were incubated for 15 minutes at room temperature.

1 million freshly isolated T cells were washed with Opti-MEM (Gibco) and re-suspended at a density of 20 million per mL. The T cells were mixed with the RNPs and transferred into a BTX Cuvette Plus electroporation cuvette (2mm gap). The cells were electroporated using a BTX ECM 830 Square Wave Electroporation System (Harvard Bioscience) at 300 V, 2 ms. Immediately following electroporation, the cells were transferred to complete RPMI media supplemented with IL2 and Dynabeads® Human T-Activator CD3/CD28 (Thermofisher) were added.

Negative selection of T cell knock-out cells

T cells with residual target protein expression were depleted by antibody staining and bead pull-down. T cells were re-suspended in MACS Buffer (PBS, 0.5% BSA, 2 mM EDTA) at a density of 100 million cells per mL. Cells were stained with 5 μL of the corresponding PE-labelled antibody per million cells for 15 minutes at 4°C, washed with MACS and re-suspended at a density of 100 million cells per mL. 1 μL of MojoSort anti-PE nanobeads were added per million cells and incubated on ice for 15 minutes. The cells were washed with MACS and the beads were pulled-down magnetically. The supernatant containing the negatively selected cells was collected.

Cellular co-culture assays

50 000 U87 cells were seeded in 100 μL of DMEM per well in a 96-well Flat-bottom plate and incubated overnight. Alternatively, 100 000 T2 cells were placed in each well of a 96-well Flat Bottom plate. Peptides were diluted in DMEM to the appropriate concentration, added to each well containing cells and incubated for 60 minutes at 37°C, 10% CO2. The media was discarded and 50 000 T cells were added to each well in 200 μL of RPMI media. Cells were incubated for 20 hours at 37°C, 5% CO2. Supernatants were collected for cytotoxicity and ELISA analysis. 25 μL of 100 mM EDTA PBS were added to each well containing the cells and samples were incubated for 5 minutes at 37°C, 5% CO2. Cells were detached by thoroughly pipetting each well and transferred to a 96-well V-bottom plate.

Flow Cytometry

Cells were stained for 20 minutes at 4°C, washed with PBS and analysed using a BD X-20 flow cytometer or Cytoflex LX Flow cytometer. Data was analysed using FlowJo v10 and GraphPad Prism.

Cytotoxicity assay

Target cell lines were engineered to express the Nluc luciferase [278]. A Coelenterazine (CTZ) 2 mM stock solution was prepared in methanol, aliquoted and stored at -80°C. Supernatant from co-culture assays was mixed in a 1:1 ratio with PBS 10 μM CTZ and luminescence was read using a SpectraMax M3 microplate reader.

Cytokine ELISA

Invitrogen Human IFNγ ELISA kits were used following the manufacturer's protocol to quantify levels of cytokine in diluted T cell supernatant. A SpectraMax M3 microplate reader was used to measure absorbance at 450 nm and 570 nm.

Surface Plasmon Resonance

All SPR experiments were carried out in the Dunn School SPR facility following the methods published on [114]. Briefly, c259 TCR/pMHC steady-state binding affinities were measured on a Biacore T200 (GE Healthcare) with a CAP chip using HBS-EP as running buffer. The CAP chip was saturated with streptavidin and biotinylated pMHCs were immobilised to the desired level. A titration of the TCR was flowed through at 37°C. CD58 was immobilised on a reference flow cell at matching levels to those of pMHCs on the remaining flow cells. The signal from the reference flow cell was subtracted (Single referencing) and the average signal from the closest buffer injection was subtracted (Double referencing). Steady-state binding affinity was calculated by fitting the one site-specific binding model (Response = B_{max}[TCR]/(K_D+ [TCR]) on GraphPad Prism to double-referenced equilibrium RU values. The B_{max} was constrained to the inferred B_{max} from the empirical standard curve, relating maximal antibody binding to maximal TCR binding.

Pooled Peptide Libraries

50 000 U87 cells in 100 μL of DMEM were seeded per well in a 96-well Flat-bottom plate and incubated overnight. The pooled peptide library was diluted in DMEM to 100 μM, added to each well containing cells and incubated for 60 minutes at 37°C, 10% CO2. 50 000 T cells were added to each well in 200 μL of RPMI media, without removing the peptide mixture. Cells were incubated for 20 hours at 37°C, 5% CO2. Supernatants were collected for cytotoxicity analysis.

Positional Scanning Peptide Library SPR

To prepare pMHC complexes presenting the local peptide library, a disulfide-stabilized variant of the human MHC-I protein HLA-A*02:01 (DS-A2) was used [334]. The DS-A2 protein was produced as described previously [334]. Briefly, the DS-A2 and 2-microglobulin (2m) subunits were produced in E. coli as inclusion bodies and solubilized in 8 M urea. The protein was then refolded in the presence of GlyLeu, a dipeptide that binds with low affinity to the peptide-binding cleft. The refolded DS-A2–2m complexes were purified by size exclusion chromatography on a Superdex S75 10/300 column (GE Healthcare/Cytiva) in HBS-EP buffer (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20). Local-library peptides were loaded by incubating the DS-A2–2m complex with each peptide for 2 h at room temperature. The pMHC complexes were stored at 4°C until use within 24 h.

Soluble c259 TCR was produced as separate TCRα and TCRβ chains in *E. coli*. Both chains were recovered as inclusion bodies, solubilised in 100 mM Tris-HCl (pH 8.0, 8M urea, 2 mM DTT) and stored in aliquots at -70^{o}C. For refolding, 30 mg of each TCR chain was added to 1 L of refolding buffer (150 mM Tris-HCl (pH 8.0) 3 M urea, 200 mM Arg-HCl, 0.5 mM EDTA, 0.1 mM PMSF) and stirred for 1 h at 4^{o}C. This was followed by dialysis in 10 L 10 mM Tris-HCl (pH 8.5) buffer for 3 days in total, with the dialysis buffer changed after 1 day. The refolded c259 TCR was purified using anion exchange chromatography (HiTrap Q HP; Cytiva), followed by size exclusion chromatography (Superdex 200 Increase; Cytiva) in HBS-EP Buffer. Purified c259 TCR was used within 48 h.

High-throughput affinity measurements of c259 TCR binding to MHC loaded with the peptide library were performed using LSA or LSA^{XT} (Carterra). Each pMHC was immobilised via biotin-streptavidin binding on a different spot of the SAHC30M biosensor (Carterra) for 20 min, resulting in immobilisation levels between 200 and 900 RUs. Measurements were performed in HBS-EP Buffer at 37 °C. A 2-fold dilution series of c259 TCR was prepared in HBS-EP buffer, with the highest

concentration between 100 - 130 μM. Starting with the highest dilution, increasing concentrations of c259 were injected over the chip for 5 min, followed by 5 -10 min of dissociation, without regeneration. Afterwards, a β2m specific antibody (clone B2M-01 (Thermo Fisher Scientific) or BBM.1 (Absolute Antibody)) was injected for 10 min. The resulting data was analysed using Kinetics Software (Carterra). Any spikes were removed from the data, before referencing against empty control spots or spots immobilised with CD86 at matching immobilisation levels. The final in a series 6 buffer injection before TCR injection was subtracted from the data for double referencing. Subsequently, the steady state binding RU was calculated by taking the average RU from over 20 seconds. Steady-state analysis was performed to obtain the $K_D$values. First, steady-state data was fitted with a one site-specific binding model (Response = B_{max}[TCR]/(K_D+ [TCR]), with K_D and B_{max} unconstrained. We then constructed an empirical standard curve using high affinity pMHCs (K_D< 20μM) to relate maximal anti-β2m binding to TCR B_{max}. Next, steady state data for all pMHCs were fitted with a one site-specific binding model with B_{max}constrained to the B_{max}inferred from the empirical standard curve. We excluded K_D values for peptides, where we observed little or no anti-β2m binding responses, indicating that the pMHC complex was unstable and lost the peptide over time (indicated as N/A in Table 4.1). We further excluded K_D values for pMHC that produced a TCR binding response of less than 5 RU (indicated as non-binders (NB) in Table 4.1).

Data analysis

EC_{50} is calculated as the concentration of antigen required to elicit 50% of the maximum response determined for each condition individually whereas P_{15} is calculated as the concentration of antigen required to elicit 15% of the maximum activation.

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