Mapping subunit organisation within the T-Cell Receptor-CD3 complex

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Abstract

In the mammalian immune system, T cells play important roles in mounting an effective and sustained immune response for protection against infection. One of the major molecules that contributes to T cell development and function is the cell surface receptor, T cell receptor (TCR). TCR recognises an antigenic peptide fragment presented by a major histocompatibility (MHC) molecule on antigen-presenting cells and subsequently initiates intracellular signalling. The T cell receptor complex is composed of ligand-sensing subunits (TCRα and TCRβ chains) and signal-transducing subunits (CD3δ, CD3γ, CD3ε and ζ chains). To transmit the extracellular ligand binding to cell interior, TCR chains communicate with the signal-transducing subunits of the receptor complex through unknown mechanisms. How subunits of the TCR-CD3 complex (hereafter referred to as the TCR complex) communicate with each other is unclear largely due to an absence of information regarding the molecular architecture of the intact TCR complex. In this thesis, I aimed to map subunit organisation within the TCR complex based on their transmembrane (TM) domain arrangement. Results presented in this thesis highlight the successful use of a cysteine crosslink screening approach to obtain TM-TM relationships within the TCR complex. I incorporated the crosslinking data along with biophysical, computational and functional methods to interrogate the architecture of the TCR complex and possible features of the TCR TM domains in mediating TCR triggering events. In chapter 3, I demonstrated that the TCRα and TCRβ subunits are closely associated in the membrane using cysteine crosslinking data and MD simulation-generated TM structures of the TCRαβ heterodimer. I described a novel polar network within the TCRαβ TM interface and demonstrated by mutagenesis that association of the TCRαβ TM domains through this polar network is crucial to maintain native TCR complex assembly and surface expression. In chapter 4, I have extended the cysteine crosslinking approach to interrogate TM-TM relationships between (1) TCRβ and CD3γ subunits and (2) TCRα and ζ subunits. Taking these data together, I proposed a model of TCR-CD3 complex architecture. Elucidating the spatial arrangement of TCR-CD3 complex subunits has provided an instructive platform to understand how these components may cooperate to transmit the crucial signals that activate T cells.
Declaration

This is to certify that:

i. This thesis comprises only my original work towards the PhD except where indicated in the Preface,

ii. Due acknowledgment has been made in the text to all other material used, and

iii. This thesis is fewer that 100,000 words in length, exclusive of tables, figures and bibliography

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Logesvaran Krshnan
Preface

Pursuant to the regulations of The University of Melbourne, the author’s contribution to each chapter was as follows;

Chapter 3 85%

Prof. Wonpil Im and Dr. Soohyung Park performed the MD simulation. Dr. Matthew Call performed the evolutionary conservation analysis. The ER microsome purification was performed along with Drs. Matthew Call and Melissa Call. General experimental design was done together with Drs. Matthew Call and Melissa Call.

Chapter 4 90%

General experimental design was done together with Drs. Matthew Call and Melissa Call.

Thus, the author’s overall contribution to the work presented in this thesis was 87.5%.
Publications

Parts of this thesis has been published previously in the form of the scientific journal article listed below:


Other publications arising from my PhD training:

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Thank you everyone.
## Abbreviations

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<thead>
<tr>
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<td>α</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<td>Ca</td>
<td>Calcium</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium Chloride</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
</tr>
<tr>
<td>CD3δ</td>
<td>Cluster of Differentiation three delta</td>
</tr>
<tr>
<td>CD3γ</td>
<td>Cluster of Differentiation three gamma</td>
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<td>CD3ε</td>
<td>Cluster of Differentiation three epsilon</td>
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<td>CuSO₄</td>
<td>Copper (II) Sulphate</td>
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<td>CuPhe</td>
<td>Copper (II) phenanthroline</td>
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<td>Connecting Peptide</td>
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<td>CPTM</td>
<td>Connecting Peptide-Transmembrane</td>
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<td>Co-IP</td>
<td>co-Immunoprecipitation</td>
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<td>CnBr</td>
<td>Cyanogen Bromide</td>
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<td>Cytomegalovirus</td>
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<td>Da</td>
<td>Dalton</td>
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<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
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<td>FACS</td>
<td>Fluorescent assisted Cell Sorting</td>
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<td>Foetal Calf Serum</td>
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<td>FRET</td>
<td>Forster Resonance Energy Transfer</td>
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<tr>
<td>GSSG</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>gram</td>
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<tr>
<td>g</td>
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<td>Isopropyl β-D-1-thiogalactopyranoside</td>
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<td>Internal Ribosomal Entry Site</td>
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<td>JM</td>
<td>Juxtamembrane</td>
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<tr>
<td>KCM</td>
<td>Potassium, Calcium, Magnesium</td>
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<td>kilo Dalton</td>
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<td>LMPG</td>
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<td>LDS</td>
<td>Lithium dodecyl sulphate</td>
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<td>L</td>
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<td>min</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MALDI-TOF</td>
<td>Matrix assisted laser desorption ionisation-time of flight</td>
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<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>MHz</td>
<td>Mega Hertz</td>
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<tr>
<td>ug</td>
<td>microgram</td>
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<tr>
<td>MD</td>
<td>Molecular Dynamics</td>
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<tr>
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<td>Definition</td>
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<tr>
<td>MW</td>
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<td>MSCV</td>
<td>Murine Stem Cell Virus</td>
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<td>nm</td>
<td>nanometer</td>
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<td>nanomolar</td>
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<td>NAMD</td>
<td>Nanoscale Molecular Dynamics</td>
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<td>ns</td>
<td>nanosecond</td>
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<td>NFkB</td>
<td>Nuclear Factor kappa B transcription factor</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<tr>
<td>PPI</td>
<td>Protein-Protein Interaction</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
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<td>PC</td>
<td>Protein C</td>
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<tr>
<td>pH</td>
<td>potential of Hydrogen</td>
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<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
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<tr>
<td>REMD</td>
<td>Replica Exchange Molecular Dynamics</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SBP</td>
<td>Streptavidin Binding Protein</td>
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<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>TMD</td>
<td>Transmembrane domain</td>
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<td>TBS</td>
<td>Tris-buffered Saline</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
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<td>TAE</td>
<td>Tris-acetate-EDTA</td>
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<td>TCRα</td>
<td>T cell receptor alpha</td>
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<tr>
<td>TCRβ</td>
<td>T cell receptor beta</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
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<tr>
<td>TFE</td>
<td>Trifluoroethylene acid</td>
</tr>
<tr>
<td>V</td>
<td>Voltage</td>
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Zeta
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Chapter 1: Introduction and literature review

1.0 Overview

The T cell receptor (TCR) is an octameric membrane protein complex expressed on the surface of a T cell and endows each T cell with its specificity for ligand. Upon recognising ligand, the TCR complex is “triggered” causing phosphorylation of intracellular domains of the receptor. While important to all T cell functions, the mechanism by which the TCR complex communicates the ligand binding to the intracellular domains is poorly understood. One of the key pieces of information that is missing from our model of TCR triggering, which would greatly aid us in understanding how the TCR works, is the architecture of the intact TCR complex in the membrane. In this introductory chapter, I will outline the important roles the TCR plays during T cell development, differentiation and T-cell mediated immunity. I then describe the components of the TCR complex and the biogenesis of the receptor complex in the endoplasmic reticulum. Finally, I describe proposed mechanisms of TCR triggering and briefly highlight the need for understanding the architecture of the TCR complex to scrutinise these models.

1.1 Adaptive immunity and αβ T cells

Adaptive immunity is characterised by the ability of immune cells to mount pathogen-specific immune responses and retain memory against the pathogens to provide long-term protection (Ahmed and Gray, 1996). The T lymphocyte (T cell) is central to this process and can be distinguished from other immune cells by the presence of an antigen receptor, the TCR, on their cell surface. T cells are divided into two major classes: the alpha-beta (αβ) and gamma-delta (γδ) T cells (Davis and Bjorkman, 1988). While γδ T cells have prominent roles in shaping our immune system (Vantourout and Hayday, 2013), this thesis will primarily focus on αβ T cells that constitute the majority of T cells in our body. Circulating αβ T cells can be further categorised into CD4 and CD8 co-receptor-expressing T cells (CD4+ and CD8+ T cells, respectively) (Cantor and Boyse, 1975, Shiku et al., 1975, Kisielow et al., 1975, Germain, 2002, Masopust et al., 2007). All αβ T cells express TCR alpha (TCRα) and TCR beta (TCRβ) chains as a
covalently linked heterodimer (TCRαβ) on the cell surface and this part of the receptor recognises processed self and foreign (antigenic) peptides presented by major histocompatibility complex (pMHC) molecules (Marrack and Kappler, 1986). The TCRαβ heterodimer is non-covalently associated with the CD3δε, CD3γε, and ζζ dimeric modules, which propagate signals through the membrane. Within the cytoplasmic domains of these modules, there are multiple repeats of a signalling motif termed the immunoreceptor tyrosine-based activation motif (ITAM) that are phosphorylated upon ligand binding by the TCRαβ heterodimer (Reth, 1989). Phosphorylation of multiple ITAMs initiates intracellular signalling that results in T cell activation (see details in section 1.4). Signalling across the membrane through the TCR plays crucial roles throughout the developmental stages of T cells in the thymus and for the T cells to perform their functions in the periphery.

1.1.1 T cell development in the thymus

The role of the thymus is to produce CD4+ and CD8+ T cells that are poised to recognise changes in MHC:peptide ligands that indicate homeostasis has been disrupted. Both CD4+ and CD8+ αβ T cells originate from multi-potent early thymic progenitor (ETP) cells that migrate into the thymus from bone marrow (Yang et al., 2010). The ETP cells do not yet express CD4 or CD8 co-receptors and are hence called double negative (DN) cells. Thymocytes undergo a series of developmental stages, from DN stage 1 (DN1) to DN4, before becoming double positive cells (DP; expressing both CD4 and CD8 co-receptors). These cells then further differentiate to only express either CD4 or CD8, termed single positive (SP) prior to exiting the thymus into the periphery.

Survival and initial differentiation of the ETPs to DN2 relies on interleukin-7 (IL-7) cytokine signalling (Murray et al., 1989, Peschon et al., 1994, Crompton et al., 1998) and Notch signalling (Radtke et al., 1999) (Fig 1.1). The fate of these thymocytes becomes more restricted towards the αβ lineage as they acquire the ability to express TCR chains from DN3 stage onwards (Fig 1.1). Genes that encode the TCRα (TCRA) and TCRβ (TCRB) proteins are organised as multiple individual gene segments in the thymocytes genome that are re-arranged (spliced and ligated) to generate a mature TCR
coding sequence (Chien et al., 1984). This process is orchestrated by recombination-activating gene (RAG) enzymes (Schatz and Baltimore, 1988, Schatz et al., 1989) and facilitates the generation of a diverse repertoire of TCR proteins on the surface of the developing thymocytes.

**Figure 1.1: Signalling through the TCR or pre-TCR is important for T cell development and function.** Schematic of T cell development in the thymus and function in the periphery highlighting the importance of signalling through the TCR complex. ETP; Early thymic progenitor, DN; Double Negative 1-4 cells (CD4⁻ and CD8⁻), DP cells (CD4⁺ and CD8⁺) and SP cells (CD4⁺ or CD8⁺).

During αβ lineage specification, the TCRβ gene segments are the first to be rearranged at the DN3 stage of differentiation (Fig 1.1) (Mallick et al., 1993). The newly generated TCRβ chains are paired with a surrogate α-chain (pre-Tα) and signalling subunits to form the pre-TCR complex that will be displayed on the cell surface (Groettrup et al., 1993, Saint-Ruf et al., 1994). Signalling through the pre-TCR complex, accompanied by Notch signalling, ensures that further TCRB gene rearrangement is halted (Hoffman et al., 1996), and promotes survival and proliferation.
of the thymocytes and differentiation into DN4 cells (Ciofani et al., 2004) (Fig 1.1). Importantly, the inability to initiate pre-TCR signalling indicates a failure in productive TCRβ chains rearrangement and prevents further progress along the αβ lineage differentiation pathway (Haks et al., 1998, Clements et al., 1998, Pivniouk et al., 1998). This process is termed the β-selection checkpoint. Successful pre-TCR signalling induces TCRA gene rearrangement and the expression of both CD4 and CD8 co-receptors, allowing cells to proceed to the double positive (DP) stage (Fig 1.1) (Ellmeier et al., 1999).

To ensure that TCRA has been correctly rearranged and that the resulting TCR is able to respond to MHC:peptide, the new TCRαβ combinations at the cell surface of DP thymocytes are tested for their ability to respond to self-peptide displayed on MHC molecules expressed on thymic epithelial cells (TECs). TECs are unique in that they are able to express a catalogue of self-antigens from essentially any tissue in our body, a feature enabled by the exclusive expression of transcription regulators Aire (Anderson et al., 2002) and Fezf2 (Takaba et al., 2015) in these cells. The interaction between TCR-expressing thymocytes and the TECs is critical to educate the thymocytes as to what is self and what is foreign (Klein et al., 2014), and while weak TCR signalling is permissive to further differentiation, a strong response to tissue antigens is not (Sebzda et al., 1994).

Hyperactivity to self MHC:peptide in the thymus will generally destine the thymocytes to death, a process termed “negative selection” (Surh and Sprent, 1994). The negative selection checkpoint is in place to prevent development of T cells that would potentially mount harmful immune responses towards self-tissues in the periphery (Teshima et al., 2003). Thymocytes that receive a strong TCR signal can also differentiate to become T-regulatory (Treg) cells, invariant Natural Killer T (iNKT) cells and intraepithelial lymphocytes (IELs) (Hogquist and Jameson, 2014). In contrast, weak signalling through the TCR supports “positive selection”, whereby thymocytes undergo CD4 or CD8 lineage specification to become single positive (SP; expressing either CD4 or CD8 co-receptors) prior to exiting the thymus (Singer and Bosselut, 2004). During this process, thymocytes that productively engage and signal upon recognising MHC class II develop into CD4+ T cells, whereas thymocytes that
productively engage and signal upon recognising MHC class I develop into CD8+ T cells (Teh et al., 1988, Hogquist et al., 1994).

1.1.2 Roles for TCR in T cell survival and function in the periphery

At homeostasis, peripheral naïve T cell populations balance death, proliferation, and survival signals to maintain their populations. Key survival signals are received through the TCR and IL-7 receptor (Goldrath and Bevan, 1999, Seddon and Zamoyska, 2003, Mackall et al., 2011). The importance of TCR engagement by MHC in maintaining the peripheral T cell populations has been demonstrated by studying survival of the adoptively transferred naïve transgenic T cells into mice that do not have the appropriate MHC recognised by the transgenic TCR, or are totally deficient in MHC (Kirberg et al., 1997, Tanchot et al., 1997, Goldrath and Bevan, 1999). In these studies, transferred transgenic naïve T cell populations diminished over time, suggesting that the unavailability of TCR-peptide-MHC interactions caused these T cells to die. Inducible deletion of the TCR gene has also demonstrated a similar loss of peripheral T cells (Polic et al., 2001).

Signalling through the TCR has also been suggested to shape naïve T cell populations with heightened responsiveness to foreign antigens. As they persist in the circulation, increased reactivity of the naïve T cells to self-peptide-MHC molecules has been correlated to increased basal phosphorylation state of the signal-transducing subunits of the TCR complex (CD3s) and the intracellular TCR signalling pathway (Persaud et al., 2014, Mandl et al., 2013). This phenomenon has been interpreted as increased preparedness of these naïve T cell populations to become activated (Hogquist and Jameson, 2014).

In the situation where an immune response must be mounted, such as during infection, antigen-specific T cell populations expand to cope with the magnitude of the insult. The critical signal that mediates T cell activation, expansion and effector function is the signalling through the TCR complex, accompanied by costimulation (Horgan et al., 1990, Chen and Flies, 2013) and cytokine signalling, in particular IL-2 signalling (Morgan et al., 1976, Watson et al., 1979, Boyman and Sprent, 2012). The
activated CD4+ and CD8+ T cells undergo transcriptional reprogramming and acquire characteristic effector functions to eliminate the threats. CD4+ T cells generally provide “help” to other immune cells such as the B-lymphocytes, by producing cell-bound or soluble factors such as cytokines (Mosmann et al., 1986, Zhu and Paul, 2008). CD8+ T cells are the direct killers of infected cells, by physically engaging with targets and releasing cell death-inducing factors such as perforin and granzymes into the infected cells (Jenne and Tschopp, 1989, Tschopp and Nabholz, 1990). After immune clearance, populations of the antigen-experienced T cells contract by programmed cell death, leaving a small number of these cells, some of which differentiate into memory T cells. Survival of the memory T cells depends on IL-7 and IL-15 cytokine signalling but has been demonstrated to be MHC-independent, precluding the need for continuous basal signalling through the TCR (Swain et al., 1999, Murali-Krishna et al., 1999, Tanchot et al., 1997, Surh et al., 2006, Leignadier et al., 2008).

In summary, signalling through the TCR complex governs almost all aspects of T cell biology. However, the precise mechanism by which the TCR complex mediates signal transduction across lipid bilayer is unclear. In the rest of this chapter, I will introduce the components of the TCR complex, describe how they assemble, and discuss what is known about how the TCR complex functions.
1.2 Components of the T cell receptor-CD3 complex

The TCR-CD3 complex is composed of six type 1 single-transmembrane (TM) proteins: TCRα, TCRβ, CD3δ, CD3γ, CD3ε, and ζ subunits (Fig 1.2). The TCRα and TCRβ subunits form a disulphide-linked TCRαβ heterodimer and non-covalently associate with the CD3δε, CD3γε, and ζζ dimeric signalling modules (Alarcon et al., 1988). The TCRαβ heterodimer lacks any intracellular signalling domains and thus relies on the CD3δε, CD3γε, and ζζ modules to engage the intracellular signalling apparatus.

Figure 1.2: Composition of the αβ TCR complex. (A) The TCR complex is composed of a ligand-sensing TCRαβ heterodimer (cyan and orange) and non-covalently associated CD3δε, CD3γε and ζζ signal-transducing dimers (CD3δ in turquoise, CD3γε in medium blue, CD3ε in magenta and ζζ in dark blue). The association of these three dimeric signalling modules with the TCR is dependent on specific basic residues (blue circles) on the TCR TM domains, acidic residues (red circles) and hydroxyl-bearing serine/threonine residues (yellow circles) on the signal-transducing TM domains. Arrows indicate the intramembrane polar network that governs the assembly of each subunit into the TCR complex. The disk represents the lipid bilayer. The signalling motifs in the cytoplasmic tails of the signal-transducing subunits, termed intracellular immuno-receptor tyrosine-based motif (ITAM), are depicted in red. The structures of EC domains of each dimer are depicted as ribbon diagrams of their respective atomic structure. Ribbon diagrams were prepared using crystal structures of a human αβ TCR (PDB ID 1QSF), and the CD3 crystal structures 1XJW (human CD3δε) and 1SY6 (human CD3γε) and NMR structure 2HAC (human ζζ).
1.2.1 TCRαβ heterodimer

The extracellular domains of the TCRαβ heterodimer form the ligand-binding module of the TCR complex. Each TCR chain is made up of two extracellular domains with immunoglobin-like (Ig) folds; the variable domains (Vα and Vβ), which are responsible for binding MHC:peptide, and the constant (Ca and Cβ) domains. These extracellular domains mediate dimerisation of TCRα and TCRβ chains and are covalently stabilised by a single inter-chain disulphide bond through a pair of cysteine residues at the start of the connecting-peptide (CP) sequences. The molecular basis of ligand binding by the TCRαβ was revealed through the x-ray crystal structures of this domain with peptide:MHC (Garboczi et al., 1996, Garcia et al., 1996) (Fig 1.3). The Ig fold of the extracellular domains allows antibody-like recognition of foreign antigens by the TCR chains through the Vα and Vβ domains. The Ca and Cβ Ig-like domains of the TCRαβ heterodimer are connected to the membrane-anchoring TM domains via CP sequences with unknown structure. CP sequences of the TCRα and TCRβ chains are intriguingly longer in length (22 amino acids in TCRα and 16 amino acid in TCRβ) than CP sequences of the signal-transducing subunits. The TCR TM domains are each approximately 20-23 amino acids in length and are thought to adopt a helical structure. A striking feature of the TCRα and TCRβ TM domains is the presence of basic residues that critically govern association with the CD3δε, CD3γε, and ζζ dimeric modules (Fig 1.4) (Alcover et al., 1990, Blumberg et al., 1990).
Figure 1.3: Interaction between a TCRαβ heterodimer and a viral peptide displayed on a MHC Class I molecule. Shown above is the ribbon representation of the x-ray crystal structure of the extracellular domains of a human TCRαβ heterodimer (A6 TCR) in complex with a HLA-A2 presenting a viral peptide (TAX peptide) (PDB ID code: 1QSF) (Ding et al., 1999).
Figure 1.4: Sequence of the juxtamembrane region of the TCR subunits. The hypothetical TM (underlined) and N- and C-terminal flanking sequences of all subunits in the TCR. The TM residues that are important for TCR-CD3 complex assembly are shown in blue (basic), red (acidic) and green (polar).

1.2.2 CD3ε, CD3δ and CD3γ subunits

The CD3ε, CD3δ and CD3γ chains share similar domain architectures. They are each composed of a single extracellular domain with an Ig fold, a short CP, a TM domain and a long cytoplasmic tail. CD3ε assembles into heterodimers with the CD3δ and CD3γ subunits (CD3δε and CD3γε, respectively). Atomic-resolution structures of extracellular domains of the CD3δε and CD3γε dimers have been individually determined (Sun et al., 2001, Arnett et al., 2004, Kjer-Nielsen et al., 2004, Sun et al., 2004) (Fig 1.5). The structures revealed a side-by-side arrangement of the two Ig domains in each CD3 dimer, and dimerisation is mediated primarily by extensive interactions between the last β-strands in each CD3 molecule (Fig 1.5). These extracellular domains are connected to the membrane-anchoring TM domains via connecting peptide (CP) sequences. The CP regions of the CD3 subunits are significantly shorter than of the TCR subunits and contain a highly conserved membrane proximal cysteine motif (RxCxxCxE; x is any amino acid) that is thought to form an intramolecular disulphide bond and is required for proper assembly and cell surface expression of the TCR complex (Xu et al., 2006, Brazin et al., 2014).
Complementing the basic residues in the TCR chains, all CD3 subunits contain conserved acidic residues in their TM domains, either an aspartic acid residue (in CD3δ and CD3ε) or glutamic acid residue (in CD3γ) (Fig 1.4). Additionally, each CD3 chain also contain another polar residue, spaced three (serine in CD3γ) or four (threonine residues in CD3δ and CD3ε) C-terminal to the acidic residues (Fig 1.4). These polar residues are critical in governing association of the CD3 subunits with the TCRα and TCRβ chains (discussed further in section 1.3) (Call et al., 2002, Call et al., 2010).

The cytoplasmic domain of the CD3 subunits are each approximately 40-50 amino acids long and contain the ITAM signalling motif (Reth, 1989). The ITAM motif is defined by the consensus amino acid sequence; Yxx(L/I)x(6-8)Yxx(L/I) (x is any amino acid), and there is one ITAM in each CD3 subunit. The two tyrosines within the ITAM are phosphorylated by the intracellular kinase Lck after ligand engagement by the TCRαβ heterodimer (see section 1.4) (Straus and Weiss, 1992). The cytoplasmic tail of the CD3ε subunit (but not the CD3δ and CD3γ subunit) contains a stretch of basic residues, adjacent to the ITAM sequence, which has been proposed to bind acidic lipid head groups of the plasma membrane inner leaflet (Xu et al., 2008). Forster resonance energy transfer (FRET) experiments in cells support this model and the solution NMR structure of the ITAM-containing CD3ε cytoplasmic peptide demonstrated that the tyrosine residues of the ITAM are inserted into the lipid bilayer, sequestering them from the kinase required for phosphorylation (Xu et al., 2008, Deford-Watts et al., 2009).
Figure 1.5: Atomic structures of the extracellular domains of the individual CD3δε and CD3γε dimers. Top: The ribbon representation of the x-ray crystal structure of the extracellular domains of the human CD3δε heterodimer (PDB ID code: 1XIW). Bottom: The solution NMR structure of the extracellular domains of the human CD3γε heterodimer (PDB ID code: 1JBJ). These structures highlighting (1) the side-by-side conformation of each CD3 subunits within the dimer and (2) the rigid interface between the two CD3 subunits within the dimers that is contributed by extensive inter-molecular interactions (contacting residues shown in stick representation).
1.2.3 ζ subunit

The ζ subunit is unique from the rest of the subunits in that it lacks a folded extracellular domain and instead has only a 9-amino-acid long stalk sequence. The ζ subunit exists as a disulphide-linked homodimer and associates with the TCRαβ through its TM domain. The structure of the ζζ TM dimer has been determined by solution NMR spectroscopy (Call et al., 2006) and remains the only atomic-scale structure of any TCR TM domain available to date (Fig 1.6). There are three important features of the ζζ TM domains that govern its homodimerisation and assembly into the TCR: (1) Two inter-helical hydrogen bonds contributed by reciprocal tyrosine-threonine pairs near the C-terminal end of the TM domains, (2) an inter-helical disulphide crosslink near the N-terminal end that stabilises the ζζ homodimer, and (3) a pair of aspartic acid residues near the N-terminal end that forms an important assembly contact point with the TCRαβ TM domains (discussed further in section 1.3) (Call et al., 2002).

The cytoplasmic tail of the ζ subunit is significantly longer than of the CD3 subunits (approximately 110 amino acid long) and contains three ITAM repeats that are phosphorylated by intracellular kinase Lck (Reth, 1989, Straus and Weiss, 1992). Like the CD3ε cytoplasmic tail, the ζ cytoplasmic tail contains a stretch of basic residues that facilitate binding of the cytoplasmic tails to the inner leaflet of the plasma membrane (Aivazian and Stern, 2000, DeFord-Watts et al., 2011, Zhang et al., 2011).
Figure 1.6: Solution NMR spectroscopy TM structure of the ζζ homodimer. Shown is the ribbon representation of the solution NMR structure of the TM domain of the human ζζ homodimer (PDB ID code: 2HAC). Dimerisation of the ζ chain is facilitated by residues highlighted in stick representation and the aspartic acid pair mediates assembly into the TCR complex.
1.3 T cell receptor-CD3 assembly in the Endoplasmic Reticulum

The subunits of the TCR complex are assembled in the endoplasmic reticulum (ER) membrane (Alarcon et al., 1988, Koning et al., 1988) through a process that requires non-covalent associations among the individual subunit TM domains (Manolios et al., 1990). It was noticed that the TCR subunits contain potentially charged residues in their TM regions, and the presence of such strongly polar amino acid residues was thought to be unusual for membrane-embedded segments of single-pass membrane proteins as they would be destabilising in the hydrophobic interior of the membrane (Clevers et al., 1988). Mutagenesis studies in transfected cell lines demonstrated that elimination of these polar residues caused defects in TCR complex assembly (Alcover et al., 1990, Blumberg et al., 1990, Rutledge et al., 1992). Subsequent studies showed that exposure of the TM polar amino acids in unassembled subunits led to ER retention and ultimately destined these for degradation (Bonifacino et al., 1989, Bonifacino et al., 1990, Bonifacino and Lippincott-Schwartz, 1991, Yang et al., 1998). Together these studies have established a framework knowledge that the TM polar residues govern both TCR complex assembly in the ER membrane and elimination of subunits that fail to assemble.

However, how these polar residues mediate proper organisation of each subunit within the receptor complex was not clear from earlier cellular studies. It was proposed that the TM polar residues facilitate formation of pairwise interactions between TCR and CD3 chains through salt bridges (Manolios et al., 1991, Cosson et al., 1991). However, it was noticed that such pairwise interactions would result in charge imbalance within the system, with three basic residues within the TCRαβ heterodimer compared to six acidic residues within the signal-transducing subunits. This led to a proposal that there are two copies of the TCRαβ heterodimer in each TCR-CD3 complex (Exley et al., 1995, Fernandez-Miguel et al., 1999). However, this proposal was not supported as co-immunoprecipitation from T cells from a transgenic mouse that was engineered to express two different TCRαβ heterodimers at the cell surface, could not be achieved (Punt et al., 1994). Furthermore, a study that utilised an in vitro translation assay to assemble and isolate radiolabelled intact TCR complexes, enabling precise quantitation of the stoichiometric relationship between each subunit, also failed...
to support the hypothesis that the TCR contained two TCRαβ heterodimers (Call et al., 2004).

A separate study using the same in vitro assembly system demonstrated that the TM lysine residue of the TCRα subunit requires both TM acidic residues within the CD3δ (aspartic acid) and CD3ε (aspartic acid) subunits to assemble the TCRα-CD3δε intermediate in the ER membrane (Call et al., 2002). Similarly, this observation was also true for the formation of the TCRβ-CD3γε intermediate, in which the lysine residues in the TCRβ subunit requires both acidic residues within the CD3γ (glutamic acid) and CD3ε (aspartic acid) subunits. ζζ also required both aspartic acid residues, the arginine residue in the TCRα TM domain, as well as the formation of hexameric CD3δε-TCRαβ-CD3γε intermediate to successfully assemble with the TCR complex. Thus, instead of pairwise interactions among basic and acidic TM residues, a trimeric arrangement of helices, where one basic residue interacts with two acidic residues was found to be central to the higher-order assembly of the TCR complex (Call et al., 2002, Engelman, 2003) (Fig 1.7).
Figure 1.7: Assembly of the full TCR complex occurs via three major assembly steps. The TM domains of the TCRα chain contain a lysine residue that requires both aspartic acids of the CD3δ and CD3ε chains for the formation of the first intermediate of the TCR complex. The TM domains of the TCRβ chain contain a lysine residue that requires both aspartic and glutamic acids of the CD3γ and CD3ε chains, respectively for the formation of the second intermediate of the TCR complex. Prior assembly of the TCRβ chain to the TCRα-CD3δε intermediate 1 may be required for the joining of the intermediate 2. The final subunit to join the TCR is the ζζ homodimer via the TCRα arginine residue and a pair of aspartic acids on the ζζ homodimer (step 3).
1.4 TCR triggering, intracellular TCR signalling and T cell activation

Despite the importance of TCR signalling in the development of T-cell mediated immunity, our understanding of the precise mechanism of TCR “triggering” is incomplete. TCR “triggering” is defined by the earliest event of molecular interaction between the TCRαβ heterodimer and peptide:MHC molecules that elicits immediate consequences within the cytoplasmic domains of the CD3δε, CD3γε and ζζ dimers, and can be measured by phosphorylation of the ITAMs. Downstream of ITAM phosphorylation, intracellular signalling events are initiated that lead to signal amplification for sustained signalling through the TCR complex and subsequent T cell activation. Due to limitations in experimental tools, dissecting TCR “triggering” from later TCR signal amplification mechanisms has been challenging. This has led to proposal of several TCR “triggering” mechanisms. Before reviewing the main models of TCR triggering, I will introduce the intracellular signalling events downstream of TCR triggering that lead to signal amplification and T cell activation (Fig 1.8).

The Src-family kinase Lck is the key intracellular kinase that phosphorylates tyrosine residues within the ITAMs, which bridge TCR triggering to the intracellular signalling cascades (Straus and Weiss, 1992). Lck is directly targeted to the inner leaflet of the plasma membrane through myristoylation and palmitoylation modifications (Marchildon et al., 1984, Paige et al., 1993, Shenoy-Scaria et al., 1993) and can also associate with the intracellular regions of the CD4 and CD8 co-receptors (Veillette et al., 1988, Rudd et al., 1988). Phosphorylated ITAMs serve as docking sites for the intracellular kinase ζ subunit-associated protein of 70kDa (ZAP70). Phosphorylation of the ζ-bound ZAP70 molecules by Lck in turn promotes ZAP70 activation (Iwashima et al., 1994) and mediates further recruitment and phosphorylation of the scaffolding/adapter proteins, Linker for Activation of T cells (LAT) (Finco et al., 1998, Zhang et al., 1998) and SH2 domain containing Leukocyte Protein of 76kDa (SLP76) (Jackman et al., 1995, Bubeck Wardenburg et al., 1996). Together, the LAT-SLP76 complex serves as a docking site for multiple intracellular TCR-induced effector proteins (Pivniouk and Geha, 2000, Yablonski and Weiss, 2001). One of the critical proteins that is recruited to the LAT-SLP76 complex is the phospholipase C gamma (PLCγ) (Sieh et al., 1994, Yablonski et al., 2001). Activated PLCγ mediates hydrolysis
of phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate the second messengers, diacylglycerol (DAG) and inositol triphosphate (IP₃) (Berridge, 1993, Berridge, 2009, Park et al., 1991, Secrist et al., 1991). DAG activates protein kinase C theta (PKCθ) and the mitogen activated protein kinase (MAPK) pathways, which lead to nuclear translocation of the transcription factor nuclear factor kappa B (NFκB) (Lenardo et al., 1988, Kang et al., 1992) and activator protein 1 (AP-1). IP₃ causes calcium mobilisation from the lumen of the ER compartment to the cytoplasm, which subsequently activates the transcription factor nuclear factor of activated T cell (NFAT) (Shaw et al., 1988, Macian, 2005). Together, these transcription factors induce gene expression that leads to proliferation and differentiation of the T cells, especially the IL2 gene for IL-2 cytokine production (Jain et al., 1995).

Very few agonist peptide:MHC ligands are required to initiate TCR signalling, but sustained TCR signalling is required for T cell activation (Huppa et al., 2003). Recently, Katz et. al., reported a mechanism by which triggered TCRs (i.e. phosphorylated) serve as sites for repeated recruitment and activation of intracellular kinase ZAP70 molecules, thus amplifying antigenic TCR signalling (Katz et al., 2016). Initial TCR signalling facilitates the organisation of a specialised signalling structure that is formed at the contact sites between the T cell and antigen-presenting cell (APC), termed the immunological synapse, which is defined by a special segregation pattern of receptor, adhesion and signalling molecules (Monks et al., 1998, Grakoui et al., 1999). Temporal regulation of formation and cessation of the immunological synapse are critical to fine-tune the duration and quality of T cell activation (Jenkins et al., 2015, Yachi et al., 2006).
Figure 1.8: Intracellular TCR signalling. (A) A schematic of intracellular signalling triggered after TCR binding to the peptide-MHC molecules. 1) Intracellular kinase ZAP70 binds to the ITAMs that have been phosphorylated by Lck. ZAP70 is also phosphorylated by Lck. 2) Activated ZAP70 recruits LAT and other signalling adaptor proteins forming a large signalosome near the plasma membrane that initiates multiple signalling pathways. 3) Activated PLCγ hydrolyses PIP2 into second messengers DAG and IP3. 4) IP3 causes IP3-sensitive Ca²⁺ channels in the ER membrane to release Ca²⁺ into the cytosol. 5) DAG activates Ras-MAPK and PKC0 pathways and Ca²⁺ acts upon calcineurin that subsequently cause translocation of transcription factors AP-I, NFκB and NFAT into the nucleus. 6) These transcription factors induce gene expression to control proliferation, differentiation and apoptosis of the T cell.
1.5 Models of T cell receptor triggering

The TCR complex has been studied for the past three decades and stemming from this work, multiple models of TCR triggering have been proposed (van der Merwe and Dushek, 2011). The variety of models can be categorised into three main groups: (1) aggregation, (2) segregation and (3) conformational change models.

1.5.1 Aggregation

Communicating the extracellular ligand-binding event to initiate intracellular signalling through ligand-induced TCR oligomerisation is an appealing idea because aggregation or clustering of the TCR complex in experimental settings cause ITAM phosphorylation. Early studies support this model of TCR triggering based on finding that soluble dimeric and oligomeric peptide-MHC molecules are able to activate T cells in culture, whereas soluble monomeric peptide-MHC molecules cannot (Stone and Stern, 2006). Ligand-induced receptor clustering or oligomerisation can also be visualized by fluorescence microscopy during the immunological synapse formation (Monks et al., 1998, Grakoui et al., 1999). However, TCR clustering in response to ligand binding cannot fully account for physiological triggering as recent studies have reported that on the surface of naïve (unactivated) T cells, the TCR-CD3 complex exists in both monomeric and pre-clustered form (Schamel et al., 2005, Kumar et al., 2011, Molnar et al., 2012, Molnar et al., 2010, Pageon et al., 2016).

1.5.2 Segregation

In T cells, it has been reported that more than 40% of Lck exists in active form and that ligand binding does not increase the proportion of the active Lck (Nika et al., 2010). To avoid constitutive T cell activation by this large pool of active Lck, the phosphatase CD45 dephosphorylates ITAM phospho-tyrosines, keeping the net kinase activity in check. Upon the establishment of molecular interaction between the TCR-antigen-MHC complex, a contact zone between the T cells and target cells is created, which results in physical segregation of large proteins on the contact site due to close apposition between the two interacting membranes (Davis and van der Merwe, 2006).
The extracellular domain of the CD45 is large and heavily glycosylated, it has been proposed that this would cause it to be excluded from the contact site (Grakoui et al., 1999, James and Vale, 2012), allowing Lck activity to dominate. Thus, such segregation could create an imbalance of phosphorylation-dephosphorylation activity at the TCR-pMHC contact site, resulting in downstream signalling. Both aggregation and segregation models depend on multivalent receptor-ligand interactions between apposing cell surfaces and therefore fail to provide a compelling explanation for the ability of a very small number of antigenic ligands to initiate signalling (see next section).

1.5.3 Conformational changes upon ligand binding

Recent findings reported that as little as 1-3 pMHC molecules can trigger calcium flux and cytokine secretion in T cells (Purbhoo et al., 2004, Huang et al., 2013). This suggests that a single TCR complex can signal intrinsically, perhaps by undergoing conformational changes. However, large-scale conformational changes have not been observed in most TCRαβ-pMHC complex structures when compared to those of the TCRαβ alone. Subtle structural differences, however, have been observed in a small number of x-ray crystal structures within a loop sequence (Cα AB loop) in the extracellular domain of TCRαβ (Ding et al., 1998, Kjer-Nielsen et al., 2003, Beddoe et al., 2009). On the intracellular side, substantial conformational changes within ITAM-containing cytoplasmic domains upon ligand binding have been documented. The critical tyrosines of ITAMs (at least for CD3ε and ζ) have been shown to be embedded in the inner leaflet of the membrane bilayer and are exposed upon ligand engagement, only then becoming susceptible to phosphorylation (Aivazian and Stern, 2000, Xu et al., 2008, Deford-Watts et al., 2009, DeFord-Watts et al., 2011). In contrast, Zhang et al. have suggested that phosphorylation of ITAM tyrosines is a pre-requisite for dislodgement of cytosolic tails of TCR subunits (at least for ζ chain) (Zhang et al., 2011). Further investigations will be required to clarify the precise sequence of events after ligand binding and reveal whether release of ITAMs from the membrane is cause or consequence of receptor triggering.
Dislodgement of the cytoplasmic tails of the CD3ε subunits has also been shown to be important for engaging the cytoplasmic Nck adapter protein, which was proposed to be involved during TCR triggering (Gil et al., 2002). Recently, it has been reported that the ζζ TM domains might undergo conformational changes upon ligand-binding (Lee et al., 2015). Taken together, these findings suggest potential ligand-induced conformational changes may occur, but the structural pathway that connects all the reported events from the ligand-binding extracellular domain to the cytoplasmic tails remains elusive.

Alternatively, it has been proposed that a large rigid-body movement of the entire TCRαβ extracellular domain upon ligand binding could occur that physically impacts the conformation of the associated CD3 dimers (Sun et al., 2001). The rigid CD3δε and CD3γε extracellular domains were proposed to have force-transducing potential, which then could result in the conformational changes of their cytoplasmic tails to allow accessibility of the ITAMs to Lck kinase. This model is supported by the discovery that the TCRαβ heterodimer acts as a “mechano-transducer” that “senses” mechanical force exerted during pMHC binding (Kim et al., 2009, Li et al., 2010).

1.5.5 Summary of mechanisms of TCR triggering

The TCR triggering models discussed here are not necessarily mutually exclusive and may all occur in concert with one another. Determination of the structures of an intact membrane-bound TCR complex with and without peptide:MHC bound would almost certainly provide deep insight into the mechanisms governing TCR triggering, however this has not yet proven feasible given the complexity of the receptor and the limitations of current biophysical methods. I have instead undertaken a crosslinking-based approach to gain insight into the membrane-embedded portions of the TCR complex to complement what is already known about the structure of the extracellular domains of individual subunits. Before presenting the hypothesis and aims of this thesis, I will now outline our current understanding of the structure of the intact TCR complex.
1.6 Understanding molecular architecture of the TCR complex

Proposals of the molecular architecture of the TCR complex outside of the membrane have largely relied on analyses of the existing individual extracellular domain structures. Using computational docking methods to assemble the published structures of extracellular domains of the TCRαβ, CD3δε and CD3γε dimers, two groups proposed that the CD3δε and CD3γε extracellular domains interact with the TCRαβ extracellular domains from opposing sites (Sun et al., 2004, Kjer-Nielsen et al., 2004). A mutagenesis analysis of evolutionarily conserved residues in the TCRαβ extracellular domains in co-immunoprecipitation experiments identified TCRα residues that mediate interactions with the CD3δε dimers and TCRβ residues that mediate interactions with the CD3γε dimer to be positioned adjacent to each other in the structure of TCRαβ heterodimer, hence suggesting neighbouring positioning of the CD3δε and CD3γε dimers in the TCR complex (Kuhns and Davis, 2007). Recently, two groups have undertaken NMR spectroscopy-based chemical shift perturbation (CSP) analyses for extracellular domains interactions between soluble fragments of the TCR and CD3 subunits, but came to different conclusions as to where the interaction points were (He et al., 2015, Natarajan et al., 2016). In both NMR studies, the estimated affinities (K_D) for TCR-CD3 interactions are in the hundreds of µM to mM range, which may explain some of the uncertainty in modelling the extracellular domains assembly of the TCR-CD3 complex. Another recent study of the intact TCR complex using negative stain electron microscopy (EM) observed a compact architecture of the TCR complex, and with the accompanying small angle x-ray scattering (SAXS) analysis of the extracellular domains of TCRαβ-CD3δε, suggested that the positioning of extracellular Ig-like domains of the CD3 dimers is underneath TCRαβ extracellular domains (Birnbaum et al., 2014). While this report highlights the feasibility of studying the intact complex using EM methods, the structural data presented in this study did not achieve sufficient resolution to allow docking of individual extracellular domains and visualising the relevant intermolecular interaction surfaces with certainty.

In the absence of atomic resolution structures of the intact receptor, a definitive answer to the spatial architecture of the TCR-CD3 complex remains elusive. The best-known contacts that relate the four dimeric modules within the complex are the polar
network within the membrane-embedded domains that link CD3δε and ζζ to TCRα and CD3γε to TCRβ, but a lack of information about how or whether the TCRα and TCRβ TM domain interact hampers our effort to precisely position these subunits. Furthermore, while the key contact points that hold the TCR-CD3 complex together have been established, very little is known how the entire TM domain of one subunit interacts with the another. Thus, obtaining additional structural information for the TM domains assembly would greatly advance our current knowledge of the TCR complex architecture.

1.7 Thesis hypothesis and aims

We hypothesised that understanding the spatial organisation of TM domains among the subunits of the TCR complex will provide insight into the overall molecular architecture of the TCR complex and provide a platform from which to generate and test new hypotheses to understand trans-bilayer TCR signalling.

Aim 1: To investigate molecular interactions between TM domains of the TCRα and TCRβ chains within the intact TCR complex.

While contact points between TCR and CD3 chains have been characterised, essentially nothing is known about TM association between TCRα and TCRβ chains. We performed cysteine crosslink screening on membrane-embedded intact TCR complexes produced in vitro to understand if TCRα and TCRβ chains interact in the membrane-embedded domains and if they do, to characterise the TCRαβ heterodimer interface. Briefly, substitution of native amino acids to cysteine residues in the TM domains allows trapping of non-covalently associating TCRα and TCRβ variants only when a pair of cysteines are in close proximity in the native structure. A collection of these proximity restraints was then used to identify interacting surfaces between TCRα and TCRβ subunits in the membrane and generate structural models. Knowing how TCRα and TCRβ chains associate would enable us to propose the spatial organisation of the rest of the subunits relative to the TCRα and TCRβ chains. Findings from this aim are presented in Chapter 3 of this thesis.
Aim 2: To map the spatial organisation between TM domains of the TCRαβ heterodimer and the signal-transducing subunits within the intact TCR complex.

We aimed to precisely map the positioning of each TCR subunit within the membrane-embedded portions of the intact TCR complex. We generated a single cysteine mutant library for every TM residues of the CD3δ, CD3γ and CD3ε chains and for a selection of ζ chain residues. Similar to the approach performed in Aim 1, these mutants were used in cysteine crosslink screening to identify contact points between subunits in the TCR complex. Findings from this aim are presented in Chapter 4 of this thesis.
Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Affinity reagents

All affinity reagents used in this thesis were purchased from commercial source and presented in Table 1.

Table 1: Affinity reagents

<table>
<thead>
<tr>
<th>Affinity reagent</th>
<th>Origin</th>
<th>Conjugation</th>
<th>Clone</th>
<th>Mono/polyclonal (for antibody)</th>
<th>Catalog number</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>anti-human CD3 mouse</td>
<td>mouse</td>
<td>agarose</td>
<td>OKT3</td>
<td>Monoclonal</td>
<td>N/A</td>
<td>WEHI antibody facility</td>
</tr>
<tr>
<td>anti-human zeta (ζ) mouse</td>
<td>mouse</td>
<td>N/A</td>
<td>6B10.2</td>
<td>Monoclonal</td>
<td>sc-1239</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>anti-mouse TCRβ hamster</td>
<td>hamster</td>
<td>PE-Cy7 fluorophore</td>
<td>H57-597</td>
<td>Monoclonal</td>
<td>109222</td>
<td>Biolegend</td>
</tr>
<tr>
<td>anti-protein C mouse</td>
<td>mouse</td>
<td>agarose</td>
<td>HPC4</td>
<td>Monoclonal</td>
<td>11815024001</td>
<td>Roche</td>
</tr>
<tr>
<td>anti-FLAG mouse</td>
<td>mouse</td>
<td>agarose</td>
<td>M2</td>
<td>Monoclonal</td>
<td>A2220</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>anti-heamagglutinin (HA)</td>
<td>mouse</td>
<td>agarose</td>
<td>HA-7</td>
<td>Monoclonal</td>
<td>A2095</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Streptavidin streptomyces avidinii</td>
<td>agarose</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>S1638</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Protein G recombinant</td>
<td>recombinant</td>
<td>agarose</td>
<td>N/A</td>
<td>N/A</td>
<td>P7700</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>

2.1.2 Bacterial strains

Bacterial strains that were used in this study are the *Escherichia coli (E.coli)* strains XL-1 Blue, Stable and BL21 (DE3). The chemically competent cells (Potassium, Calcium, Magnesium) were used for all cloning and plasmid preparation in this study.
2.1.3 Oligonucleotides

Due to large amounts of oligonucleotides used in this study, details about the oligonucleotides can be provided upon request from the Call laboratory. The sequences of these oligonucleotides have been deposited onto a password-secured wiki page (CallWiki). All oligonucleotides were purchased from Integrated DNA Technologies.

2.1.4 Plasmid vectors

All plasmid vectors that were used in this study are presented in Table 2.

*Table 2: Plasmid vectors*

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Original plasmid</th>
<th>Antibiotic selection</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>pIVT</td>
<td>pSP64</td>
<td>Ampicillin</td>
<td>Cloning DNA sequences of TCR complex subunits for T7-promoter driven <em>in vitro</em> mRNA production</td>
</tr>
<tr>
<td>pTrpLE</td>
<td>pMM-LR6</td>
<td>Kanamycin</td>
<td>Cloning DNA sequences of TM peptides for IPTG-inducible protein expression</td>
</tr>
<tr>
<td>pMIG ΔGFP</td>
<td>pMIG</td>
<td>Ampicillin</td>
<td>Cloning DNA sequences of TCR proteins for stable retrovirus-mediated genome integration in mammalian cells</td>
</tr>
<tr>
<td>pHAGE-mCherry</td>
<td>pHAGE</td>
<td>Ampicillin</td>
<td>Cloning DNA sequences of CD3ζ and ζ subunits for stable lentivirus-mediated genome integration in mammalian cells</td>
</tr>
<tr>
<td>pGAG and pENV</td>
<td>pGAG and pENV</td>
<td>Ampicillin</td>
<td>Retrovirus packaging and envelop</td>
</tr>
<tr>
<td>pRSV, pMDL, pVSV-G</td>
<td>pRSV, pMDL, pVSV-G</td>
<td>Ampicillin</td>
<td>Lentivirus (second generation) packaging and envelop</td>
</tr>
</tbody>
</table>

2.1.5 Chemical stocks, media and solutions

Compositions of all chemical stocks, media and solutions are summarised in Table 3 (see next page).
<table>
<thead>
<tr>
<th>Buffer/Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer</td>
<td>100mM Tris, 500mM KCl, 15mM MgCl₂, 1% triton X-100, pH8.8</td>
</tr>
<tr>
<td>KCM solution</td>
<td>0.2M KCl, 0.06M CaCl₂, 0.1M MgCl</td>
</tr>
<tr>
<td>TAE</td>
<td>40mM Tris, 20mM acetic acid, 1mM EDTA</td>
</tr>
<tr>
<td>NEB restriction enzymes</td>
<td>refer to New England Biolab (NEB) website for details.</td>
</tr>
<tr>
<td>broths</td>
<td></td>
</tr>
<tr>
<td>Lysogeny broth</td>
<td>10g/l tryptone, 5g/l yeast extract, 10g/l NaCl</td>
</tr>
<tr>
<td>Super broth</td>
<td>32g/l tryptone, 20g/l yeast extract, 5g/l NaCl, 5mM NaOH</td>
</tr>
<tr>
<td>Complete DMEM</td>
<td>DMEM (without L-Glutamine) from Lonza, 10% FBS, 2mM L-Glutamine</td>
</tr>
<tr>
<td>Complete RPMI</td>
<td>RPMI (without L-Glutamine) from Lonza, 10% FBS, 2mM L-Glutamine</td>
</tr>
<tr>
<td>Iso-osmotic buffer</td>
<td>10mM HEPES/KOH pH7.5, 0.25M Sucrose</td>
</tr>
<tr>
<td>Hypo-osmotic buffer</td>
<td>10mM HEPES/KOH pH7.5</td>
</tr>
<tr>
<td>Hyper-osmotic buffer</td>
<td>10mM HEPES/KOH pH7.5, 0.6M Sucrose</td>
</tr>
<tr>
<td>Membrane storage buffer</td>
<td>50mM HEPES/KOH pH7.5, 0.25M Sucrose, 1mM DTT</td>
</tr>
<tr>
<td>TBS</td>
<td>20mM Tris-HCl, 150mM NaCl, pH8.0</td>
</tr>
<tr>
<td>EndoH buffer</td>
<td>1x G5 buffer (NEB), 0.5% SDS</td>
</tr>
<tr>
<td>Digitonin extraction solution</td>
<td>1% (w/v) digitonin, 0.1% (w/v) BSA, 10mM Iodoacetamide in TBS pH8.0</td>
</tr>
<tr>
<td>Digitonin wash solution</td>
<td>0.5% (w/v) digitonin in TBS pH8.0</td>
</tr>
<tr>
<td>CaCl₂-digitonin buffer</td>
<td>0.5% (w/v) digitonin in TBS pH8.0, 1mM CaCl₂, 0.1% (w/v) BSA, 10mM Iodoacetamide</td>
</tr>
<tr>
<td>EDTA-digitonin buffer</td>
<td>0.5% (w/v) digitonin in TBS pH8.0, 5mM EDTA, 0.1% (w/v) BSA, 10mM Iodoacetamide</td>
</tr>
<tr>
<td>NuPAGE® LDS sample buffer</td>
<td>106mM Tris-HCl, 141mM Tris-base, 2% LDS, 10% Glycerol, 0.51mM EDTA, 0.22mM SERVA® Blue G250, 0.15mM Phenol Red, pH8.5</td>
</tr>
<tr>
<td>NuPAGE® MES SDS running buffer</td>
<td>50mM MES, 50mM Tris-base, 0.1% (w/v)SDS, 1mM EDTA, pH7.3</td>
</tr>
<tr>
<td>Glycine transfer buffer</td>
<td>2mM Tris-HCl, 200mM Glycine</td>
</tr>
<tr>
<td>CuPhe solution</td>
<td>30mM cooper (II) sulphate, 100mM o-Phenanthroline</td>
</tr>
<tr>
<td>NMR sample buffer</td>
<td>20mM sodium phosphate, 0.02% sodium azide, pH6.8</td>
</tr>
<tr>
<td>Refolding buffer</td>
<td>8M Urea in 20mM MES buffer (50mM NaCl, pH5.5)</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>PBS, 0.5% (w/v) BSA, 5mM EDTA</td>
</tr>
</tbody>
</table>
Continued from previous page.

<table>
<thead>
<tr>
<th>Buffer/Media</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixing buffer</td>
<td>PBS, 0.5% (w/v) BSA, 5mM EDTA, 1% (v/v) PFA</td>
</tr>
<tr>
<td>Coomassie staining solution</td>
<td>30% (v/v) 2-Propanol, 10% (v/v) acetic acid, 0.01% (w/v) R250 Coomassie brilliant blue</td>
</tr>
<tr>
<td>Coomassie destaining solution</td>
<td>20% (v/v) 2-Propanol, 10% (v/v) acetic acid</td>
</tr>
<tr>
<td>M9 minimal media (in 1L)</td>
<td>100µl 1M CaCl₂, 1ml 2M MgSO₄, 7.5g Na₂HPO₄, 3.0g KH₂PO₄, 1.0g NH₄Cl, 4.0g glucose, 4ml of centrum solution, 50µg/ml (final) kanamycin</td>
</tr>
<tr>
<td>¹⁵N labelled M9 media</td>
<td>Same as M9 minimal media, but use ¹⁵N-labelled NH₄Cl</td>
</tr>
<tr>
<td>²H (80%),¹⁵N,¹³C labelled M9 media</td>
<td>Same as M9 minimal media, but use ¹⁵N-labelled NH₄Cl, ¹³C-labelled glucose and D₂O (80%)</td>
</tr>
</tbody>
</table>

2.2 Methods

2.2.1 In vitro translation and TM cysteine crosslinking assay

2.2.1.1 Generation of cysteine mutant constructs

Full-length sequences of subunits of the TCR complex were cloned into a modified pSP64 vector with C-terminal peptide affinity tags including hemagglutinin, streptavidin-binding peptide, FLAG and Protein C affinity tags (pIVT-HA, pIVT-SBP, pIVT-FLAG and pIVT-PC). For each mutation, a pair of complementary and overlapping oligonucleotides/primers with a single mismatched codon at the position of interest were designed and purchased from Integrated DNA Technologies (IDT). DNA inserts of each mutant were generated using either QuikChange® Site-Directed Mutagenesis or by overlapping PCR.

2.2.1.2 QuikChange® Site-Directed Mutagenesis

QuikChange® Site-Directed Mutagenesis was adapted from the manufacturer protocol (Stratagene). Briefly, 125ng of the complementary primers and 25ng template plasmid were used in a 50µl PCR reaction with 1X Phusion PCR buffer, 1µl of 10mM dNTPs
and 1µl of Phusion Polymerase. The PCR reaction cycling conditions were 94°C for 2min (denaturing the DNA), followed by 25 cycles of 94°C (30s), 52°C (30s) and 72°C (1min) and a final single step at 72°C for 7min. After PCR reaction, the template plasmid was digested with restriction enzyme DpnI (New England Biolabs) at 37°C for 1 hour. The resultant plasmid was transformed into chemically-competent (Potassium-Calcium-Magnesium; KCM) XL-1 Blue E.coli cells.

### 2.2.1.3 Overlapping PCR strategy

Alternatively, mutagenesis was carried out by two-step overlap PCR reaction. Initially, two fragments of DNA were generated. The first fragment was generated using a common forward primer from the 5’ end of the gene (5’-GAAGCTAAAAACAAATCAATC -3’) and a reverse primer encoding the cysteine mutations (provided upon request, Call laboratory). The second fragment was generated using the complementary forward primer encoding the cysteine mutations (provided upon request, Call laboratory) and a common reverse primer from the 3’ end of the gene (5’-GGGCTCAAAATGTTCCTTTACGATGCTATC -3’). These fragments were purified and used as templates in the third PCR reaction to amplify the entire gene (with inserted mutations) using the common forward and reverse primers. Final DNA inserts were separated on 1% agarose gel (in 1X TAE, 125V for 20min). DNA inserts were extracted and purified using Zymoclean™ Gel DNA Recovery Kit (Zymo Research). Purified DNA inserts were then digested using the restriction enzymes, NotI and BamHI at 37°C for an hour and ligated into NotI/BamHI-digested pIVT vector (see appendix 2) using the Quick Ligation™ kit (New England Biolabs). The resultant plasmid was transformed into chemically-competent (Potassium-Calcium-Magnesium; KCM) XL-1 Blue E.coli cells and plated on an ampicillin-containing agar plate ([Ampicillin] = 100mg/ml).

### 2.2.1.4 DNA plasmid purification

Bacterial colonies selected by antibiotic resistance on agar plate were inoculated in 10ml of ampicillin/kanamycin-containing Lysogeny Broth (LB) media (WEHI media kitchen) overnight at 37°C in the shaking incubator (180rpm). Cells were grown
overnight at 37°C in the shaking incubator (180rpm). Bacterial cells were pelleted and DNA plasmids were purified using QIAprep® Spin Miniprep Kit or Hispeed® Plasmid Midi Kit based on the manufacturer’s protocol (Qiagen).

2.2.1.5 Endoplasmic reticulum (ER) microsomes preparation

In vitro translation assays performed in this thesis were based on a previously published protocol with several modifications (Call et al., 2002). The source of ER microsomes used in this thesis was purified from IVD12 cells (murine B cell hybridoma cell line). IVD12 cells were maintained in Dulbecco’s modified Eagle medium (DMEM) (Lonza BioWhittaker®, 4.5g/L glucose, without L-Glutamine) supplemented with 10% Fetal Bovine Serum (ScientifixLIFE; origin = France) and 2mM L-glutamine (Sigma). 12L culture of IVD12 cells at 1.5 x 10⁶ cells/ml was pelleted by centrifugation (400g, 15min, without brake). The pellets were washed twice by re-suspending in ice-cold iso-osmotic buffer (0.25M sucrose) and pelleted by centrifugation. Next, the cell pellets were weighed, resuspended in hypo-osmotic buffer (5ml/g of pellet) and incubated on ice for 10min to allow swelling. Cells were returned to iso-osmotic conditions by addition of 30ml of hyper-osmotic buffer (0.60M sucrose). Protease Inhibitor Cocktail P8340 (Sigma) was added to the solution to prevent protease activity. Cell suspensions were transferred to a Dounce homogeniser tube and processed with an overhead motor-driven Duall pestle (Kontes) in 25ml aliquots at speed setting 6-8. For efficient disruption, homogenisation was done by 2x 5 slow up and down movements. The crude homogenates were centrifuged (10,000g, 4°C, 20min) to pellet nuclei and large organelles. The resultant pellets were re-suspended in iso-osmotic buffer (with protease inhibitor) and homogenisation was repeated. The resultant supernatants were centrifuged (Beckman Ti 60 rotor, 150,000g, 40min, 4°C) to pellet the microsomal fraction. The total microsomal suspension was mixed with Opti-Prep solution (60% Iodixanol) to a final concentration of 20% Iodixanol. The mixtures were centrifuged (Beckman VTi 65.2 rotor, 363,000g, 2 hours, 4°C, without brake) to allow fractionation through a self-generating Iodixanol gradient. Without disrupting the generated gradient, fractions were collected under positive pressure using peristaltic pump into 10x 1ml fractions. The fractions were diluted in iso-osmotic buffer, pelleted by centrifugation (21,130g, 4°C, 10min) and subsequently, were resuspended in
membrane storage buffer. Each fraction was adjusted to an OD$_{280\text{nm}}$ of 100 and the activity of the membranous components in each fraction was tested in \textit{in vitro} translation reactions.

\textbf{2.2.1.6 \textit{In vitro} Transcription}

Messenger RNA (mRNA) was synthetically produced from pIVT constructs using RibomAX® T7 Large-Scale RNA Production kit (Promega) and methyl-G cap analog (Promega). The \textit{in vitro} transcription reactions were performed at 37°C for 3.5 hours. The resultant mRNAs were then purified using the Qiagen RNeasy® Mini kit (Qiagen) according to the manufacturer’s protocol.

\textbf{2.2.1.7 \textit{In vitro} Test Translation}

All synthetic mRNAs made in this thesis were individually test-translated to ensure proper translation and ER insertion (judged by glycosylation pattern). The experiments were performed in 12.5µl translation reactions containing 8.25µl of nuclease treated rabbit reticulocyte lysate (Promega), 1µl of purified ER microsomes (see section 2.2.1.5), 1µl of $^{35}$S-labelled cysteine and methionine (Perkin Elmer), 0.25µl of amino acid mixture minus cysteine and methionine (Promega) and 0.25µl of recombinant Rnasin® ribonuclease inhibitor (Promega). Translation reactions were performed for an hour and further translation was halted by the addition of 500µl of ice-cold tris-buffered saline (TBS, pH8.0) into the lysates. Lysates were spun down (21,130g, 4°C, 10min) and supernatants were carefully discarded without disrupting the membrane pellets. Pellets were washed twice in TBS. Next, the pellets were dissolved in 27µl of glycoprotein denaturing buffer (New England Biolabs) and the samples were boiled at 95°C for 5min. The samples were then spun down (20,238g, RT, 1min) and 3µl of 10X G5 buffer (New England Biolabs) were added to the samples. The samples were then equally divided into two 1.5ml centrifuge tubes. Into one of the tube, 0.5ul of Endoglycosidases H (EndoH) were added and the enzyme digestions were performed at 37°C for an hour and the other tube was left untreated. Following EndoH digestion, electrophoretic analysis was undertaken (see section 2.2.1.12). After autoradiography imaging (see section 2.2.1.12), band intensity of each translated product was quantitated.
using densitometry analysis. Intensity was divided by the cysteine and methionine content in the protein sequence and these values were used to determine the amount of each mRNA required to produce equivalent molar amounts of each protein in the full TCR complex assembly reactions.

2.2.1.8 In vitro Translation and Assembly of the TCR complex

All mRNAs for subunits of the TCR complex were co-translated and assembled in 25µl translation reactions containing 17.5µl of nuclease treated rabbit reticulocyte lysate (Promega), 2µl of purified ER microsomes (see section 2.2.1.5), 2µl of 35S-labelled cysteine and methionine (Perkin Elmer), 0.5µl of amino acid mixture minus cysteine and methionine (Promega) and 0.5µl of recombinant Rnasin® ribonuclease inhibitor (Promega). For two-step immunoprecipitation analysis (see section 2.2.1.9), subunits of the TCR complex were co-translated and assembled in 50µl translation reaction. Translation reactions were performed for 30min at 30°C. Then, oxidised glutathione (GSSG) was added to each translation reaction (final concentration at 4mM) to allow oxidative folding and assembly. The reactions were allowed to proceed for 4 hours and further translation was halted by the addition of 500µl of ice-cold TBS (pH8.0) into the lysates. Lysates were spun down (21,130g, 4°C, 10min) and supernatant was carefully discarded without disrupting the membrane pellets. Pellets were washed twice in TBS. Pellets were kept cold throughout the procedure and following detergent extraction and immunoprecipitation steps.

2.2.1.9 Detergent extraction and Immunoprecipitation

ER membrane pellets were thoroughly resuspended in 400µl digitonin extraction solution (TBS + 1% digitonin + 0.1% BSA + 10mM Iodoacetamide). Protein complexes were extracted for at least 30min at 4°C on a centrifuge tube rotator. Digitonin-solubilised protein complexes were cleared by centrifugation (21,130g, 4°C, 10min) to remove insoluble materials. The supernatants were transferred into fresh 1.5ml centrifuge tubes that contain affinity resins for immunoprecipitation reactions. 20µl of 50% slurry of affinity resin in TBS (pH8.0, 2% BSA) was used for each immunoprecipitation reaction. Immunoprecipitation reactions were carried out for 4
hours at 4°C on a rotator. Protein complexes bound to the affinity resin were harvested by centrifugation (21,130g, 4°C, 10min) and the resin was washed twice with 0.5% digitonin containing TBS (pH8.0), with 30min mixing at 4°C for each wash step. Residual wash solution was removed from the resin using a 50μl Hamilton syringe.

2.2.1.10 Two-step sequential immunoprecipitation

Two-step sequential immunoprecipitation (IP) reactions involve a non-denaturing elution step after the first immunoprecipitation step. In this thesis, two types of two-step IP were employed, namely (1) PC-to-FLAG and (2) SBP-to-HA. In the PC-to-FLAG IP, the anti-PC antibody recognises the PC affinity tag installed on desired subunits, in a calcium (Ca\textsuperscript{2+})-dependent manner. After digitonin extraction of protein complexes from ER membrane, the first IP was performed in the presence of 1mM CaCl\textsubscript{2}. After 4 hours of incubation at 4°C on a rotator, anti-PC antibody resin was collected by centrifugation and washed twice in CaCl\textsubscript{2}-containing wash solution (0.5% digitonin). Protein complexes were eluted from anti-PC antibody resins by the addition of 5mM EDTA-containing digitonin solution (TBS + 1% digitonin + 0.1% BSA + 10mM Iodoacetamide). The elution reactions were allowed to proceed for at least 30min at 4°C on a rotator. The supernatants were collected after centrifugation (21,130g, 4°C, 10min) and transferred into fresh 1.5mL centrifuge tubes that contains affinity reagents for the second IP reactions.

In the SBP-to-HA IP, the first IP step was performed with streptavidin-agarose pull-down in the standard digitonin extraction buffer. After 4 hours of incubation at 4°C on a rotator, streptavidin-agarose was collected by centrifugation and washed twice in wash solution (0.5% digitonin). Protein complexes were eluted from streptavidin-agarose by the addition of 100μM biotin-containing digitonin solution (TBS + 1% digitonin + 0.1% BSA + 10mM Iodoacetamide). The elution reactions were allowed to proceed for at least 30min at 4°C on a rotator. The supernatants were collected after centrifugation (21,130g, 4°C, 10min) and transferred into fresh 1.5mL centrifuge tubes that contains affinity reagents for the second IP reactions.

The second IP step was performed as described in the section 2.2.1.9.
2.2.1.11 Endoglycosidases H digestion

Protein complexes were eluted from affinity resins in 20µl of EndoH buffer (0.5% SDS in 1X G5 buffer, without DTT) at 95°C. Then, 0.5µl of EndoH were added and the enzyme digestion was performed at 37°C for an hour.

2.2.1.12 Electrophoretic analysis

Following EndoH digestion, 6µl of 4X NuPAGE® LDS Sample Buffer (1X final concentration) was added to the reactions, boiled at 95°C for 5min and centrifuged (20,238g, RT, 1min). The samples were carefully pipetted up and down using cut pipet tips and transferred to a 0.22µm SpinX® Centrifuge Tube Filters (Sigma) and spun down (20,238g, RT, 1min) to filter out the beads. 15µl of each sample was loaded onto 12% NuPAGE Bis-Tris gels (Life Technologies) and proteins separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in MES SDS running buffer (Life Technologies) at 180V for 50min under non-reducing conditions. The separated proteins on SDS-PAGE gels were transferred onto PVDF membrane using XCell SureLock® transfer system (Thermo Fisher) (35V, 60min) in glycine transfer buffer (tris-glycine + 20% methanol). The PVDF membranes were then air-dried and exposed to phosphor imager plates (GE Life Science) overnight (short exposure) or a week (for longer exposure). The images were analysed using Fujifilm FL-3000 reader (Fujifilm).

2.2.1.13 Cysteine linkage by disulphide bond formation

After the assembly step in the in vitro translation assay prior to washing the ER microsomes, the reactions were halted by the addition of 500µl of ice-cold TBS (pH8.0) supplemented with a membrane-penetrating oxidant, 1X copper (II) phenanthroline (stock is 100X; 200µM CuSO4/1000µM o-phenanthroline [Sigma]). Then, the lysates were frozen at -80°C for an hour and thawed at 30°C for 5min. TM disulphide bond formation proceeded during a further 30-minute incubation period on ice.
2.2.2 Protein expression and purification for solution NMR spectroscopy

The protocol to express and purify TM peptides performed in this thesis was based on previously published protocol with minor modifications (Sharma et al., 2013).

2.2.2.1 Generation of protein expression constructs

DNA inserts encoding sequence of the TM peptide were designed and purchased in the form of a G-Block from IDT. These DNA inserts were digested with restriction enzymes HindIII and BamHI and ligated into HindIII/BamHI-digested pTrpLE vector (Call et al., 2006) using the Quick Ligation™ kit (New England Biolabs). The resultant plasmid was transformed into chemically-competent XL-1 Blue E.coli cells and plated on a kanamycin-containing agar plate ([Kanamycin] = 50mg/ml).

2.2.2.2 E.coli expression system

TM peptide constructs shown below were used in this thesis.

TCRa: SDVKLVEKSFETDNLNFQNLGSVIGFRILLLVVAGFNLLVTTLRLWSS
TCRβ: SGFTSVSYQQGVLSATILYEILLGVATLYAVLVSALVLLVA
CD3δ: ELDPATVAGIIVTDVIATLLLALLGVFSAGHETGRL
CD3γ: ELNAATISGFLFAEIVSIFVLAVGYFIAGQDGVRQ
CD3ε: ELDVASVATIVVDISITGGLLLLVYYWSKRNKAKA
CD3ε short: ELDVASVATIVVDISITGGLLLVYYWSK

The TM peptide expression constructs were transformed into KCM competent BL21 (DE3) E.coli cells. The cells were plated onto kanamycin-supplemented LG-agar plates and incubated overnight at 37°C. For peptide expression, the transformed cells were inoculated into a starter culture of 100ml M9 minimal media supplemented with Centrum A-Z vitamins (M9 media) and grown overnight in the 37°C shaker. The culture was scaled up to 1L on the next day and were grown at 37°C to an OD600 of 0.6. At this point, the cultures were cooled for one full hour to 18°C and protein expression was
induced with 0.1mM final concentration of IPTG (Sigma). Protein expression proceeded at 18°C overnight with shaking.

For 2H, 15N-labelled peptide expression, 15N-labelled M9 media was used (see table 3). For triple-isotope labelling, transformed cells were inoculated into a starter culture of 50ml M9 media (no isotope) and grown overnight in the 37°C. Next morning, 1ml of the starter culture was added to a 50ml 15N, 13C, 2H (80%) M9 media and grown all day (37°C). In the evening, 1ml of this culture was added to a 100ml 15N, 13C, 2H (100%) M9 media and grown overnight (37°C). The culture was scaled up to 1L of 15N, 13C, 2H (100%) M9 media and the culture was split into two 2L baffled flasks and grown to OD600 of 0.6. At this point, the cultures were cooled for one full hour to 18°C and protein expression was induced with 0.1mM final concentration of IPTG (Sigma). Protein expression proceeded at 18°C overnight with shaking.

2.2.2.3 Protein purification

Inclusion bodies (IB) were purified by pelleting cultures (5000g, 4°C, 20min) and resuspending them in 25ml lysis buffer supplemented with 20mM 2-mercaptoethanol (β-ME) for sonication on ice (Misonix Sonicator 3000, setting 10, 1min x 3). IB pellets were harvested (20000g, 4°C, 15min) and resuspended with sonication (Misonix Sonicator 3000, setting 10, 1min x 3) in guanidine lysis buffer supplemented with 5mM β-ME. The IB lysates were cleared by centrifugation (75000g, 10°C, 1 hour) and sterile-filtered. Protein of interest was purified from IB lysates by batch binding to Ni-NTA resin (pre-washed in H2O and equilibrated in guanidine buffer) overnight. The Ni-NTA resin suspension was washed with urea wash buffer under gravity flow before elution. Bound protein was eluted in 6ml 100% trifluoroacetic acid (TFA). The final concentration of TFA was adjusted to ~80% by adding H2O before hydrolysis with cyanogen bromide (CNBr).

To cleave at the unique methionine residue that links the trpLE fusion protein to the peptide of interest, 1g/ml CNBr crystal were added to Ni-NTA eluate (in TFA) and the hydrolysis reaction proceeded for 4 hours at RT. After CNBr cleavage, samples were injected into 2kDa cut-off dialysis cassette (Thermo Fisher) and dialysed against 4L...
H$_2$O overnight. Dialysed samples were collected, frozen in liquid nitrogen and lyophilised.

Reverse-phase high performance liquid chromatography (RP-HPLC) was used to purify the peptide of interest. RP-HPLC separation of the CNBr-digested TCR$_\alpha$-CPTM and TCR$_\beta$-CPTM peptides were performed on C3 PrepHT column with linear gradient from 100% buffer A (60% H$_2$O + 40% acetonitrile + 0.3% TFE) to 100% buffer B (75% 2-Propanol + 25% acetonitrile + 0.3% TFE). RP-HPLC separation of CNBr-digested CD3$_\delta$, CD3$_\gamma$, CD3$_\varepsilon$ TM peptides were performed on C3 Semi-Prep column with linear gradient from 100% buffer A (60% H$_2$O + 40% acetonitrile + 0.3% TFA) to 100% buffer B (75% 2-Propanol + 25% acetonitrile + 0.3% TFA). RP-HPLC separation of CNBr-digested CD3$_\varepsilon$short was performed on C8 Semi-Prep column with linear gradient from 100% buffer A (60% H$_2$O + 40% acetonitrile + 0.3% TFA) to 100% buffer B (90% 2-Propanol + 10% TFE + 0.3% TFA).

2.2.2.4 Electrophoretic analysis of IPTG induced E.coli lysates and purified peptide samples

For whole E.coli cell lysates samples, an aliquot of bacterial culture that is equivalent to 150µl of aliquot from culture with OD$_{600nm}$ = 1.0 was collected. The aliquot was spun down (20,238g, RT, 1min) to pellet the bacterial cells. Supernatant was discarded and the pellet was resuspended in 40µl of H$_2$O. To this, 14µl of the 4X NuPAGE® LDS Sample Buffer and 6µl of 1M DTT were added, boiled at 95°C for 5min and centrifuged (20,238g, RT, 1min). Then, sample was sonicated in a bath sonicator to shear the genomic DNA and spun down (20,238g, RT, 1min). For lyophilised peptide samples, 50µl of 1X NuPAGE® LDS Sample Buffer was added to each protein sample, boiled at 95°C for 5min and centrifuged (20,238g, RT, 1min). 20µl of each sample was loaded and separated on 12% NuPAGE Bis-Tris gels (Life Technologies) by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in MES running buffer (Life Technologies) at 180V for 50min under non-reducing conditions. Protein gels were stained in Coomassie stain solution and destained in Coomassie destain solution. Protein gels were visualised in Gel Doc™ EZ Imager (BioRad).
2.2.2.5 Solution NMR sample preparation

1.0-2.0mg of purified, lyophilised and stable-isotype labelled-TM peptides of TCRα, TCRβ, CD3δ, CD3γ and CD3ε were dissolved in detergent/lipid containing NMR sample buffer (250mM lysomyristoyl phosphatidylglycerol [LMPG], 0.04% sodium azide, 20mM phosphate buffer [pH6.8] and 5% (vol/vol) D₂O – final volume of 340µl). ¹⁵N-labelled peptides were utilised for experiments in section 2.2.2.6 and ¹⁵N, ¹³C and ²H-labelled peptides were utilised for experiments in section 2.2.2.7. Peptide samples were transferred into Shigemi Advanced NMR microtubes with 5mm sample chamber.

2.2.2.6 NMR sample preparation by urea refolding step

For the Urea refolding, 250mM LMPG and 0.5mM CD3ε TM peptide were separately prepared in 300µl of hexafluoroisopropanol (HFIP). Both solutions were then transferred into in a single 50mL polypropylene centrifuge tube. By slowly rotating the tube on almost vertical angle under the nitrogen gas flow, a thin film of the lipid+peptide mixture was created on the wall of the tube. The mixture was lyophilised and subsequently re-dissolved in 4mL of 8M Urea in 20mM MES buffer (pH5.5; 50mM NaCl). The urea-dissolved lipid+peptide mixture was transferred into a pre-wetted dialysis cassette (3.5kDa cut-off) and was dialysed extensively in 20mM MES buffer (pH5.5; 50mM NaCl) overnight. After dialysis, peptide sample volume was reduced to 250µl using centrifugal concentrator. Concentrated peptide sample was prepared for NMR experiment by adding 20µl of D₂O and 70µl of MES NMR buffer (20mM MES buffer, pH5.5, 50mM NaCl and 0.02% azide) to bring the final volume to 340µl.

2.2.2.7 ¹H-¹⁵N Heteronuclear Single Quantum Coherence (HSQC) NMR spectroscopy

¹H-¹⁵N HSQC experiments were recorded and collected on a Bruker Avance III spectrometer operating at ¹H frequency of 600MHz and equipped with a cryogenic probe. The resultant spectra were decoded by Fourier transformation and processed using TopSpin® NMR software (Bruker).
2.2.2.8 Triple-resonance NMR spectroscopy

Amide backbone assignments were obtained using a standard suite of triple-resonance experiments recorded on uniformly $^{15}\text{N}$, $^{13}\text{C}$ and $^2\text{H}$ (~80%)-labelled protein samples. To obtain complete connectivity between these backbone atoms in the protein sequences, transverse relaxation-optimised (TROSY) versions of the HNCO, HN(CO)CA, and HNCACB experiments were recorded. These experiments were recorded on a Bruker spectrometer operating at a $^1\text{H}$ frequency of 600MHz and equipped with a cryogenic probe. These experiments were analysed on CARA software package (http://cara.nmr.ch/doku.php) to assign the identity of peaks on $^1\text{H}$-$^{15}\text{N}$ HSQC spectra. From backbone assignments, chemical shift values for all C, N and H atoms along the peptide backbone were extracted and used to predict phi (Φ) and psi (Ψ) backbone torsion angles of each amino acid to give information about secondary structure of the protein using Torsion angle likelihood obtained from chemical shift and sequence similarity (TALOS+) software (https://spin.niddk.nih.gov/bax/software/TALOS/+).

2.2.3 MD simulation

2.2.3.1 Replica Exchange Molecular Dynamics (REMD) simulation of TCRαβ TM structure in an implicit bilayer

REMD simulations were performed to determine the likely TM structures of the TCRαβ heterodimer. TM sequences of the human TCRα and TCRβ (see below, TM helix limits were highlighted in grey) were used to build helices based on backbone chemical shift analysis (see section 2.5.4).

TCRα: LNFGQNLSVIGFRKILLLKVAGFNLLMTLRL
TCRβ: LSATILYEILLGKATLYAVLVSAVLMA

Arginine and lysine residues of the TCRα sequence and lysine residue of the TCRβ sequence (bold and underlined above) were substituted with leucine residues to ensure stability of the helices during simulation. The two helices were initially separated from
each other by 30Å and simulated in Generalised Born with a simple SWitching (GBSW) implicit membrane (Im et al., 2003) model using Chemistry at Harvard Macromolecular Mechanics (CHARMM) force fields (Brooks et al., 2009). The simulation was performed for 10ns with random exchange of a total of 32 replicas between different temperatures that ranged from 300-750K. The initial temperatures for each replica were also randomised and exchanges were attempted at every 1ps. TCRα and TCRβ TM helices were simulated to form either disulphide-linked TCRαβ heterodimer through αF26 to βE20 or satisfy distance restraints based on all inter-helical TCRαβ crosslinks identified from the cysteine crosslinking screen (see chapter 3). For the distance-restrained simulation, a distance of 3.7Å to 6.0Å between Cβ atoms of the crosslinked positions was applied. Resulting structures were analysed through cluster analysis (see section 2.2.3.3).

2.2.3.2 MD simulations in explicit lipid bilayer

The centroid TM structure of the TCRαβ heterodimer from distance-restrained REMD simulations (see section 2.2.3.1) was inserted into an explicit lipid bilayer consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). This bilayer was generated using CHARMM-GUI (web-based graphical user interface) Membrane Builder function to contain 60 POPC lipids in each leaflet of the bilayer and bulk water with 150mM KCl (Jo et al., 2008, Wu et al., 2014). Upon equilibration of the restrained-TCRαβ heterodimer in the lipid bilayer, a 200ns restraint-free simulation using Nanoscale MD (NAMD) simulation was performed (Phillips et al., 2005). The simulation was performed under the constant temperature and pressure condition at 303.15K and 1bar.

2.2.3.3 Cluster analysis for modelling

Cluster analysis of the TCRαβ structure obtained from REMD simulations was performed based on hierarchical clustering. Every conformation of the TCRαβ TM structure obtained was randomly assigned into a different cluster. Pairs of random clusters were merged when the root mean square deviation (RMSD) between all Cα
atoms of these pairs was less than 3Å. Structures from the 3 to 10ns REMD simulation trajectory at 300K were subjected to cluster analysis.

2.2.4 Cell culture

2.2.4.1 Maintenance of cell lines

Cell lines utilised in this thesis are human embryonic kidney 293T cells (HEK293T) (RRID: CVCL_0063), and murine thymoma cell line BW5147 (RRID: CVCL_3896). HEK293T cells were maintained in DMEM (Lonza BioWhittaker®, 4.5g/L glucose, without L-glutamine) supplemented with 10% Fetal Bovine Serum (ScientifixLIFE; origin = France) and 2mM L-glutamine (Sigma). BW5147 cells were maintained in Roswell Park Memorial Institute-1640 (RPMI-1640) media (Lonza BioWhittaker® without L-glutamine) supplemented with 10% Fetal Bovine Serum (FBS) (ScientifixLIFE; origin = France) and 2mM L-glutamine (Sigma). Cells were maintained at 37°C in humidified 5% (BW5147 cells) or 10% (HEK293T cells) CO₂ incubators and passaged when 80-90% confluent. To passage 293T cells, cells were pipetted vigorously to disrupt the adherent monolayer of cells on the bottom of the tissue culture plates. For suspension cells (BW5147 cells), cells were simply pipetted up and down to avoid clumps of cells. Roughly 20% of cells from the original culture were harvested and spun down (1500g, 5 min, 4°C). Cell pellets were subsequently re-suspended in fresh culture media and transferred into new tissue culture plates/flasks.

2.2.4.2 Generation of retroviral constructs

We used the MSCV-IRES-GFP-containing (pMIG) plasmid for retro-transduction of cells. This plasmid was modified to remove the green fluorescent protein (GFP) gene (pMIG ΔGFP) by restriction digest with XhoI and SalI restriction enzymes to enable future experiments with a transcription factor GFP reporter cell-line (that has not been discussed in this thesis). The DNA sequences of the full-length murine OT-I TCRα and TCRβ chains were given as a gift by Professor Stephen Turner (Monash University, Australia). An expression cassette of TCRβ-2A-TCRα with new restriction enzymes-recognition sites to aid cloning into pMIG ΔGFP plasmid was generated using
overlapping PCR strategy (see appendix 3). XhoI and SalI were introduced at the 5’ and 3’ end of the designed cassette, respectively. Unique EcoRI or XbaI restriction sites were also introduced in the 2A peptide sequence to allow shuffling of each TCR gene independently of the other. Wild-type or mutant TCRβ-2A-TCRα cassettes were digested with appropriate restriction enzymes and ligated into the pMIG ΔGFP plasmid using the Quick Ligation™ kit (New England Biolabs).

A modified pMIG plasmid encoding mCherry gene instead of GFP gene was given as gift by Dr. Doug Fairlie (Olivia Newton John Cancer Research Institute). This plasmid was adapted to contain XhoI/EcoRI cloning sites. Murine CD3δ-2A-ζ cassette was designed as a G-Block fragment (IDT) and amplified with oligonucleotides to incorporate 5’-XhoI and 3’-EcoRI restriction sites (see appendix 4). The resultant DNA insert was then digested with XhoI/EcoRI and ligated into XhoI/EcoRI-digested pMIG-mCherry plasmid using a Quick Ligation™ kit (New England Biolabs). The resultant plasmid was transformed into chemically-competent NEB® Stable E.coli strain and selected by antibiotic resistance on agar plates. Bacterial colonies were inoculated in 10ml of ampicillin-containing starter LB media for 8 hours, then expanded into 50ml ampicillin-containing LB culture. Cells were grown overnight at 37°C in the shaking incubator (180rpm). Bacterial cells were pelleted and DNA plasmids were purified using QIAprep® Spin Miniprep Kit or Hispeed® Plasmid Midi Kit based on manufacturer’s protocol (Qiagen).

2.2.4.4 Establishing CD3γ+TCRαβ+ BW5147 cell lines

Original BW5147 cells were deficient in components of the TCR complex namely the TCRα, TCRβ, CD3δ, and ζ subunits. In this thesis, several lines of the BW5147 cells were generated. In section 3.1.2.2, TCRα’TCRβ’CD3δζ+ and TCRα’TCRβ’CD3δζ- BW5147 cell lines were generated. First, the BW5147 cells were retrovirally transduced with a pMIG-mCherry retroviral transfer plasmid (see section 2.1.5) that encodes either CD3δ-2A-ζ-INES-mCherry or CD3δ-INES-mCherry cassettes. The CD3δζ+ and CD3δζ- BW5147 cells were then retrovirally transduced with the modified pMIG plasmids (pMIG ΔGFP) (see section 2.1.5) that encode for full-length OT-I TCRα and TCRβ sequences (WT or cysteine-less). In section 3.1.12, above-mentioned CD3δζ+
BW5147 cells were retrovirally transduced with the modified pMIG plasmids (pMIG ΔGFP) that encode for full-length OT-I TCRα and TCRβ sequences (WT or TCRαβ TM interface mutants).

2.2.4.5 Retroviral transduction

For retroviral production, retroviral packaging (Gag; polyprotein), envelope (Ecotropic-ENV), and transfer (pMIG ΔGFP) plasmids were transiently transfected into HEK293T cells by calcium phosphate transfection. Briefly, 215ng of the envelope, 215ng of the packaging and 1.72µg of the transfer plasmids were prepared in 2X HEPES-buffered saline (HBS) pH7.1. The plasmids mixture was sterile filtered using a 0.22µm SpinX® Centrifuge Tube Filters (Sigma). The filtered mixture was supplemented with 0.125M CaCl₂ (final concentration) solution to precipitate the plasmids. The entire solution was then carefully dispersed onto HEK293T cells. After 4 hours of incubation, media were replaced with fresh DMEM (10% FBS + 2mM L-Glutamine). Transfected HEK293T cells were incubated for at least 36 hours and media containing virus particles were harvested and filtered through 0.45µm syringe filter to remove contaminating HEK293T cells. Healthy BW5147 cells were prepared at ~30% confluency in 1ml volume per well using 12-well tissue culture plate and supplemented with 4µg/ml polybrene. Virus particles-containing media were then added onto BW5147 cells at 1:1 volume ratio (i.e. 1mL of virus-containing media + 1mL of BW5147 cells). Retroviral transduction/infection was carried out by centrifuging the tissue culture plates at 1500g for 45 min at 32°C on a pre-warmed centrifuge. Cells were incubated for at least 48 hours at 37°C in 5% CO₂ incubator. Polybrene-containing media were replaced with fresh RPMI-1640 (10% FBS + 2mM L-glutamine). After several passages to remove remaining retroviral particles. Fluorescent assisted cell sorting (FACS) method was used to sort cells of interest. Desired cell lines were frozen down in FBS (10% DMSO) and stored at -80°C freezer (for short-term storage) or in liquid nitrogen (for long-term storage).
2.2.4.6 Flow cytometry

For analysis by flow cytometry, cells were stained with PE/Cy7 fluorophore-conjugated antibody against extracellular epitope on TCRβ chain. Prior to antibody staining, cells were harvested from tissue culture plate and centrifuged at 1500g for 5min in a pre-chilled centrifuge. Cells were washed once with phosphate-buffered saline (PBS) supplemented with 0.5% Bovine Serum Albumin (BSA) and 5mM EDTA. Cells were spun down and subsequently cell pellets were re-suspended in PE/Cy-conjugated anti-TCRβ antibody-containing PBS (0.5% BSA + 5mM EDTA). Cells were kept on ice all the time. Antibody staining was performed on ice for an hour. Cells were then washed twice in ice-cold PBS (0.5% BSA + 5mM EDTA) and cell pellets were re-suspended in ice-cold 2% PFA-containing PBS (0.5% BSA + 5mM EDTA) for flow cytometry analysis.
Chapter 3: A conserved αβ transmembrane interface forms the core of a compact TCR-CD3 structure within the membrane

3.0 Introduction

To interrogate the structural assembly of protein complexes in the membrane, structure determination techniques such as x-ray crystallography and electron microscopy have been commonly applied. Recently, Birnbaum et al., (2014) reported the first effort in using electron microscopy (negative stain) on the intact TCR complex that was expressed and purified from mammalian cell lines (Birnbaum et al., 2014). Low-resolution EM images obtained from this study were utilised to deduce the rough placement of the CD3 extracellular (EC) domains underneath of the TCRαβ EC domains and allowed the authors to propose a preliminary model of architecture of the TCR complex. This study showed a promising avenue for future studies in using EM for TCR complex structure determination.

We sought an alternative strategy that would allow us to determine the architecture of the TCR complex in a native membrane environment. Assembly of the complete TCR complex occurs in the ER membrane, governed by a cluster of polar amino acid residues in the TM domains (Call and Wucherpfennig, 2005). The importance of the TM domains as the site of major interactions among subunits of the TCR complex has motivated us to determine the spatial organisation of the TM domains as a guide to the architecture of the intact TCR complex. For this, we envisioned using a cysteine crosslinking approach while the receptor complex is intact in the native membrane. Cysteine crosslinking would provide information on residue proximity between pairs of interacting TM domains within the TCR complex. A large collection of such residue proximity information could then be utilised to develop a model of the TCR complex.

Here, I interrogated the relationship between the TCRα and TCRβ TM domains using cysteine crosslinking. While we knew that the TCRα and TCRβ subunits directly contacted the signal-transducing subunits, essentially nothing was known about whether TCRα and TCRβ interact in the membrane. Based on crystallographic studies, it is known that the extracellular domains of the TCRα and TCRβ chains form a
heterodimer that mediates the recognition and discrimination of peptide antigen displayed by the MHC. The TCR\(\alpha\) and TCR\(\beta\) chains are disulphide-linked through a pair of cysteine residues in the CP regions, which is positioned approximately 16-20 amino acid residues N-terminal from the TM domains. If the CP segments exist in extended conformation within the TCR complex, this would mean that the TM domains of the TCR\(\alpha\) and TCR\(\beta\) could be separated as far as 100Å in the membrane, which would impact how the signal-transducing subunits are positioned within the TCR complex. Hence, understanding the relationship between the TM domains of the TCR\(\alpha\) and TCR\(\beta\) will allow us to obtain a more complete picture of TM assembly within the intact TCR complex.

Generally, cysteine crosslinking of membrane proteins has been performed in cell lines transfected with the cysteine-substituted variants of the protein of interest (Luo et al., 2004, Brooks et al., 2014). Cellular transfection will necessitate the use of T cell lines that are deficient in expression of certain subunits of the TCR complex, which may limit which subunits of the TCR complex can be interrogated. Alternatively, the use of non-T cell lines will require expression of six different TCR complex subunits, which is technically challenging. Moreover, generating and maintaining cell lines carrying single TM cysteine variants would be laborious and rate limiting. In this study, we have utilised a cell-free protein translation and assembly approach, termed \textit{in vitro} translation (IVT), which offers ease of analysis, high reproducibility and higher throughput when compared to cellular systems. The IVT assay has previously been utilised to assemble the intact TCR complex with correct stoichiometry (Call et al., 2002). The IVT assay recapitulates cellular membrane protein biosynthesis using rabbit reticulocyte lysate to translate mRNAs encoding subunits of the TCR complex in the presence of purified ER microsomes, where subunits of the TCR complex can be cotranslationally inserted into the ER membrane, allowing the luminal, cytosolic and TM segments to cooperatively fold and assemble (Fig 3.1).

Previously (during my undergraduate Honours research project, 2012), I generated a TM cysteine mutant library of the TCR\(\alpha\) and TCR\(\beta\) chains and performed preliminary cysteine crosslink screening between these TM domains. I identified a small number of inter-helical TCR\(\alpha\)-TCR\(\beta\) crosslinks, which indicated that the TCR\(\alpha\) and TCR\(\beta\) TM domains are physically associated in the membrane. In this chapter, I
validated the compatibility of these inter-helical crosslinks within an intact TCR complex assembly and utilised crosslinking data in combination with solution NMR spectroscopy and computational modelling to determine the TM structure of the TCRαβ heterodimer.

3.1 Results

3.1.1 Experimental setup for cysteine crosslinking of the TCR complex assembled in the in vitro translation system

For all IVT assay performed in this thesis (chapter 3 and 4), I utilised full-length TCRα and TCRβ sequences from the human A6 TCR that recognises the viral TAX peptide in the context of HLA-A2 (LLFGYPVYV/HLA-A2). The A6 TCR was chosen because of its efficient assembly with the signal-transducing subunits, which had been validated in previous IVT studies of the TCR complex assembly (Call et al., 2002, Call et al., 2004). Affinity tags were engineered to the C-terminus of these proteins to facilitate immunoprecipitation (IP) with high specificity antibodies. The human A6 TCRα protein was tagged with a hemagglutinin tag (TCRαHA) that can be immunoprecipitated with agarose-conjugated to the monoclonal anti-HA antibody (clone HA-7). The human A6 TCRβ was tagged with a streptavidin binding peptide (TCRβSBP) that can be immunoprecipitated with streptavidin-conjugated agarose. The invariant human CD3δ and CD3γ subunits were tagged with a Protein C peptide (CD3δPC) and FLAG (CD3γFLAG), respectively. The CD3δPC subunit can be immunoprecipitated using a calcium (Ca²⁺) dependent monoclonal anti-PC antibody (clone HPC4) conjugated to agarose. The CD3γFLAG subunit can be immunoprecipitated with the monoclonal anti-FLAG antibody (clone M2) conjugated to agarose. The human CD3ε and ζ proteins were not engineered with any affinity tag but these subunits can be immunoprecipitated using the monoclonal anti-CD3 antibody (clone OKT3) and monoclonal anti-ζ antibody (clone 6B10), respectively. The OKT3 antibody recognises an epitope within the folded extracellular domains of the CD3 dimeric modules, whereas the anti-ζ antibody recognises an epitope within the cytoplasmic tail of the ζ subunit.
Synthetically produced mRNAs for the TCRα and TCRβ subunits were co-translated along with mRNA for the invariant human CD3δ, CD3γ, CD3ε and ζ subunits in the IVT reaction. To enable detection of protein synthesised in this assay, we supplemented with 35S-labelled methionine and cysteine amino acids. The assembled TCR complexes were extracted out from the membrane into digitonin micelles for subsequent IP. To screen for cysteine crosslinks, I utilised two IP strategies; (1) single IP using the OKT3 antibody and (2) non-denaturing sequential IP targeting the CD3δPC subunit, followed by targeting the CD3γFLAG subunit (hexameric CD3εδPC-TCRαHAβSBP-CD3εγFLAG complex selection). In the sequential IP, the assembled and digitonin-extracted TCR complexes were captured using an anti-PC antibody in the presence of Ca²⁺ ions, followed by non-denaturing elution using the calcium chelator, EDTA. The eluted complexes were then captured using anti-FLAG antibody in the second IP step. One IP experiment in the chapter was done in the presence of reducing agent and utilised streptavidin-conjugated agarose to target the TCRβSBP subunit (experiment performed in Fig 3.7B). This strategy was chosen because reducing agent would compromise the function of antibodies due to reduction of key disulphide bonds that are critical in maintaining the native antibody structure.

All IP reactions were followed by non-reducing SDS-PAGE analysis and transferred onto PVDF membrane for 35S-detection. A brief illustration of the IVT protocol is shown in figure 3.1 and further experimental details can be found in chapter 2.
3.1.2 Design and characterisation of cysteine-less TCRα and TCRβ chain constructs to aid cysteine crosslinking screening

The TCRα and TCRβ chains exist as disulphide crosslinked heterodimer within the TCR complex. Each TCR chain has a cysteine residue in the CP region that mediates covalent linkage between these two chains. This poses the problem that the formation
of new TM crosslinks in our cysteine crosslinking screen will be masked by the constitutive disulphide-linkage of TCRαβ in the CP region (Fig 3.2A). Therefore, to overcome this issue, we proposed to remove the native disulphide bond, by designing a TCRα and TCRβ subunits with the CP cysteine residues substituted with serine residues, termed “cysteine-less” TCR chains (despite retaining the cysteines required for Ig domain folding). It has previously been reported that the CP disulphide bond formation between the TCRα and TCRβ subunits is not required for cell surface expression or function, but the ability to co-IP ζζ module was compromised (Arnaud et al., 1997). Because the cysteine-less TCRαβ would be resolved as individual monomeric chains in the non-reducing SDS-PAGE analysis, our readout for a successful TM crosslink between the TCRα and TCRβ chains would be the formation disulphide-linked TCRαβ heterodimer protein product.

3.1.2.1 Both cysteine-less TCRα and TCRβ chains assemble with folded CD3 dimers

To investigate the ability of the cysteine-less TCR subunits to assemble with the signal-transducing subunits in the IVT assay, I assembled both wild type (WT) and cysteine-less TCR subunits in the IVT assay in the context of full TCR complex assembly and immunoprecipitated the assembled and digitonin-extracted TCR complexes using the OKT3 antibody (Fig 3.2B; left panel). This IP strategy resulted in the recovery of both cysteine-less TCRα and TCRβ subunits, suggesting these subunits interact with the CD3 dimers. Moreover, as anticipated, the cysteine-less TCRα and TCRβ subunits were unable to form disulphide-linked heterodimer as determined by the absence of TCRαβ heterodimer band on the non-reducing SDS-PAGE. To determine if the recovered cysteine-less TCRα and TCRβ subunits formed non-covalent heterodimer in the IVT assay, I employed a hexamer selection IP strategy (Fig 3.2B; right panel). In the hexamer selection, it is expected that only interacting TCRα and TCRβ subunits can be immunoprecipitated upon sequentially targeting the two different CD3 dimeric modules. Indeed, I observed recovery of both cysteine-less TCRα and TCRβ subunits in this experiment (Fig 3.2B; right panel). These experiments demonstrated that the cysteine-less TCR complex assembles with the folded CD3 dimeric modules similarly to the WT TCR complex.
In contrast, I noticed that the recovery ζζ modules was not evident in either IP strategy, similar to that which was reported in cells expressing cysteine-less TCR (Arnaud et al., 1997). Because the recruitment of the ζζ homodimer into the TCR complex is final step in TCR complex biogenesis and indicates the other CD3 modules have correctly joined, I decided to investigate whether the altered ζζ association within the cysteine-less TCR complex (section 3.1.2.2) is because ζζ was no longer part of the TCR complex, or whether it was being lost during IP after detergent extraction.

**Figure 3.2: C cysteine-less TCRα and TCRβ construct design.** (A) Cartoon representation of: (Left) WT TCRαβ heterodimer with the intact CP disulphide bond (red connecting line), (middle) cysteine-less non-covalent TCRαβ heterodimer and (right) hypothetical heterodimerisation of the TM cysteine mutants of the cysteine-less TCRα and TCRβ subunits through a TM disulphide bond (red connecting line). (B) IVT assembly assay of the WT and cysteine-less TCR chains in the context of full TCR complex assembly. The resultant TCR complexes were immunoprecipitated with the conformational-dependent anti-CD3 antibody (OKT3), separated with the non-reducing SDS-PAGE, transferred onto PVDF membrane, exposed to phosphor screen to detect 35S isotope radioactivity.
3.1.2.2 *Cysteine-less TCRαβ forms within the intact TCR complex but has altered detergent sensitivity*

There are two possibilities that could result in failure to recover ζζ homodimer in the cysteine-less TCR complex assembly: (1) Failure of the ζζ homodimer to assemble within the cysteine-less TCR complex or (2) loss of the ability to co-IP ζζ homodimer due to acquired detergent-sensitivity of the ζζ-containing cysteine-less TCR complex. To address these possibilities, I employed a cell-based assay to assess the ability of a cysteine-less TCR complex to be cell surface-expressed. Using the TCRαβCD3ζζCD3γε+ murine BW5147 cell line, ζ subunit-dependent cell surface expression of the WT and cysteine-less TCR complexes were assessed.

I began by reintroducing missing CD3 chains into BW5147 cells by either introducing the CD3δ and ζ subunits (+ζ) or CD3δ subunit alone (-ζ) (Fig 3.3A). These stable cell lines were then transduced with retroviruses carrying either WT or cysteine-less (Cys-less) murine OT-I TCRα and TCRβ subunits, which share approximately 80% amino acid conservation with the A6 TCR (Appendix 1) in the TM domain. The ability of these TCR complexes to be cell surface expressed was assessed by flow cytometry after staining with a PE/Cy7-conjugated anti-TCRβ antibody (clone H57-597). I found that both WT and cysteine-less TCRs could not be detected on the cell surface when these subunits were transduced into ζ-deficient recipient BW5147 cells (Fig 3.3B). In contrast, expression of both WT and cysteine-less TCRs, albeit at lower levels, were observed on the cell surface when these subunits were transduced into ζ-sufficient recipient BW5147 cells (Fig 3.3B). This experiment indicates that the cysteine-less TCR complex can associate with the ζζ homodimer as ζζ was required for its surface expression. Therefore, the finding that ζζ does not immunoprecipitate with cysteine-less TCR is because the complex is not stable after detergent extraction. However, in our approach, cysteine crosslinking will be induced while the TCR complexes are still intact resident in the ER membrane prior to detergent extraction, and therefore any crosslinks identified will occur in TCR complexes that are permissive to ζζ association. Thus, we proceeded to generate a TM cysteine mutant library on TCR chains in which the native CP cysteine residues are absent.
Figure 3.3: Cell surface expression of the cysteine-less TCR constructs. The mouse OT-I TCR was encoded in a TCRβ-2A-TCRα cassette and cloned into a retroviral construct using pHAGE-IRES-ZsGreen vector. WT and cysteine-less versions of the OT-I TCR were transduced into a TCRα, TCRβ, CD3δ and ζ deficient BW5147 murine thymoma line, which had been stably reconstituted with either CD3δ chain only (-ζ) or both CD3δ and ζ chains (+ζ). ZsGreen+ cells were stained PE-Cy7-conjugated anti-mouse TCRβ antibody (clone H57-597) and assessed for surface TCR expression by flow cytometry.
3.1.3 Design of the TM cysteine mutant library

Our cysteine mutant library consists of 26 of TCRα and 27 of TCRβ mutants (Fig 3.4). Cysteine substitutions were introduced for every position in the TM domains as predicted by the TMHMM prediction tool. We also included approximately 3 to 5 amino acid residues on N- and C-terminal juxtamembrane regions. Because our initial aim was to probe TM-TM relationships between the TCRα and TCRβ chains within the intact TCR complex, we excluded from cysteine substitution all TM polar amino acid residues that govern assembly of the signal-transducing subunits within the TCR complex. These are the arginine 27 (R27) and lysine 32 (K32) of the TCRα chain and lysine 25 (K25) of the TCRβ chain.

![Diagram of TCRα and TCRβ cysteine mutant library]

*Figure 3.4: The TCRαβ TM cysteine mutant library.* Shown above are the sequences of human TCRα and TCRβ connecting peptide (CP), predicted transmembrane domain (TM) and cytosolic domain. Asterisks (*) indicate positions where single-cysteine substitutions were made for TCRα-to-TCRβ cysteine crosslinking screening. The critical basic residues (bolded and highlighted in blue) were not mutated.
3.1.4 Cysteine crosslinking screen between TCRα and TCRβ TM domains

Interrogating proximity of every single TCRα TM cysteine mutant against all of the TCRβ TM cysteine mutants would be laborious and unnecessary as residues at very different depths in the membrane are unlikely to interact. Instead, we strategically screened single mutants of one TCR chain against at least four mutants of the other TCR chain, to scan approximately one helical turn. This strategy interrogated 104 combinations of TCRα/TCRβ cysteine mutant pairs. In each crosslinking reaction, a pair of TCRα/TCRβ cysteine mutants was co-translated and assembled in the IVT system, along with the signal-transducing subunits. To facilitate disulphide bond formation in the non-aqueous membrane environment, I treated each assembly-completed reaction with the membrane-penetrating oxidant, copper (II) phenanthroline (CuPhe). Our readout for a successful TM crosslink between the TCRα and TCRβ chains was the formation disulphide-linked TCRαβ heterodimer product. Because the CD3ε and CD3δ subunits each contain a single native cysteine residues (εC117 and δC103) in their TM domains, TCRα or TCRβ cysteine substituted positions that form interface with these signal-transducing subunits could also recover crosslinks in the SDS-PAGE analysis. However, these crosslinks can be distinguished from those of TCRα-to-TCRβ due to their difference in molecular weight.

In the primary cysteine crosslinking screen, I subjected all digitonin-extracted reaction to OKT3 IP, enabling the recovery of potential TCRα-to-TCRβ crosslinks that are compatible with at least one of the folded dimeric CD3δε and CD3γε modules. I identified several TCRα/TCRβ cysteine mutant combinations that resulted in the recovery of TCRαβ crosslinked products (see appendix 2). I selected 21 combinations from this screen to present on a summary panel based on the ability to recover (1) TCRα-to-TCRβ crosslinks and (2) TCRα or TCRβ crosslinked to CD3δ or CD3ε subunits.

In addition to the OKT3 antibody IP analysis, the assembled and digitonin-extracted TCR complexes were also analysed by hexamer (CD3δε-TCRαβ-CD3γε) selection (Fig 3.5C). Hexamer selection was performed for two specific reasons. First, IP analysis with the OKT3 antibody can precipitate intermediates of the TCR complex that form during the assembly process in the ER membrane, and we wanted to confirm
that the identified TCRα-to-TCRβ crosslinks were compatible with complete TCR complex assembly. Secondly, while it would have been ideal to perform this IP with anti-ζ antibody (because the ζ subunit is the last subunit to join the TCR complex), our earlier observation indicated that association of the ζ subunit into the TCR complex containing CP-cysteine substituted TCRα/TCRβ combinations was sensitive to detergent extraction.

In both OKT3 and hexamer selection IP, I identified 17 TCRα/TCRβ cysteine mutant pairs that resulted in recovery of assembled TCR complexes containing crosslinked products (Fig 3.5B and C, marked with *). These crosslinks were more abundant at the N- and C-terminal end of the predicted TCRα and TCRβ TM domains, which might indicate that these regions are flexible and possibly unstructured. Importantly, crosslinks that formed between T16-A26 positions of the TCRα subunit and V23-A34 regions of the TCRβ subunit within the predicted TM domains displayed helical periodicity in their crosslinking pattern, spaced 3-4 residues apart. This pattern of crosslinks is expected between helical TM domains and suggests a discrete stable structure is formed between the TCRα and TCRβ TM domains. Crosslinks within the helical TM domains will be the most useful crosslinks to use to model the TCRαβ TM interface.

Four of the TCRα/TCRβ cysteine mutant combinations that we included in the summary panel resulted in the recovery of crosslinked products that have lower MW than the crosslinked-TCRαβ product in the SDS-PAGE analysis (Fig 3.5. marked with #). I speculated that these crosslinked products are formed between the TCR cysteine mutants and the native TM cysteines in either CD3ε or CD3δ subunits. The native cysteine in the CD3ε subunits is located towards the middle of the TM domain, whereas the native cysteine in the CD3δ subunit is located towards the C-terminal end of the TM domain. Thus, as the residues in the C-terminal end of the TCRα and TCRβ TM domain were involved in these crosslinks, the lower molecular weight products most likely represent TCRα-to-CD3δ and TCRβ-to-CD3δ crosslinks.
Figure 3.5: A selected panel of TCRα/TCRβ cysteine mutant pairs from the primary TCRα-to-TCRβ cysteine crosslinking screen. (A) summary of observed TCRα-TCRβ TM crosslinks indicated by solid lines between cysteine-substituted positions. (B) IVT assembly assay and CuPhe-mediated cysteine crosslinking of the WT TCR, cysteine-less TCR and selected TCRα/TCRβ TM cysteine mutant combinations analysed with OKT3 IP. (C) The same assembly reactions from B were re-analysed with snIP targeting CD3δPC followed by CD3γFLAG to isolate TCR complexes that contained both CD3 heterodimers. The precipitated TCR complexes from both analyses were separated with the non-reducing SDS-PAGE, transferred onto PVDF membrane, exposed to phosphor screen to detect ³⁵S isotope radioactivity. Crosslink-positive combinations are marked with (*). TCRα/TCRβ combinations that resulted in crosslinks to other receptor subunits are indicated with (#). A combination that displayed weak crosslinking after OKT3 IP, but was found to be incompatible with TCR assembly is shown by “^”. The FxE crosslink that uniquely associates with the ζζ homodimer is highlighted in red.
Interestingly, although the ability to co-IP the ζζ homodimer within TM crosslinked cysteine-less TCR complexes was compromised in most combinations, I found that a single TCRα/TCRβ cysteine mutant pair, αF26CxβE20C (termed “FxE” hereafter) that resulted in TCRα-to-TCRβ crosslink that restored ζζ co-IP. This data argues that the FxE crosslink has recapitulated a TM conformation that is similar to that of the WT TM conformation and permissive for native-like ζζ association. In addition to the FxE crosslink, the αF26C cysteine could also crosslink with two other neighbouring TCRβ cysteine mutants; βY19C and βI21C mutants (FxY and FxI, respectively), but neither of these restored ζζ co-IP. Out of these three crosslinks, the FxI was the weakest crosslink in the primary screen (see Appendix 2, marked ^), but this crosslink could not be reproduced in the summary panel analysis (Fig 3.5B, marked ^). Importantly, hexamer selection IP analysis of the FxI TCR complex resulted in a complete loss of TCR complex recovery, suggesting incompatibility crosslinking at this position with the hexameric TCR complex assembly and that the previously observed crosslink was likely to have been derived from a partially assembled product.

3.1.5 Characterising the FxE TM crosslinks

The FxE crosslink uniquely restored native-like ζζ homodimer association to the TCR complex after IP analysis in both OKT3 and hexamer IP strategies. First, I confirmed that ζζ association was dependent on the crosslink being formed, and not simply a consequence of mutating both αF26 and βE20 to cysteines. I demonstrated that co-IP of ζζ with the FxE heterodimer only occurred when the membrane penetrating oxidant CuPhe was added to the IVT assay, indicating the crosslink was indeed what had restored the native-like ζζ association (Fig 3.6A, compare lanes 3 and 4). Detergent-stable association of ζζ suggests that the FxE crosslink mimics the native structure and therefore should be able to be formed at the same time as the native CP crosslink. I designed an experiment in which this could be tested. To this end, I introduced the αF26C and βE20C mutations onto the WT TCR sequence (FxEWT) and repeated the cysteine crosslinking (Fig 3.6B). The polar reducing agent tri(2-carboxyethyl)phosphine (TCEP) was used to selectively reduce only disulphide bonds that are solvent-exposed (i.e. extracellular domains), while leaving the micelle-embedded TM disulphide bond intact. Because our protocol necessitates TCEP
treatment during the IP step, an alternative non-antibody-based pull-down was performed using streptavidin to target the SBP tag on the TCRβ subunit. In this analysis, we observed disulphide-crosslinked TCRαβ heterodimer in the WT TCR complex assembly was completely reduced upon TCEP treatment (Fig 3.6B; lane 4), while recovery of the FxE-crosslinked product was unchanged between untreated or TCEP-treated samples (Fig 3.6B; compare lanes 2 and 5), thus validating the selective reducing property of the TCEP. I found that FxE crosslink was detected with similar efficiency whether the native disulphide was present (FxEWT) or absent (FxE) (Fig 3.6B; comparing lanes 5 and 6), suggesting that the presence of the native CP disulphide bond does not interfere with the propensity of the FxE crosslink to form.

Taken together, these data show that assembly of an intact ζζ-containing TCR complex requires both close association of TCRα and TCRβ TM domains, presumably stabilised by the extracellular CP crosslink in the WT TCR, and a specific TM conformation that is uniquely replicated by the FxE TM crosslink in the cysteine-less TCR.

3.1.6 Summary of the TCRα-to-TCRβ cysteine crosslinking screen

In summary, identification of crosslinking between the TCRα and TCRβ TM domains indicated that their TM domains interact. Because our cysteine crosslinking screen only identified a small number of TCRα-TCRβ crosslinks that fall within the predicted TM domains and display an approximately helical periodicity in their crosslinking pattern, we believed that the TCRα and TCRβ TM domains must assemble through a specific structure. We can therefore determine the structure of the interacting TCRα and TCRβ TM domains and understand features of their interaction that may be responsible for governing the TCRαβ heterodimer assembly within the intact TCR complex. To this end, we considered NMR spectroscopy and molecular modelling approaches.
Figure 3.6: The αF26C x βE20C TM crosslink requires CuPhe to form and is required for ζζ homodimer association. (A) IVT assembly assay of the WT TCR, cysteine-less TCR and FxE TCR TM cysteine mutant combination. The completed assembly reactions were either treated with CuPhe (CuPhe +) or untreated (CuPhe -). These assembly reactions were analysed by snIP (CD3δPC to CD3γFLAG) to isolate hexameric assemblies. (B) Comparison of the αF26C x βE20C TM crosslinking in the presence and absence of the native CP disulphide bond. WT, FxE (CP cysteine-less background) and FxEWT (intact CP cysteines) TCR complex were assembled and treated with CuPhe. The resultant TCR complexes were immunoprecipitated with streptavidin beads targeting the TCRβSBP chain. The final wash step of the IP was performed with (+) or without (-) TCEP in the IP buffer to selectively reduce extracellular but not TM disulphide bonds.
3.1.7 Preliminary study for atomic resolution structure determination of the TCRαβ TM domains

Having identified that the TCRα and TCRβ TM domains interact through a specific interface, we explored the possibility of utilising solution NMR spectroscopy to determine the three-dimensional (3D) structure of TCRαβ TM domains. Solution NMR spectroscopy has been used successfully to elucidate the structures of a number of transmembrane proteins (Call and Chou, 2010). Additionally, this method will allow us to obtain preliminary secondary structure information on individual TM peptides, which will aid molecular modelling (see section 3.1.8). The major steps involved in determining a membrane protein structure via NMR are (1) expression and purification of ^15^N stable-isotope labelled protein of interest, (2) identification of membrane-mimicking conditions that support the membrane protein solubilisation, (3) obtaining high-quality 2D ^1^H-^15^N heteronuclear single quantum correlation (HSQC) spectra (4) data collection from triple-resonance experiments for secondary structure calculation and ^15^N- and ^13^C- NOESY experiments for distance restraints acquisition and (5) structure calculation using computational tools that incorporate above-mentioned data.

3.1.7.1 TCRα and TCRβ peptides design, expression and purification

TCRα and TCRβ TM domain peptides for study by solution NMR spectroscopy were expressed and purified based on a previously published protocol (Sharma et al., 2013). Our TCRα peptide construct (TCRα CPTM peptide) contained all amino acid residues from the native cysteine in the extracellular CP regions to the native C-terminus of the full-length protein. For TCRβ CPTM peptide, we truncated the last six residues to limit the number of unstructured residues in the C-terminus. This resulted in TCRα and TCRβ CPTM peptides that were 47 and 40 amino acid long, respectively. DNA encoding these peptides was cloned into a protein expression plasmid and expressed as a C-terminal fusion to the 9x histidine tagged-trpLE sequence (Fig 3.7A). The trpLE fusion directs expressed protein to inclusion bodies and allows \textit{E.coli} expression of these very hydrophobic peptides, which would be otherwise toxic to cells. The fusion protein can be purified from solubilised inclusion bodies using Ni-NTA chromatography. I introduced a unique methionine residue between the TCR CPTM
peptide and the trpLE fusion protein that allows digestion with cyanogen bromide (CNBr) treatment (Fig 3.7A). To aid in production and purification, I made several amino acid substitutions to the peptide sequences: (1) N-terminal cysteines were substituted to serine to prevent homo-oligomerisation, (2) the central lysine residues that make contacts to the signal-transducing subunits in intact TCR complex assembly were mutated to valine to prevent peptide instability in detergent micelles, and (3) one native methionine each in TCRα CPTM and TCRβ CPTM was mutated to valine to eliminate secondary CNBr cleavage sites.

I successfully induced expression of both fusion constructs in E.coli (Fig 3.7B; left panel). After inclusion body extraction and Ni-NTA chromatography, I eluted the fusion proteins in 100% formic acid (Fig 3.7B; right panel, CNBr-). We tracked the efficiency of CNBr digestion by comparing the intensity of coomassie blue staining of the fusion protein compared with the newly observed protein band corresponding to free trpLE (Fig 3.7B; right panel, CNBr+). Hydrophobic peptides tend to stain poorly with coomassie blue, but we did observe appearance of diffuse low molecular weight bands corresponding to the TCRα CPTM and TCRβ CPTM peptides (Fig 3.7B; right panel, marked *) We, then separated these CPTM peptides from uncleaved fusion proteins and free trpLE products in a single reverse-phase HPLC step (Fig 3.7C). The TCRα CPTM and TCRβ CPTM peptides were eluted towards the end of the hydrophobicity gradient, consistent with their hydrophobic nature. These peptides were then lyophilised and subsequently used in solution NMR experiments.
Figure 3.7: TCRα and TCRβ peptides design, expression and purification. (A) 47 and 40 amino acids-long peptides corresponding to the CP and predicted TM domains of the TCRα (TCRα-CPTM) and TCRβ (TCRβ-CPTM) chains, respectively, were designed as C-terminal fusion protein to the trpLE fragment for inclusion body expression in E. coli. (B) SDS-PAGE/coomassie staining analyses of (left) IPTG induced expression and (right) cyanogen bromide digestion of 15N stable-isotope labelled TCRα-CPTM and TCRβ-CPTM peptides. (C) RP-HPLC separation of the CNBr-digested TCRα-CPTM and TCRβ-CPTM peptides on C3 PrepHT column with linear gradient from 100% buffer A (60% H2O + 40% acetonitrile + 0.3% TFE) to 100% buffer B (75% 2-Propanol + 25% acetonitrile + 0.3% TFE).
3.1.7.2 Determination of secondary structure of the TCRα and TCRβ CP-TM peptides by solution NMR spectroscopy

Preliminary NMR analysis of $^{15}\text{N}$-labelled peptides in 250mM lyso-myristolphosphatidylglycerol (LMPG) micelles yielded excellent $^1\text{H}^{-^{15}\text{N}}$ heteronuclear single-quantum correlation (HSQC) spectra and the NMR samples were stable over at least a week at room temperature. I prepared NMR samples for the $^2\text{H}^{-^{15}\text{N}^{-^{13}\text{C}}}$-triple labelled TCRα and TCRβ CPTM peptides to assign HSQC peaks to amino acids in the peptide sequence, which is required to determine secondary structure. I recorded a standard suite of TROSY-triple resonance experiments for backbone assignment, namely TROSY-HNCA, -HN(CO)CA and –HNCACB. The well-dispersed $^1\text{H}^{-^{15}\text{N}}$ HSQC spectra from this experiment indicated properly folded peptides (Fig 3.8). Sequential backbone assignments were made using the computer aided resonance assignment (CARA) software package (Fig 3.8). Full backbone resonance assignments were obtained for residues V3→S47 of the TCRα-CPTM peptide and F3→A40 of the TCRβ-CPTM peptide. I then analysed the secondary backbone chemical shift information that was obtained from triple-resonance experiments in the TALOS+ web server, a computational tool that provides prediction of secondary structure of a given protein sequence based on NMR-determined data (Fig 3.9). Using TALOS+ tool, the region of the TCRα and TCRβ CPTM peptides that adopt α-helical structure was determined (Fig 3.9).

The TCRα and TCRβ CPTM regions are crosslinked in the full-length proteins through the native CP cysteine residues. Thus, to recapitulate the physiological interaction between these two peptides, I envisioned to express and purify the native CP cysteine-containing CPTM peptides of the TCRα and TCRβ subunits and crosslink these peptides. Initial experiments have determined that inclusion of the native cysteines in the CP region complicates peptide purification and significant challenges will need to be overcome to make the appropriate samples required for structure determination. Nevertheless, the secondary structural information obtained here was valuable and utilised in molecular dynamics (MD) simulations to model the TM structure of the TCRαβ heterodimer (see section 3.1.8).
Figure 3.8: Solution NMR spectroscopy analysis of the TCRα and TCRβ CPTM peptides. (A) Peptide sequences of TCRα-CPTM and TCRβ-CPTM used in the solution NMR spectroscopy experiments. The $^{1}H$-$^{15}N$ HSQC spectra of the $^{15}N$-, $^{13}C$-, and $^2H$ (70%)-labelled of the (B) TCRα-CPTM and (C) TCRβ-CPTM peptides. 0.5mM peptides were reconstituted in 250mM LMPG and 20mM phosphate buffer (pH6.8). $^{1}H$-$^{15}N$ HSQC spectra were recorded at 600mHz $^1H$ frequency and 35°C. The residues assignment for each peak is labelled on the spectra.
Figure 3.9: TALOS+ prediction of TM helix limits for TCRα and TCRβ CPTM peptides. Identification of helical regions of the (A) TCRα-CPTM and (B) TCRβ-CPTM peptides from TALOS+ analysis. Confidence scores of helical secondary structure or unstructured (coil) are plotted on the x-axis. Residues marked with asterisks (*) were substituted to facilitate production of the peptides and NMR analysis: C→S to block homodimerisation of these peptides during purification; K→V to stabilise unassembled TM peptides in lipid micelles; M→V to avoid internal cleavage by cyanogen bromide.
3.1.8 Modelling TCRαβ TM structure using molecular dynamics (MD) simulations

The inter-helical crosslinks from cysteine scanning experiments and secondary structure information from the NMR-derived TALOS+ predictions presented us with an excellent opportunity to interrogate the TCRαβ TM structure using molecular dynamic (MD) simulation methods. To this end, we engaged collaborators from Lehigh University, USA to perform replica exchange MD (REMD) simulations. In general, the REMD simulation allows multiple replicas of a single simulation at multiple temperatures to be performed to avoid trapping of all structures in the simulations in a local energy minima that does not represent the physiological structure. Additionally, structures from different replicas are randomly exchanged between replicas to experience a new environment throughout the simulation. To model TCRαβ TM structure, we asked our collaborators to model the FxE crosslinked TCRαβ heterodimer that resulted in WT-like TCR complex assembly in the IVT assay.

3.1.8.1 Structure of the FxE disulphide-bonded TCRαβ TM heterodimer

The core TM helical domains of TCRα and TCRβ subunits, as determined by the solution NMR spectroscopy analysis described earlier, were modelled in an implicit membrane bilayer model (Im et al., 2003) and a disulphide bond was installed between positions α26 and β20. The modelling was performed as a 10ns REMD simulation of 32 replicas (see methods for details). We surveyed tens of thousands of structures obtained from the simulation. To analyse these structures, a cluster analysis was performed, whereby 500 structures were randomly chosen and binned based on RMSD (i.e into clusters of a more similar structure). The analysis identified a single dominant cluster that contains 469 of 500 structures, indicating that a single unique TM packing orientation was consistent with the crosslinked product (Figure 3.10B). The centroid structure representing this cluster revealed a left-handed coiled-coil with contacts along the entire length of the two helices. Several features of this structure indicated that it faithfully represents the TCRαβ TM interface identified in our initial cysteine
crosslinking screen. First, we observed that the CD3-interacting polar residues are all facing outward, away from the TCRαβ interface (Figure 3.10C).

![Figure 3.10: Structure of the TCRαβ TM interface within the receptor complex.](image)

(A) Protein sequences of the TM domains of the TCRα and TCRβ chains. αF26C and βE20C positions were disulphide bonded (orange connecting line) during the simulation (B) The centroid structure from the major cluster in a 10ns REMD simulation of the FxE crosslinked TCRαβ TM heterodimer in an implicit lipid bilayer model. The TCRα TM domain is coloured in turquoise and the TCRβ TM domain is coloured in orange. (C) Top-down view of the structure shown in B. The basic residues that recruit the dimeric signal-transducing subunits are labelled in blue, highlighting the exclusion of these residues away from the interface formed by the TCRα and TCRβ TM domains.
Secondly, while we utilised only the FxE crosslink in this simulation, we could map all the other positions that resulted in crosslinked products in our initial screen to the simulated TCRαβ interface (Figure 3.11). We therefore asked whether a structure could be determined that included all the measured crosslinks and whether it would be the same or different to the structure found using the FxE crosslink (see below).

**Figure 3.11:** TM positions that resulted in TCRα-TCRβ crosslinks in the cysteine crosslinking screen can be mapped to the TCRαβ TM interface. (A) Protein sequences of the TCRα and TCRβ chains with the TM segments indicated. TCRα-TCRβ TM crosslinks that were identified in the cysteine crosslinking assay are indicated by the connecting lines between cysteine-substituted positions. (B) The FxE crosslinked TCRαβ TM structure from Fig 3.12 (Turquoise for the TCRα TM domain and orange for the TCRβ TM domain). The FxE disulphide bond is labelled in orange and the critical basic residues that recruit the dimeric signal-transducing subunits are labelled in blue. The native TM positions that resulted in TCRα-TCRβ crosslinks when substituted to cysteine in the cysteine crosslinking screen are shown in magenta.
3.1.8.2 Structure of the TCRαβ heterodimer TM domain determined with distance restraints

The second simulation strategy was aimed at obtaining the TCRαβ TM structure that would satisfy all the inter-helical crosslinks. In this simulation, we defined the crosslinking positions as distance restraints rather than as an actual disulphide bond. This process is similar to NMR structure determination using nuclear Overhauser enhancement (NOE)-derived distances. We set the distance restraint between the Cβ atoms of the crosslinking positions to be between 3.7 and 6.0Å. There are eight crosslinks that lie within the helical structure of the TCRα and TCRβ TM domains. We obtained a similar structure for “distance-restrained” TCRαβ TM domains and the two structures overlaid extremely well with a RMSD value of only 0.5Å (Figure 3.12).

![Figure 3.12: Overlay of the FxE and “distance-restrained” TCRαβ TM structures.](image)

(A) The FxE crosslinked TCRαβ TM structure from Fig 3.12. (B) The TCRαβ TM structure obtained from the “distance-restrained” simulation (C) The aligned centroid structures from the FxE disulphide bond simulation (Turquoise for the TCRα TM domain and orange for the TCRβ TM domain) and the distance-restrained simulation (gray). The backbone RMSD between these two structures is 0.5Å.
3.1.8.3 A 200ns unrestrained MD simulation of the TCRαβ TM structure

Because both simulation strategies utilised restraints for structure determination, we asked whether the structure would be stable without these restraints. This prompted us to undertake an unrestrained simulation of the TCRαβ TM structure that we obtained from the restrained simulation. The rationale for this approach is that if a stable structure has been determined, it would be maintained upon removal of all restraints. Conversely, the structure will collapse in the simulation if it has been forced into high-energy conformation. In this simulation, we moved away from the implicit membrane environment and instead inserted the interacting TM structure into an atomistic model of a POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine) bilayer, which is more realistic, but computationally “expensive”. The unrestrained simulation was performed for 200ns and RMSD deviations from the starting structure throughout the simulation were recorded (Fig 3.13). There was minimal change in the backbone of the structure as judged by Cα RMSD (Fig 3.13C). We also specifically measured the distances between the Cβ atoms of the eight positions that were previously used as distance restraints. The RMSD between these positions deviated only 1-2Å from the starting Cβ-Cβ distance, except for the most N-terminal crosslink (αS22-βA15) (Fig 3.13B). This simulation data suggests that the TCRαβ TM domains structure we have determined is stable in the membrane, with potential structural flexibility in the N-terminus of the TM domain structure.
Figure 3.1: Stability of the TCRαβ TM interface in a lipid bilayer. The TCRαβ TM centroid structure from the distance-restrained REMD simulations was placed in explicitly modelled POPC bilayer and subjected to a 200ns MD simulation in which distance restraints were removed. (A) Protein sequences of the TCRα and TCRβ chains with the TM segments indicated and the inter-helical crosslinks identified from the cysteine crosslinking assay shown. Summary of the TCRα-TCRβ TM crosslinks used for the distance-restrained TM structure simulation are indicated by solid lines (orange). (B) Cβ atoms distances between the original restrained positions over the 200ns simulation re plotted. Each plot is colour-coded to the lines connecting crosslinked positions in the sequence above (A). (C) RMSD for the unrestrained TCRαβ TM structure over the course of the simulation measured by the distance between all Cα atoms.
3.1.9 Identification of a putative hydrogen bond network in the C-terminal TCRαβ TM interface

In the 200ns-simulation, the entire TCRαβ TM structure adopts a stable structure. While we did observe small structural variations at the N-terminal end of the structure, this was not the case for the C-terminal half of the structure. Upon closer inspection, we found a polar network within the interface of the two interacting helices that could account for enhanced stability at the C-terminus of the TM domain structure (Figure 3.14A). Two hydrogen bonds were formed within this network: (1) between asparagine 37 (N37) and the backbone carbonyl oxygen atom of alanine 26 (A26) of the TCRβ chain, and (2) between threonine 41 (T41) of the TCRα chain and tyrosine 29 (Y29) of the TCRβ chain. This polar interface maintained consistent hydrogen-bonding distances throughout the simulation. Interestingly, despite the TCRα and TCRβ TM domains forming an interface throughout its length, I have never recovered any crosslinks within the C-terminal helical portion of the TCRαβ TM domains (see Fig 3.6). Crosslinks are unlikely to be found between residues that are critical to forming the interface and the absence of crosslinks in this region suggested that mutation of the residues in the polar network might be important to TCR complex structure.
Figure 3.14: Identification of a putative hydrogen bond network in the C-terminal TCRαβ TM interface. (A) Close-up view of the polar network formed by αN37, αT41 and βY29 at the C-terminal end of the TCRαβ TM domain. (B) Protein sequences of the TCRα and TCRβ chains summarising the observed TCRα-TCRβ TM crosslinks from the cysteine crosslinking screen indicated by solid lines (orange) between cysteine-substituted positions. The blue oval indicates an area in the TCRα-TCRβ TM where crosslinks were not detected. Embedded in this area are αN37, αT41 and βY29, which are indicated in red.
3.1.10 Evolutionary conservation analysis

TM polar residues have already been shown to have important roles for receptor complex assembly (Call and Wucherpfennig, 2005). To gain insight into the roles of the polar residues of the TCRαβ TM interface, I first wanted to know whether these polar residues are a shared feature of TCR sequences from different species. An evolutionary conservation analysis was performed by comparing more than 30 vertebrate species that have recognisable TCR genes and aligning their TM sequences. Related TCRδ and TCRγ subunits (32 and 28 sequences, respectively) were also included in the analysis. As reported previously, the key TCRα and TCRβ TM residues that contact and recruit the signal-transducing subunits into the TCR complex (αR27, αK32, and βK25) are absolutely conserved (Fig 3.15A and B). Additionally, the βY19 residue that was previously implicated in assembly with ζζ (Fuller-Espie et al., 1998, Kunjibettu et al., 2001), was completely conserved (Fig 3.15B). More importantly, the polar residues of the TCRαβ TM interface identified in this study are also completely conserved across the evolution of the TCRαβ and TCRγδ systems. This finding suggests that the C-terminal region of the TCRαβ TM interface plays an important role in TCR structure and/or function.
Figure 3.15: Evolutionary conservation analysis of the TCRα and TCRβ TM sequences. Sequence logos illustrate the degree of amino acid conservation (represented by the height of each letter on the sequence logo) within TM sequences based on (A) 65 sequences of the TCRα/TCRδ vertebrate chains and (B) 66 sequences of the TCRβ/TCRγ vertebrate sequences. The sequences of human (A) TCRα and (B) TCRβ are shown below sequence logos for reference. Colours represent basic (blue), acidic (red), hydroxyl/thiol-containing (yellow), carboxamide (purple), small (green), and aromatic/hydrophobic (black) amino acids.
3.1.11 Mutagenesis of the TCRα and TCRβ interface

To further our investigation into potential roles of the polar residues of the TCRαβ TM interface, I asked whether mutations in this region would have an effect in TCR complex assembly. To this end, I designed two panels of mutants and assembled these mutants along with all CD3 subunits in the IVT assay. In the first panel of mutants, I designed mutations that would cause steric hindrance to the TM interface at the C-terminal region, and were thus predicted to alter the structure or stability of the interface. The N37 position of the TCRα chain was substituted to leucine and phenylalanine (N37L and N37F), which have large hydrophobic side chains. I also designed a TCRβ mutant, V33F, to introduce steric hindrance because of its positioning within the TCRαβ TM interface and proximity to the polar network. The second panel was of mutants designed to eliminate the hydrogen bond-forming capability of the conserved TCRαβ TM interface residues αN37, αT41, and βY29 without causing additional steric constraints. I substituted these residues with alanine at N37 and T41 (N37A and T41A) and with phenylalanine at Y29 (Y29F). These mutants were cloned onto the CP-intact (WT) human A6 TCR and assembly of these mutants with all the CD3 components was measured with the IVT assay.

First, I examined mutant TCR chains for their ability to form TCRαβ heterodimers, analysed by TCRβ-SBP to TCRα-HA sequential with non-denaturing biotin elution. None of the mutants exhibited a significant defect in the formation of disulphide-linked TCRαβ heterodimers (Fig 3.16A and B). This finding is unsurprising because folding and dimerisation of the TCRαβ extracellular domains in structural studies have been done in the absence of their TM domains.

Strikingly, we observed large variations in the recovery of completely assembled TCR complexes. I analysed hexameric assembly by CD3δ-PC to CD3γ-FLAG sequential IP, with non-denaturing EDTA elution (Figure 3.16C and D). Assembly of αN37L and αN37F mutants resulted in severe reductions of complex recovery (13% and 1.4% of WT complex recovery, respectively). We also observed a significant loss of complex recovery in the βV33F-containing TCR complex assembly (38% of WT complex recovery). Interestingly, while the βV33 position is not highly conserved, we noted that phenylalanine never occurs at this position in our evolutionary
conservation analysis (see Fig 3.15B), consistent with evolutionary selection to allow close TM-TM contact within the C-terminal region. I found that assembly of αN37A tended towards lower complex recovery (average 55% of WT complex recovery), but did not reach statistical significance in the context of this analysis. Assembly of αT41A- and βY29F-containing TCR complex resulted in significant loss of the TCR complex recovery (38% and 44% of WT complex recovery, respectively). However, analysis of the TCR complex assembly that contains both αT41A and βY29F mutations resulted in TCR complex recovery similar to the WT control, suggesting that the assembly defect in the individual mutants was due to unpaired polar groups in the membrane rather than loss of the tyrosine-threonine hydrogen bond. Additionally, assembly of a mutant TCR complex, whereby all N-T-Y residues have been substituted to A-A-F (“3mut”) also resulted in TCR complex recovery equivalent to the WT control. Taken together, we concluded that the ability of the TCRαβ TM domains to adopt a closely packed structure impacts on their ability to productively assemble with the CD3δε and CD3γε dimer in the TCR complex.
Figure 3.16: Mutations in the C-terminal conserved TCRαβ TM interface impair TCR complex stability. WT or the indicated mutants TCR chains were assembled in the context of full TCR complex assembly in the IVT assay. The resultant TCR complexes were analysed using either (A; left) snIP targeting TCRββ SBP followed by TCRαHA or (C; left) hexamer selection snIP (CD3δPC→CD3γFLAG). The precipitated TCR complexes were processed for SDS-PAGE/35S-detection. (B and D) Quantitative analysis of four independent experiments (N=4) from densitometry data (A and C, respectively). Each plotted value represents the raw intensity of the TCRαβ band of the mutant, expressed as a percentage of WT in that experiment. Statistical analysis was performed as an ordinary one-way ANOVA uncorrected Fischer’s least significant difference test with single pooled variance. *P <0.05 **P < 0.01, ***P < 0.001, ****P < 0.0001.
3.1.12 Cell surface expression analysis of the TCRαβ interface mutants

Next, I tested whether the TCR mutants would display similar defects reflected in their surface expression in cells. I utilised the previously generated TCRαβ-deficient CD3δζ+ BW5147 cell line (see section 3.1.2.2) to establish T cell lines with retroviral transduction that carried OT-I TCR TM interface mutants. TCR mutant-carrying BW5147 cells were stained with an anti-TCRβ antibody (clone H57-597) and were analysed by flow cytometry. All cell lines stained for TCR (albeit at varying levels), suggesting all mutants had some capacity to assemble into TCR complexes and be expressed at the cell surface (Fig 3.17).

To assess the efficiency of TCR complex formation of each mutant, I measured the median fluorescent intensity (MFI) of anti-TCR staining for each of the cell line (Fig 3.17). I observed that all mutants had a lower level of anti-TCRβ staining when compared to the WT TCR control cell line. I found that increasingly more disruptive mutations within the TCRαβ TM interface had increasingly lower MFI, exemplified by the αN37L and αN37F-carrying cell lines. (Fig 3.17A). The TCRαβ TM interface mutants that were designed to remove hydrogen bonds at the C-terminal of the TCRαβ TM domains (αT41A, βY29F, αT41A-βY29F and 3mut), were also expressed at a lower level on the cell surface (Fig 3.17B). I also noticed that the pattern of cell surface expression of these mutants mirrored the pattern of TCR complex recovery observed in the IVT assay.
**Figure 3.17: Cell surface expression analysis of the TCRαβ interface mutants.** The mouse OT-I TCR was encoded in a TCRβ-2A-TCRα retroviral construct using the pMIG viral expression plasmid. WT and interface mutants of the OT-I TCR were transduced into a TCRα, TCRβ, CD3δ and ζ deficient BW5147 murine thymoma line that has been stably reconstituted with CD3δ, and ζ. The transduced cells were stained PE-Cy7-conjugated anti-mouse TCRβ antibody (clone H57-597) and assessed for surface TCR expression by flow cytometry. Level of cell surface expression is presented as percentage of PE-Cy7 median fluorescence intensity (MFI) for BW5147 cells expressing TM interface mutant TCRs that have been normalised to the WT TCR-transduced cells. Cell surface expression comparisons for interface disruptive mutants: N37L and N37F mutants are shown in (A) and cell surface expression comparison for polar network-removing mutants are shown in (A) for N37A mutants and in (B) for T41A, Y29F, T41A+Y29F and 3mut mutants. Data presented here were obtained from two independent transduction experiments (N=2). Error bars represent standard error of the mean (SEM). Statistical analysis was performed as an ordinary one-way ANOVA. *P <0.05 **P < 0.01, ***P < 0.001, ****P < 0.0001.
3.2 Summary

TM residues of the TCR complex play a critical role in governing TCR complex assembly in the ER membrane. Earlier studies have identified the critical contact points that facilitate recruitment of the signal-transducing subunits (CD3δ, CD3γ, CD3ε and ζ) to the TCRα and TCRβ chains (Call and Wucherpfennig, 2005). An important piece of information that was missing about TM-TM association among the subunits of the TCR complex was the relationship between TM domains of the TCRα and TCRβ chains. We hypothesised that a more complete picture of TCR complex assembly would be established if we could determine if and how TCRα and TCRβ TM domains interact.

In this study, we showed that TCRα and TCRβ chains do interact through their membrane-embedded domains and that this interaction occurs through a specific interface. We reported the identification of eight inter-helical crosslinks between TM domains of the TCRα and TCRβ chains. Converting these crosslinks into distance restraints, we determined the structure of the interacting TCRαβ TM domains using computational modelling. We performed two separate simulations in which; (1) TCRα and TCRβ TM domains were disulphide-crosslinked between α26 and β20 positions, and (2) the distances between the TCRα and TCRβ TM domains where we recovered the eight crosslinks were restrained. Both simulation strategies resulted in nearly identical structures.

From these structures, we noticed the presence of a hydrogen bond network within the C-terminal end of the TCRαβ TM domains. This network was formed by three polar residues (αN37, αT41 and βY29), which we showed to be absolutely conserved across the evolution of the TCR chains. In the IVT assay, I showed that alteration of the TCRαβ TM interface by mutagenesis resulted in loss of integrity of the digitonin-extracted TCR complex. In cells, I demonstrated that these TCRαβ interface-altering mutants can be surface-expressed in our TCR-transduced T cell lines, however, these mutants had lower surface expression levels (MFI of TCR staining), suggesting that assembly had been impaired.
Taken together, the findings presented in this chapter identified an interface between the TCRα and TCRβ TM domains that forms a structured hub for the octameric receptor complex within the membrane. The spatial organisation of the signal-transducing subunits around the TCRαβ TM structure can be inferred based on the locations of CD3δε-recruiting TCRα lysine, CD3γε-recruiting TCRβ lysine and ζζ-recruiting TCRα arginine residues. This model fixes the positions of the two CD3 dimers on opposite sides of the TCRαβ within an intimately associated eight-TM-helix bundle (Fig 3.18). The ζζ homodimer bridges and possibility contacts both CD3 dimeric modules as well as the TCRαβ, which may provide a possible explanation for the observation that the ζζ homodimer is the last to join the complex and “senses” a properly assembled hexameric TCR complex.

However, this model still has uncertainty with respect to the CD3 heterodimers. Is CD3δ or CD3ε closer to the ζζ homodimer in the CD3δε heterodimer when associated with the TCRα TM lysine? Is CD3γ or CD3ε closer to the ζζ homodimer in the CD3γε heterodimer? Answers to these questions require further experimentation. We believe that the way in which the CD3 heterodimers associate with TCRα and TCRβ matters. One example whereby specific positioning of the CD3 heterodimers within the TCR complex important is their functional proximity to the Lck-bearing co-receptor CD4 or CD8 molecules. Determination of the x-ray crystal structure of the extracellular domains of the TCR-peptide-MHCII-CD4 complex indicated a specific spatial organisation between the TCR complex and co-receptor while engaging the MHC:peptide complex (Yin et al., 2012) Because TCR and co-receptor are spatially constrained, it might be functionally critical which signal-transducing subunits has the closest access to the co-receptor bound Lck, hence more likely to be phosphorylated, a notion that has recently been addressed (Glassman et al., 2016).

In the future, extending the cysteine crosslinking screen to obtain TM-TM contact information for all TM domains of the TCR complex would aid in understanding the TCR complex architecture. Such effort has already been initiated and preliminary data are presented in chapter 4. Additionally, whether conformational changes within the TCRαβ TM interface are important in mediating signal transfer across the membrane upon TCR triggering will need to be explored further in functional assays.
Figure 3.18: Model of TM arrangement within the octameric TCR complex. Top-down view of the proposed spatial organisation of the TCR complex with the TM structure of the TCRαβ heterodimer in the middle (TCRα is coloured in turquoise and TCRβ is coloured in orange) and the signal transducing subunits are represented as gray circles. Placement of the signal-transducing subunits within the intact TCR complex are inferred from the positioning of the basic residues (shown in blue) on the TM structure of the TCRαβ heterodimer. Red crescents represent acidic residues involved in assembly with the TCR chains.
Chapter 4: Mapping subunit organisation within the TCR complex in the membrane

4.0 Introduction

pMHC binding to the extracellular domains of the TCR\(\alpha\) and TCR\(\beta\) chains causes the ITAMs of the signal-transducing subunits to become phosphorylated through an unknown mechanism. In the previous chapter, our cysteine crosslinking analysis of the TCR complex assembly revealed the existence of a specific interface between the TM domains of the TCR\(\alpha\) and TCR\(\beta\) subunits that could plausibly form part of a structured pathway for transmission of conformational changes between extracellular and intracellular receptor domains. Extending from this finding, I hypothesise that, upon antigen recognition, modification of the TCR\(\alpha\β\) TM interface could cause conformational alterations to the closely associated TM domains of the CD3 subunits.

High-resolution structures of the intact TCR complex will be important to understand how each TM domain within the TCR might communicate with others upon ligand binding. The TCR complex is one of the most complex membrane receptors, containing eight single pass TM proteins in four dimeric modules that are held together by a non-covalent polar network in the membrane, and as such no atomic-resolution structure of an intact TCR complex has been determined. In chapter 3, I utilised an intramembrane cysteine crosslinking screen to identify contact points between the TCR\(\alpha\) and TCR\(\beta\) subunits and determined the structure of TCR\(\alpha\β\) TM heterodimer. Based on this structure, I mapped a plausible organisation of the CD3\(\delta\varepsilon\), CD3\(\gamma\varepsilon\) and \(\zeta\zeta\) dimers (Fig 3.18). While this provides a preliminary model of TM-TM arrangement within the TCR complex, the precise positioning of the individual CD3 subunits remains unclear, and potentially interacting surfaces among CD3 and \(\zeta\) TM domains have yet to be identified. In this chapter, I will present cysteine crosslinking studies that have been completed between TCR\(\alpha\) and \(\zeta\), and TCR\(\beta\) and CD3\(\gamma\) TM domains to demonstrate significant progress toward obtaining crosslink data for every TM-TM helix interaction within the TCR complex that may in future be used to calculate models of the complete eight helix assembly.
**4.1 Results**

### 4.1.1 CD3 TM peptides construct design, expression and purification

Prior to undertaking the cysteine crosslinking screen, I first determined the TM helix limits within the CD3δ, CD3γ and CD3ε subunits using NMR spectroscopy, allowing me to select residues within the helices for cysteine scanning that are most informative for future modelling efforts. To this end, I cloned and expressed peptides corresponding to the predicted TM domains of each of the CD3δ, CD3γ and CD3ε chains in the *E.coli* expression system as described in chapter 3 and in our previous publication (Sharma et al., 2013). Modifications to the native protein sequence were made to aid the expression and purification of the peptides and are shown in Fig 4.1 (comparing WT and NMR peptide).

The His-tagged trpLE-CD3δTM and -CD3γTM fusion proteins were successfully expressed in the *E.coli* expression system (Figure 4.2A), purified on a nickel affinity column (Fig 4.2B, CNBr– lanes) and cleaved using cyanogen bromide (Fig 4.2B, CNBr+ lanes). The cleaved CD3δ and CD3γ TM peptides were then purified on RP-HPLC to isolate the peptide (Fig 4.2C and D, peak 3) from the uncleaved fusion proteins (Fig 4.2C and D, peak 2) and the free trpLE fragment (Fig 4.2C and D, peak 1). The CD3 TM peptides were eluted in the final peak of the RP-HPLC elution, which is consistent with the fact that they are the most hydrophobic products. The identity and purity of the peptides were confirmed by SDS-PAGE (Fig 4.2E and F). Each peptide was lyophilised and analysed by solution NMR spectroscopy (section 4.1.2).
Figure 4.1: CD3 peptide constructs used in solution NMR spectroscopy studies. NMR TM peptide sequences of human CD3δ (top), CD3γ (middle), and CD3ε (bottom). For each CD3 TM peptide, we designed a 36 amino acids long peptide, with sequences starting from the most C-terminal residue of the tetracysteine motif, RxCxxCxE (not shown). Mutations were made to cysteines and methionine residues to prevent dimerization and spurious cleavage during CNBr digestion, respectively, and are marked in yellow.
Figure 4.2: Expression and purification of CD3δ and CD3γ TM peptides. (A and B) SDS-PAGE/Coomassie staining analyses of *E. coli* expression (A) and cyanogen bromide digestion of 15N stable-isotope labelled CD3δ and CD3γ TM peptides. (A) IPTG induction of CD3δ and CD3γ visualised from *E. coli* lysates. (B) Ni-NTA purified (CNBr-) and CNBr digested (CNBr+) peptide were analysed by SDS-PAGE. RP-HPLC separation of CNBr-digested CD3δ (C) and CD3γ (D) TM peptides on C3 Semi-Prep column with linear gradient from 100% buffer A (60% H2O + 40% acetonitrile + 0.3% TFA) to 100% buffer B (75% 2-Propanol + 25% acetonitrile + 0.3% TFA). Peaks corresponding to each expected product in CNBr digest were collected for SDS-PAGE analysis (peak 1; trpLE, peak 2; trpLE-fusion, peak 3; peptide). The purity of each product in the (E) CD3δ and (F) CD3γ purification are shown.
Expression and purification of the CD3ε TM peptide was more challenging (Fig 4.3). Evidence for expression of the His-tagged trpLE-CD3εTM fusion protein could not be detected by Coomassie blue staining after SDS-PAGE (Figure 4.3A, comparing IPTG-/+ lanes). Because hydrophobic TM peptides often stain poorly with Coomassie blue and do not always resolve well by SDS-PAGE, I continued to perform the nickel column purification and subsequent cyanogen bromide digestion. SDS-PAGE analysis of the cyanogen bromide-digested sample showed the clear presence of liberated trpLE indicating that the CD3ε TM peptide had indeed been expressed (Fig 4.3A, comparing -/+ CNBr lanes). However, I again failed to observe the presence of a discrete band corresponding to the CD3ε TM peptide. I did however, observe the appearance of a higher molecular weight protein species, which could be aggregated form of the CD3ε TM peptide (Fig 4.3A, +CNBr, marked “? ”).

To obtain pure CD3ε TM peptide product, I then performed RP-HPLC (Fig 4.3B). Three peaks were successfully separated (Fig 4.3B) and the last two peaks (fraction 1 and 2) were assessed by SDS-PAGE (Fig 4.3C). Fraction 1 stained well with Coomassie blue and migrated at a molecular weight equivalent to the full-length trpLE-CD3εTM fusion protein product. SDS-PAGE analysis of Fraction 2 exhibited a slight hint of Coomassie blue staining of the CD3ε TM peptide (Fig 4.3C, labelled monomer). However, I still observed appearance of a high molecular weight smear in the SDS-PAGE analysis of this fraction, which may suggest aggregation (Fig 4.3C, fraction 2 on lanes 3 and 4). SDS sometimes can induce aggregation, which may not be present in the original sample. I lyophilised the peptide-containing peak and proceeded to analysis by solution NMR spectroscopy (section 4.1.3).
Figure 4.3: CD3ε TM peptide expression and purification. (A) SDS-PAGE/Coomassie blue staining analyses of expression and cyanogen bromide digestion of $^{15}$N stable-isotope labelled CD3ε TM peptides. The trpLE-CD3ε TM fusion proteins expression could not be detected after IPTG induction (IPTG+ lane). Inclusion bodies from *E. coli* contain trpLE- CD3ε expression constructs were nevertheless run over Ni-NTA. Protein that eluted from Ni-NTA is shown in CNBr- lanes. CNBr digest this eluate resulted in the appearance a protein band corresponding to the free trpLE fragment (CNBr+ lanes). (B) RP-HPLC separation of CNBr-digested CD3ε on C3 Semi-Prep column with linear gradient from 100% buffer A (60% H₂O + 40% acetonitrile + 0.3% TFA) to 100% buffer B (75% 2-Propanol + 25% acetonitrile + 0.3% TFA). Peaks corresponding to each expected product in CNBr digest were collected (peak 1; trpLE-fusion, peak 2; peptide). (C) The purity of each product in the CD3ε purification was assessed by SDS-PAGE/Coomassie blue staining analysis.
4.1.2 Solution NMR spectroscopy analysis of the CD3 peptides

Preliminary NMR analysis of $^{15}$N-labelled peptides in 250mM LMPG micelles yielded excellent $^1$H-$^{15}$N HSQC spectra for the CD3$\delta$ and CD3$\gamma$ TM peptides (data not shown). The NMR samples were stable over at least a week at room temperature. I therefore produced and prepared NMR samples for the $^2$H-$^{15}$N-$^{13}$C-triple labelled CD3$\delta$ and CD3$\gamma$ for triple resonance experiments. I recorded a standard suite of TROSY-triple resonance experiments for backbone assignment, namely TROSY-HNCA, -HN(CO)CA and –HNCACB. The well-dispersed $^1$H-$^{15}$N HSQC spectra for the CD3$\delta$ and CD3$\gamma$ TM peptides from this experiment indicated properly folded peptides (Fig 4.4). Sequential backbone assignments were made using the computer aided resonance assignment (CARA) software package (Fig 4.4). The full backbone resonance assignments were obtained for residues D3→L36 of the CD3$\delta$ TM peptide and L2→Q36 of the CD3$\gamma$ TM peptide. I used the TALOS+ web server and the secondary backbone chemical shift information to identify the helical limits of these peptides (Figure 4.5).
Figure 4.4: Analysis of CD3δ and CD3γ TM peptides by solution NMR spectroscopy. $^1$H-$^{15}$N HSQC spectra of 70% $^{15}$N-, $^{13}$C-, and $^1$H-labelled CD3δ (A) and CD3γ (B) TM peptides. 0.5mM peptides were reconstituted in 250mM LMPG and 20mM phosphate buffer (pH6.8). $^1$H-$^{15}$N HSQC spectra were recorded at a $^1$H frequency 600mHz and at 35°C. Full backbone resonance assignments were obtained for residues L2$\text{→}$L36 of the CD3δ peptide and L2$\text{→}$Q36 of the CD3γ peptide using standard triple-resonance experiments.
Figure 4.5: Secondary structure determination of CD3δ and CD3γ TM peptides. Identification of the helical regions of the (A) CD3δ and (B) CD3γ TM peptides from TALOS+ analysis based on backbone secondary chemical shift analysis obtained from triple-resonance NMR experiments (Figure 4.4). Plotted on the x-axis are the confidence scores for helical secondary structure (positive values) or random coil (negative values).
4.1.3 Optimisation of NMR sample preparation and conditions of the CD3ε TM peptide

In contrast, I was unable to obtain good quality NMR spectrum for the CD3ε TM peptide (Fig 4.6). I noticed the presence of additional chemical shifts that cannot be accounted for by the number of amino acids present in the peptide sequence. There were at least 52 chemical shift peaks compared to 36 amino acids in the peptide. The presence of twinned peaks in several places in the spectrum suggested the existence of at least two conformations, perhaps from a dimer or higher order oligomers. This observation is consistent with the initial observation that this peptide forms higher molecular weight oligomers when analysed by SDS-PAGE (Fig 4.3C).

Figure 4.6: $^1$H-$^{15}$N HSQC spectrum of the $^{15}$N -labelled of CD3ε TM peptide. 0.5mM peptide was reconstituted in 250mM LMPG and 20mM phosphate buffer (pH6.8). $^1$H-$^{15}$N HSQC spectrum was recorded at a $^1$H frequency 600mHz at 35°C.
To reduce aggregation of the CD3ε TM peptide, I applied several modifications to our typical NMR sample preparation conditions. First, I tried to promote monodispersity in the sample by including a refolding step in 8M urea buffer prior to reconstituting the peptide into LMPG micelles. I also reasoned that presence of basic residues in the C-terminal of the peptide (NRKAKA) and acidic residues in the N-terminal of the peptide could form N-to-C terminal (“head-to-tail”) oligomers. Thus, along with the urea re-folding step, I investigated how pH alters the behaviour of the NMR sample. I solubilised urea-refolded CD3ε TM peptide in LMPG-MES buffer (pH5.5) instead of our original LMPG-phosphate buffer (pH6.8). Unfortunately, NMR analysis of the CD3ε TM peptide sample indicated no improvement in the quality of the $^1$H-$^{15}$N HSQC spectrum (Figure 4.7).

$^1$H-$^{15}$N HSQC of CD3ε-TMD (optimizing sample prep)

![Figure 4.7: $^1$H-$^{15}$N HSQC spectrum of the $^{15}$N-labelled of the CD3ε TM peptide after Urea treatment.](image)

1.0mM CD3ε TM peptide in 500mM LMPG was refolded in 8M Urea, dialysed and lyophilised and redissolved in 20mM MES buffer (pH5.5; 50mM NaCl). $^1$H-$^{15}$N HSQC spectrum was recorded at a $^1$H frequency 600mHz at 35°C. The $^1$H-$^{15}$N HSQC spectrum of the refolded sample (Red) was overlaid with the $^1$H-$^{15}$N HSQC spectrum from figure 4.6 (Blue).
I also designed a shorter form of the protein sequence by truncating six C-terminal amino acids from the original construct (termed CD3ε\text{short} TM). Expression of the CD3ε\text{short} peptide was successful and could be seen by SDS-PAGE (Fig 4.8A). While inclusion body extraction, and nickel column affinity purification and cyanogen bromide digestion proceeded without incident, separation of the products by RP-HPLC was challenging. In the RP-HPLC analysis, we observed three peaks (fractions 1, 3 and 5). SDS-PAGE analysis of these fractions determined that fraction 1 contained free trpLE and fraction 3 most likely to contain the undigested fusion protein that appeared as smear on the SDS-PAGE (Fig 4.8B; HPLC fraction 1 and 3). The peak that most likely contains CD3ε\text{short} peptide eluted as a small peak (fraction 5), but also contained higher order aggregates by SDS-PAGE analysis (Fig 4.8B; HPLC fraction 5). The alternative expression, purification and NMR sample preparation strategies presented here did not improve CD3ε TM peptide analysis. Thorough optimisation of the RP-HPLC protocol to purify the CD3ε\text{short} peptide has not been completed yet and will be the future direction of this part of the project.

4.1.4 Summary of solution NMR spectroscopy experiments for the CD3 TM peptides

Here, I have initiated structural characterisation of the TM peptides of the CD3δ, CD3γ and CD3ε. I have completed secondary structure determination for the CD3δ and CD3γ TM peptides, whereas structural characterisation of the CD3ε TM peptide is still underway. The secondary structure information obtained here will be utilised in future for computational modelling of the entire TM assembly of the TCR complex. I have also utilised the information regarding the TM helix limits to guide our TM cysteine crosslinking study (section 4.1.5).
Figure 4.8: Expression and purification of the CD3ε_{short} TM peptide. SDS-PAGE/Coomassie staining analyses of (A) expression and (B) purification of the 15N stable-isotope labelled CD3ε_{short} TM peptides. (A) Expression of the trpLE-CD3ε TM fusion protein could not be clearly detected from E.colli lysates (IPTG+ lane), but inclusion bodies were nevertheless made for Ni-NTA chromatography. (B) Eluted protein from Ni-NTA chromatography ran as a smear (CNBr- lanes), but CNBr digest of the fusion proteins resulted in the appearance a protein band corresponding to the free trpLE fragment (CNBr+ lanes) indicating expression had been successful. (C) RP-HPLC separation of CNBr-digested CD3ε on C8 Semi-Prep column with linear gradient from 100% buffer A (60% H2O + 40% acetonitrile + 0.3% TFA) to 100% buffer B (90% 2-Propanol + 10% TFE + 0.3% TFA). Peak separation on RP-HPLC was suboptimal and collected fractions did not clearly resolve on SDS-PAGE (see panel B).
4.1.5 TM cysteine crosslinking between the TCR and signal-transducing subunits

From previous studies, it has been established that the interactions between the TM domains of the TCR complex are governed by intramembrane polar contacts. Here, we sought to determine what additional TM contact points exist between the TCR and the signal-transducing subunits in order to map complete interacting surfaces. In section 4.1.5.1, I will present the cysteine crosslinking screen between the TCRβ and CD3γ TM domains. Then, in section 4.1.5.2, I will present the data from targeted cysteine crosslinking screening between the TCRα and ζ subunits.

In chapter 3, the TCRα and TCRβ TM cysteine mutants were generated on the background where the conserved cysteine residues in the CP region were substituted to serine residues. These substitutions were necessary to enable detection of TM-crosslinked TCRαβ heterodimer recovery that served as the readout for the TCRα-to-TCRβ TM cysteine crosslinking screening. Considering that the TCR and the signal transducing subunits interact non-covalently in the membrane, the use of cysteine-less TCRα and TCRβ subunit variants here is not necessary. Thus, in this chapter, I used the WT human A6 TCRα and human A6 TCRβ sequences (with intact CP cysteine) to generate TM cysteine mutants of the TCRα and TCRβ subunits. New cysteine mutants were also generated in the invariant human CD3γ-PC (PC-tagged) and human ζ (no tag) sequences.

4.1.5.1 TM cysteine crosslinking between the TCRβ and CD3γ subunits

I generated a library of TM cysteine mutants, in which every TM residue of TCRβ and CD3γ were individually substituted to cysteine (Fig 4.9). To perform the screening in the context of full TCR complex assembly, the TM residues that have been shown to disrupt formation of the TCR complex were excluded from cysteine substitutions (Fig 4.9). For the TCRβ TM cysteine mutant library, the critical CD3γε dimer-interacting lysine (K25) residue was excluded from cysteine substitution. For the CD3γ TM cysteine mutant library, the critical TCRβ-interacting glutamic acid (E100) and serine (S103) residues were excluded from cysteine substitution as they have been shown to interact with TCRβ K25 (Call et al., 2010).
**Figure 4.9: TM cysteine mutant library of the TCRβ and CD3γ complex subunits.**

Shown are sequences for the TCRβ and CD3γ TM and juxtamembrane domains. Residues numbering is shown above the protein sequences. Underlined protein sequences are either experimentally-determined TM segment (TCRβ in chapter 3 and CD3γ in section 4.1.2 above). The residues that governs TCR complex assembly are coloured in blue (basic amino acids) and red (acidic amino acids). The TM serine residue in the CD3γ subunit, which also contributes to the TCR complex assembly is coloured in green. Bolded protein sequences are TM residues that were individually substituted to cysteine residue for the TCRβ-to-CD3γ cysteine crosslinking screen.
4.1.5.1.1 Primary cysteine crosslinking screen

The TM cysteine crosslinking screen between the TCRβ and CD3γ cysteine mutants were performed based on the protocol described in chapter 3. Individual TCRβ TM cysteine mutants were tested against 4-5 CD3γ TM cysteine mutants with similar depth in the membrane, covering approximately one helical turn. This resulted in interrogating 107 TCRβ/CD3γ cysteine combinations and these were assembled with all other subunits, CuPhe-treated and digitonin-extracted. The resulting TCR complexes were immunoprecipitated using the anti-CD3ε OKT3 antibody (Fig 4.10 A-H). In the primary screen, many TCRβ-to-CD3γ crosslinks were identified but most of them were weak and displayed no helical periodicity in the crosslinking pattern (Fig 4.10 A-H).

To directly compare these crosslinks, I determined the crosslinking efficiency value for every combination tested by taking a ratio between the crosslinked product and the TCRαβ heterodimer bands (Fig 4.11). Crosslinks were identified at both N-terminal and C-terminal juxtamembrane regions, suggesting that the TCRβ and CD3γ subunits interact throughout the length of their TM domains (Fig 4.10 and 4.11, A and H). At the C-terminal juxtamembrane region, crosslinks were recovered in almost all TCRβ/CD3γ combinations tested (Fig 4.10 and 4.11, panel G and H), suggesting a flexible and unstructured C-terminus, consistent with the NMR data (Fig 3.10; TCRβ CPTM and Fig 4.5; CD3γ TM).

Within the TM domains, I found that several crosslinks that were formed between neighbouring cysteine-substituted positions at the N-terminal end, and displayed no helical periodicity. These are the crosslinks that are contributed by the TCRβ cysteine mutants L18C, Y19C, I21C, L22C and L23C, and the CD3γ cysteine mutants G95C, F96C and A99C (Fig 4.10, panels A-D). In contrast, no crosslinks were identified at the C-terminal half of the TM domains (Fig 4.10 and 4.11, panels E and F).
(Fig 4.10) Continuing to the next page.
Continued from previous page.

Figure 4.10: TM cysteine crosslinking screen between the TCRβ and CD3γ TM domains after anti-CD3 IP. (A-H) Combinatorial cysteine crosslinking screening between the TCRβ and CD3γ TM cysteine mutants was performed. The assembled and CuPhe-treated TCR complexes were extracted into digitonin micelles, immunoprecipitated with the indicated IP strategy and visualised with 35S-detection after non-reducing SDS-PAGE analysis. Crosslink-positive combinations are marked with (*).
Figure 4.1: Quantitation of crosslinking efficiency from TM cysteine crosslinking screening between the TCRβ and CD3γ TM domains after anti-CD3 IP. (A-H) Crosslinking efficiency was calculated as a ratio of the intensities of the TCRαβ and TCRαβ-CD3γ bands. Crosslink-positive combinations that were selected for further analysis are marked with (*).
As this part of the screen was performed using OKT3 IP, it is possible some of the crosslinks identified are from assembly intermediates and are not compatible with the full TCR complex assembly. To determine which crosslinks could form in the context of the full TCR complex assembly, I chose 11 TCRβ/CD3γ crosslinking combinations based on the quantitative analysis in Fig 4.11 (Fig 4.10 and 4.11, marked *). A single strongest C-terminal juxtamembrane crosslink, βV37C-to-γI114C was also included as a positive control in the subsequent analysis.

4.1.5.1.2 Compatibility of the TCRβ-to-CD3γ crosslinks within the intact TCR complex

Next, I asked whether the selected TCRβ-to-CD3γ crosslinks identified in the primary screen were compatible within the hexameric TCR complex. I assembled the 11 TCRβ/CD3γ cysteine mutant pairs in the context of full TCR complex assembly in the IVT assay, CuPhe-treated and performed sequential CD3γPC-to-CD3δFLAG IP. I found that all 11 TCRβ-to-CD3γ crosslinks were recovered in the hexamer selection (Fig 4.12).

ζζ homodimer association with the assembling TCR complex is the final step in TCR complex biogenesis. Unlike the scan in Chapter 3, we expect ζζ to be able to join these crosslinked products because the CP disulphide bond of the TCRαβ is intact. However, the efficiency of disulphide bond formation is not 100% and thus the ζζ homodimer may exclusively associate with the TCRαβ heterodimer that has not formed crosslinks with the CD3γ cysteine mutants. To understand whether the crosslinked products could associate with the ζζ homodimer, I re-analysed all 11 TCRβ-to-CD3γ crosslink-positive combinations in the IVT assay and performed IP against the ζ subunit using the anti-ζ antibody (clone 6B10). Strikingly, we found that only one of the TCRβ/CD3γ cysteine mutant combinations, the βL18C-to-γG95C (termed “LxG” hereafter), resulted in the recovery of significant amounts of TCRαβ-CD3γ crosslinked product (Fig 4.12C, marked #). The LxG crosslink is spaced 4 residues apart from the key βK25-γE100-containing polar network, suggesting it is one helical turn above this critical contact site. Taken together, these results indicated the presence of a unique TCRβ/CD3γ TM interface and that alteration to this interface could impact the assembly efficiency of the rest of the complex.
Figure 4.12: Compatibility analysis of the TCRβ-to-CD3γ TM cysteine crosslinks within the intact TCR complex. (A) Summary of observed TCRβ-to-CD3γ TM crosslinks indicated by either dotted lines (only formed in hexamer selection) or solid lines (formed in both hexamer and full TCR complex selection) between cysteine-substituted positions. Crosslink-positive combinations from the primary screening (OKT3 IP) were re-analysed with (B) hexamer selection (CD3γPC→CD3δFLAG snIP) or (C) full TCR complex selection (anti-ζ IP) in the IVT assay. The only crosslink that is compatible with ζ-containing TCR complex, LxG crosslink marked in (#), crosslinks that were not compatible with ζ-containing TCR complex are marked in (*) and TCRβ/CD3γ cysteine combinations that resulted in the loss of TCR complex recovery are marked in (^). All assembled and CuPhe-treated TCR complexes were extracted into digitonin micelles, immunoprecipitated with the indicated IP strategy and visualized with 35S-detection after non-reducing SDS-PAGE analysis.
4.1.5.2 TM cysteine crosslinking between the TCRα and ζ subunits

Next, I investigated TM-TM contacts between the TCRα and ζ subunits. Unlike the CD3 subunits, which heterodimerise based on extracellular domain folding, residues within the TM domain of ζ are known to facilitate ζζ homodimerisation. I therefore carefully selected ζ residues to mutate that are outside the dimerisation interface based on the solution NMR structure of the ζζ TM homodimer (Call et al., 2006). The dimerisation of the ζ subunit is driven by a pair of inter-helical hydrogen bonds between a tyrosine and threonine within the C-terminal half of the TM domains. The ζζ homodimer is stabilised through a disulphide bond within the N-terminal stalk region. The N-terminal juxtamembrane region contains the critical aspartic acid residue (D15) that is required for the homodimer assembly into the TCR complex (Call et al., 2002). Taking this into consideration, I generated individual cysteine substitution for eight TM residues near the C-terminal end of the ζ TM domain that is devoid of any critical residues for the formation of an intact TCR complex (Fig 4.13; bolded).

![TCRα-to-CD3ζ TM cysteine crosslinking](image)

**Figure 4.13: TM cysteine mutant library of the TCRα and ζ subunits.** The protein sequences for the TCRα and ζ TM and flanking regions are shown. The true the C-terminus (--WSS) of the TCRα sequence is shown and the mature N-terminal sequence of ζ is also shown (QSF--). Residues numbering is shown above the protein sequences. Underlined protein sequences depicted the experimentally-determined TM segments (for the TCRα in chapter 3 and for ζ (Call et al., 2006)). The critical residues that governs TCR complex assembly are coloured in blue (basic amino acids) and red (acidic amino acids). Bolded protein sequences are the TM residues that were individually substituted to cysteine residue for the TCRα-to-ζ TM cysteine crosslinking screen.
These ζ TM cysteine mutants were assayed against six TCRα TM cysteine mutants with similar depth in the membrane (Fig 4.13; bolded). All possible combinations of the TCRα/ζ cysteine mutants (48 combinations) were assembled in the IVT assay in the context of full TCR complex assembly and immunoprecipitation with the OKT3 antibody was performed. The crosslinked products will be identified as a protein complex with higher molecular weight than the TCRαβ heterodimer.

In the screening, I identified 23 of the TCRα/ζ cysteine mutant combinations resulted in the recovery of crosslinked products, which involved crosslinking of the αL42C, αR43C and αL44C to almost all the ζ TM cysteines tested (Fig 4.14A-D). In contrast to the expected crosslink of TCRαβ-ζζ, some of these reactions contain two crosslinked products with higher molecular weight than the disulphide-linked TCRαβ heterodimer (Fig 4.14A-D). To better estimate the molecular weight of these crosslinked species, I analysed αL42C/ζL30C (predominantly the highest molecular weight crosslinked product) and αL42C/ζK33C (containing both crosslinked products) by non-reducing tris-acetate SDS-PAGE, which is better at separating proteins with high molecular weight. I estimated that the largest protein complex migrates roughly at 110kDa while the lower crosslinked product migrates roughly at 80kDa (Fig 4.15; *** and **, respectively). The expected molecular weight for TCRαβ-ζζ is 99kDa and TCRαβ-ζ is 83kDa, suggesting that the higher molecular weight product is the expected product and the lower molecular weight band corresponds to the TCRαβ crosslinked to a ζ monomer.

Only 4 out of the 23 crosslinks resulted in the recovery of exclusively TCRαβ-ζζ crosslinked product; αL42C-ζL30C, αL42C-ζF34C, αL44C-ζL30C and αL44C-ζF34C (Fig 4.14B and C). Based on the ζζ TM homodimer structure, position F34 of the ζ subunit is outside of the helical TM domain. Similarly, position L44 of the TCRα subunit is in the juxtamembrane region. Hence, the αL42C-ζL30C, which most likely to form within the helical region of the TCRα and ζ TM domains will be utilised in future computational modelling studies.
**Figure 4.14: Cysteine crosslinking screen between the TCRα and ζ TM domains after anti-CD3 IP.** Protein sequences of TCRα and ζ are shown with the TM domain underlined. Summary of observed TM crosslinks between αL42C mutant (within TM helix limit) and ζ cysteine mutants are indicated by solid lines (orange) between cysteine-substituted positions. Crosslinks contributed by the αR43C and αL44C mutants are not shown because their positions in the non-helical region. (A-D) Combinatorial cysteine crosslinking between the TCRα and ζ TM cysteine mutants that were performed in the IVT assay with 35S-detection after non-reducing SDS-PAGE analysis. Crosslink-positive combinations are marked with (*).
Figure 4.15: Analysis of the molecular weight of TCRα-ζ TM crosslinked species using Tris-Acetate protein gel electrophoresis. WT, αL42C-ζL30C and αL42C-ζK33C were assembled in the IVT assay, CuPhe-treated and analysed by OKT IP after digitonin extraction. The precipitated samples were analysed with 35S-detection after non-reducing Tris-Acetate gel analysis. The TCRαβ-ζζ is marked ***, the TCRαβ-ζ is marked **, and the TCRαβ heterodimer is marked *. Other lower molecular weight protein species in the TCR complex are marked #.
4.2 Summary

Our primary aim for this chapter was to map subunit organisation within the TCR complex providing crosslinks that can be utilised in the computational simulations to obtain a model of TM assembly of the TCR complex. The most informative crosslinks are those that form within the helical limits of the TM domain. In this chapter, I determined the TM helical limits of CD3δ and CD3γ using TALOS+ secondary structure prediction tool based on data obtained from triple resonance NMR experiments. The CD3ε TM peptide was not amenable to secondary structure prediction but a number of alternative expression and purification strategies could be explored. The TM helix limit data presented here will be utilised in future computational modelling studies and here I used these data to interpret cysteine crosslinking data between the TCRβ and CD3γ subunits (TCRβ-to-CD3γ) and the TCRα and ζ subunits (TCRα-to-ζ).

In the TCRβ-to-CD3γ TM cysteine crosslinking screen, I initially demonstrated that 11 selected crosslinks could be co-immunoprecipitated in the hexamer TCR complex selection IP. In further characterising the compatibility of these crosslinks within the ζ-containing full TCR complex, I performed IP targeting the ζ subunit and found that only one of these crosslinks, LxG TM crosslink was recovered, suggested that the TCRβ and CD3γ TM domains interact through a specific interface that is compatible with the formation of the LxG TM crosslink. In future computational modelling studies, the inter-helical restraints between the TCRβ and CD3γ TM domains are (1) the LxG TM crosslink and (2) the key polar network participated by the βK25, γE100 and γS103 residues.

The cysteine crosslinking screen between the TCRα and ζ subunits was limited to residues within the C-terminal end of the TM domain due to known critical TM residues for either homodimerisation of the ζ subunit or its recruitment into the TCR complex. Within this region, I identified strong crosslinks between L42C and L44C of the TCRα subunit and the L30C and F34C of the ζ subunit, but only the crosslink between the αL42C and ζL30C mutants lie within the helical TM domain and this crosslink will serve as the most informative crosslink for computational modelling.
studies. Interestingly, I observed recovery of either TCRαβ-ζζ or TCRαβ-ζ or both crosslinked products in the TCRα-to-ζ cysteine crosslinking screen. Monomeric ζ is not known to join the TCR complex and we cannot discriminate whether we have crosslinked ζ monomers that have joined the complex or non-covalent ζ dimers in which the disulphide bond between the ζ chains has yet to form. The significance of this result can only be determined with further experimentation.

Data from the cysteine crosslink screen in the IVT assay has contributed to our understanding how the TM domains of TCR complex subunits interact with one another. Cysteine crosslinking screens among the rest of the TM domains within the TCR complex have been initiated and together with secondary structural information from NMR spectroscopy will be utilised in computational modelling to generate a full picture of the TM domain arrangement within an intact TCR complex.
Chapter 5: Discussion and concluding remarks

Emerging ideas in the field of TCR biology have suggested that understanding the architecture of the TCR complex would provide key insights into how this intricate receptor machinery functions (Kuhns et al., 2006, Kuhns and Badgandi, 2012, Birnbaum et al., 2014). Structural analysis of the intact TCR complex has been hampered by the complexity of the receptor and its nature as a membrane-embedded protein complex (Baker, 2010). Here, I used several complementary techniques to gain insight into the structural basis of TCR complex assembly. To obtain residue-specific TM-TM contact points between the TCRα and TCRβ chains, I used intramembrane cysteine crosslinking. In the absence of detailed structural information, cysteine crosslinking has been successfully utilised to determine information about residue proximity and has uncovered novel biology in other membrane proteins (Brooks et al., 2014, Shiota et al., 2015). To perform our crosslinking study, I used the well-established cell-free membrane protein translation, assembly and isolation protocols (Call et al., 2002). These techniques had allowed us to investigate the TM-TM relationship between the TCRα and TCRβ chains within a fully assembled membrane-embedded native TCR complex. Using this system, TM cysteine mutants of the TCRα and TCRβ chains were expressed in combinations and probed for formation of interhelical crosslinks. Resultant crosslinks provided low-resolution structural information regarding residue proximity and TM-TM interface. To probe the TM structure of the TCRαβ heterodimer based on the crosslinks, we combined secondary structural information from solution NMR spectroscopy experiments and highly validated computational modelling methods (Jo et al., 2016). The key advantages of our strategy were:

1) Information on residue proximity within a TCR complex assembly while it resides in a native membrane environment.

2) Direct experimental evidence of the TM helix limits to aid structure determination by computational modelling.
3) Computational techniques that account for the hydrophobic nature of the bilayer and simulate TM domains in a realistic membrane-like environment (Wu et al., 2014).

Specific discussion points that arose from findings presented in this thesis are highlighted below.

5.1 Mapping of spatial organisation of subunits of the TCR complex based on TCRαβ TM structure

In chapter 3, we determined the structure of the interacting TCRα and TCRβ TM domains. The positioning of the CD3δε and CD3γε dimers was then inferred around the TCRαβ heterodimer based on the direction of the key basic residues αK32 and βK25, respectively (Fig 3.19). The ζζ homodimer can be placed based on the position of αR27 and falls between the two CD3 heterodimers. In chapter 4, I further identified the βL18C-γG95C (Fig 4.13B) and the αL42C-ζL30C (Fig 4.15B) crosslinks, which provide additional inter-helical restraints between the TCRβ and CD3γ subunits and TCRα and ζζ subunits, respectively. Additionally, in chapter 3, I also identified a crosslink between the TCRα and a native cysteine residue in the CD3δ TM domain (αR43-δC103). Together these crosslinks allow us to further refine the orientation of the placement of the CD3 heterodimers such that CD3ε of CD3δε and CD3γ of CD3γε lie closest to the ζζ homodimer. This model is presented in figure 5 and was generated using pymol. We used the TCRαβ TM structure (chapter 3), the solution NMR structure of the ζζ TM domains (Call et al., 2006) and pymol-generated helices of CD3δ, CD3γ and CD3ε TM domains. This model suggests that the two CD3 dimers are most likely to be placed on the opposite sites of the TCRαβ heterodimer, separated by the ζζ homodimer (Fig 5). Our finding is consistent with previous studies using antibody epitope mapping and computational docking (Kim et al., 2009, Sun et al., 2004), however contradicting with mutational analysis of TCR-to-CD3 interaction surface, which suggested that the two CD3 heterodimers co-localise on a single interface of the TCRαβ heterodimer (Kuhns and Davis, 2007). Two recent solution NMR-based studies in mapping extracellular domain interactions between the TCR and CD3 molecules reported contradicting results with one study supporting opposing CD3 positioning (as
proposed in this thesis) (Natarajan et al., 2016) and the other study supports colocation of the CD3 dimers on one interface of the TCRαβ heterodimer (He et al., 2015). These NMR studies, however utilised chemical shift perturbation data from very low affinity interaction between TCR and CD3 extracellular domains in solution, hence its relevance to infer architecture of the membrane-embedded TCR complex is questionable.

It is important to note that the proposed models of TCR complex architecture in this thesis can only definitively report on the arrangement of the TM domains. The presence of long CP sequences in the TCRα and TCRβ chains connecting the TM and EC domains could result in a different spatial organisation of the extracellular domains. In fact, the recent EM analysis of the intact TCR complex suggested that the extracellular domains might not interact laterally, but are instead placed underneath the TCRαβ extracellular Ig-like domains, sandwiched next to the CP regions of TCRαβ. High-resolution structural information of the intact TCR complex would be required to understand how extracellular domain assembly is related to our proposed TM domain assembly.
Figure 5: Model of the spatial organisation of the TM domains of the TCR complex. A hypothetical model of the TCR complex assembly was generated using the Pymol computer program. The TM structure of the TCRαβ heterodimer (deep teal and orange) was determined in chapter 3 using MD simulation and the TM structure of the ζζ homodimer (bright blue) was previously determined structure (Call et al., 2006). The TM structures of the CD3δ (turquoise), CD3γ (dark blue) and CD3ε (magenta) subunit were generated using the helix builder function in the Pymol program. The front view (A) and top view (B) of the model of TCR complex generated in from pymol modelling exercise are shown above. Key TM polar residues are presented in stick representation and coloured blue for basic residues, red for acidic residues, yellow for hydroxyl-bearing residues, and green for the N-T-Y polar network identified in chapter 3.
5.2 Identification of the conserved intramembrane polar network

In chapter 3, we identified an inter-helical hydrogen bond network between the TCRα and TCRβ TM domains. N37 and T41 residues of the TCRα subunit and Y29 residue of the TCRβ subunit participated in this polar network and we found that these residues are evolutionarily conserved. Previous studies have examined mutations at the conserved TCRβ Y29 position (Y29L or Y29F), and demonstrated that these mutations result in defective TCR signalling and memory T cell formation (Rodriguez-Tarduchy et al., 1996, Sahuquillo et al., 1998, Teixeiro et al., 1999, Teixeiro et al., 2002, Fuller-Espie et al., 1998, Kunjibettu et al., 2001). Although previously identified as functionally important, these studies did not propose a mechanistic explanation for how βY29 regulates the TCR signalling. In this thesis, I demonstrated a potential role for the βY29 as part of a polar network that lies within a specific TCRαβ TM interface. Given the previous functional data, this suggests that there is a specific role for TM domain conformation in TCR triggering. We have planned in vitro and in vivo T cell functional experiments to investigate the role of the intramembrane polar residues identified in this chapter to address this hypothesis.

5.3 ζζ association to the TCR complex

Throughout my experiments, I observed that association of the ζζ homodimer was impaired in many contexts. First, co-IP of the ζζ homodimer in the cysteine-less TCRαβ receptor complex assembly was compromised but could be rescued by introducing an intramembrane TM crosslink between the αF26C and βE20C mutants. Secondly, in the TCRβ-to-CD3γ cysteine crosslinking screen, the βL18C-γG95C crosslink was the only crosslink compatible with a ζ-containing TCR complex. These observations suggested that the ζζ homodimer is exquisitely sensitive to the packing of the rest of the TM domains within the hexameric TCR-CD3 subcomplex. This is consistent with the model of the TCR complex that I proposed above, which indicated a large protein-protein interaction surface that the ζζ homodimer can “sense”, contributed by both TCRαβ and CD3 dimers. Such sensitivity may also provide a mechanistic explanation for a previously observed property of the ζζ homodimer, which was described to be “loosely” associated with the constitutively active pre-TCR...
(Groettrup et al., 1992) and has been shown to dissociate from the αβTCR complex after ligand-binding (La Gruta et al., 2004). Assuming the αF26C-βE20C and βL18C-γG95C crosslinks stabilised a TM-TM architecture within the TCR complex that is receptive for ζζ association, it will be interesting to assess the signalling ability of these crosslinked TCR complexes upon ligand binding.

5.4 Implications for the TCR triggering mechanism

Recent studies have proposed that the TCR complex is a mechano-transducer that “senses” mechanical force exerted by antigen-loaded MHC molecules and translates this information to intracellular biochemical signalling (Kim et al., 2009). While a mechanistic description of how TCR chains convert the mechanical force into chemical signals has not been established, it has been suggested that force experienced by the TCR chains can cause rigid body movement of the extracellular domains of the TCR chains that alters protein-protein interactions between the TCR and signal-transducing subunits. Such physical distortion of the TCR complex could possibly cause conformational changes at the cytoplasmic tails of the signal-transducing subunits to expose ITAMs for phosphorylation. More recently, evidence that the TM domains of the TCR complex could undergo conformational changes was reported for the ζζ homodimer (Lee et al., 2015). From our investigation into the TM-TM relationship between the TCRα and TCRβ chains in chapter 3, I found that small alterations in a specific TCRαβ TM interface can have substantial effects on the nature of interactions with ζζ and CD3 signalling dimers (Krshnan et al., 2016). This finding suggests that forces exerted on the ligand-binding module could propagate to signalling modules and across the membrane at least in part through lateral communication of structural alterations among the membrane-embedded regions of the intact receptor complex. Future studies using my new structural models to examine the functional consequences of altering specific TM interfaces will provide further evidence to support or discard such a mechanism.
5.5 Concluding remarks

This study has contributed to further our understanding about the architecture of the TCR complex in the membrane. Experimental strategies described in this thesis provided framework to further characterise the TM assembly of the TCR complex. I believe that understanding the nature of the structural alterations will be key to deciphering the mechanism of TCR triggering. Crosslinks that we reported in this thesis represents proximity between TCR TM domains in the untriggered state. Further investigation into either changes in the crosslinking pattern upon activation of the TCR complex or ability of the TCR complex to be triggered upon restraining the complex by crosslinking it in cells would be key to demonstrate that alteration to TM-TM interactions is critical to TCR triggering.
Appendix 1: Sequence homology between human A6 TCR and murine OT-I TCR.

A. A6 versus OT-I TCRα sequence alignment

EMBOSS_001 1 17
EMBOSS_001 1 1
EMBOSS_001 1 5
EMBOSS_001 1 6
EMBOSS_001 1 10
EMBOSS_001 1 11
EMBOSS_001 1 14
EMBOSS_001 1 16
EMBOSS_001 1 19
EMBOSS_001 1 21
EMBOSS_001 1 24

B. A6 versus OT-I TCRβ sequence alignment

EMBOSS_001 1 21
EMBOSS_001 1 5
EMBOSS_001 1 6
EMBOSS_001 1 9
EMBOSS_001 1 9
EMBOSS_001 1 11
EMBOSS_001 1 14
EMBOSS_001 1 16
EMBOSS_001 1 19
EMBOSS_001 1 21
EMBOSS_001 1 24

Appendix 1: Sequence alignment between human A6 and murine OT-I TCRs. Protein sequences of the human A6 TCRα and β were compared to the mouse OT-I TCRα and β. (A) Alignment of the TCRα chain of the human A6 (Top, reference) and mouse OT-I (Bottom). (B) Alignment of the TCRβ chain of the human A6 (Top, reference) and mouse OT-I (Bottom). Highlighted in yellow are residues of the highly conserved connecting peptide and TM region.
Appendix 2: TCR$\alpha$-to-TCR$\beta$ cysteine crosslinking screening.

A.

**Figure appendix 1.** (continued)
B.

```
TCRβ  --SATILYEILLGKATLYAVLVSALVMAM--
TCRα  --FQNLSVICFRILLKVAGFNLLMTLRLWSS--
```

**Figure appendix 1. (continued)**
C.

**Figure appendix 1. (continued)**

D.
Figure appendix 1. (continued)
Appendix 2 (A-E): Primary cysteine crosslinking screen between the TCRα and TCRβ TM domains. The regions being interrogated in each panel are highlighted in orange on the primary sequence at the top of each figure. These combinations were assembled in the IVT assay, CuPhe-treated, digitonin-extracted and IP with OKT3 antibody. Lanes are labelled with the combinations tested. Quantitation to determine crosslinking efficiency for each panel is presented on the bottom of the figure and was calculated from a ratio between the crosslinked product and free TCRβ.
Appendix 3: Murine OT-I TCRα-2A-TCRβ retroviral expression cassette

CTCGAGGGTCAGGCGGGCCACGATGGTACAGGCTGTGCTGCTGTGTGGGCTGCTGCGCTGACTGACACGGG
                            OT-I TCRβ
                         LEASAAATMVPCLLLALAPTQR
                                      OT-I TCRβ
                                      ADSGVVQSPRHIKEKGGRRSVLTCP
                                      OT-I TCRβ
                                      IGSHSNVWYQQTGLKELKFLIQHYEK
                                      OT-I TCRβ
                                      VERDKGFLPSRFVQGFDDYHSEMNS
                                      OT-I TCRβ
tgccttggaactgaggtacctcttgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgctgct
Appendix 4: Murine CD3δ-2A-ζ retroviral expression cassette


GROETTRUP, M., BARON, A., GRIFFITHS, G., PALACIOS, R. & VON BOEHMER, H. 1992. T cell receptor (TCR) beta chain homodimers on the surface of immature but
not mature alpha, gamma, delta chain deficient T cell lines. *EMBO J*, 11, 2735-45.


SAHUQUILLO, A. G., ROUMIER, A., TEIXEIRO, E., BRAGADO, R. & ALARCON, B. 1998. T cell receptor (TCR) engagement in apoptosis-defective, but interleukin 2 (IL-


STONE, J. D. & STERN, L. J. 2006. CD8 T cells, like CD4 T cells, are triggered by multivalent engagement of TCRs by MHC-peptide ligands but not by monovalent engagement. *J Immunol*, 176, 1498-505.


