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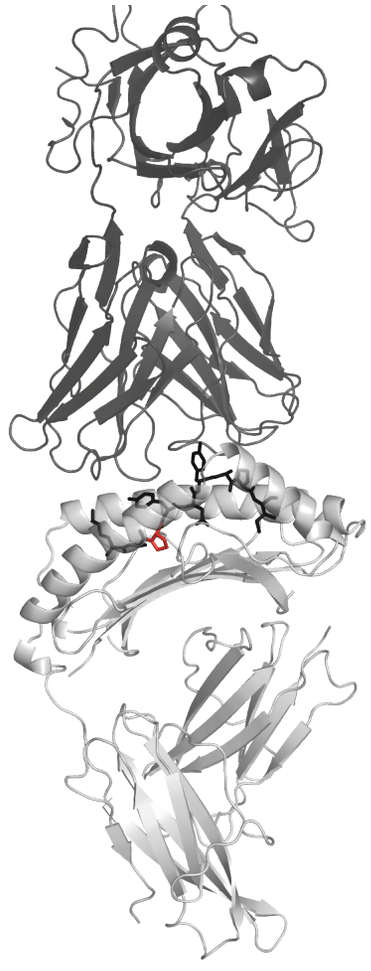

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Molecular Basis for Enhanced T-cell Recognition and Cross-reactivity

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Stockholm 2014

The cover figure shows a crystal structure of a T-cell receptor (dark grey) bound to a peptide (black) with the third residue, proline, colored in red, in complex with a Major Histocomatibility Complex (MHC) (light grey).

Artistic illustrations by Kimi Drobin

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“It always seems impossible until it´s done”.

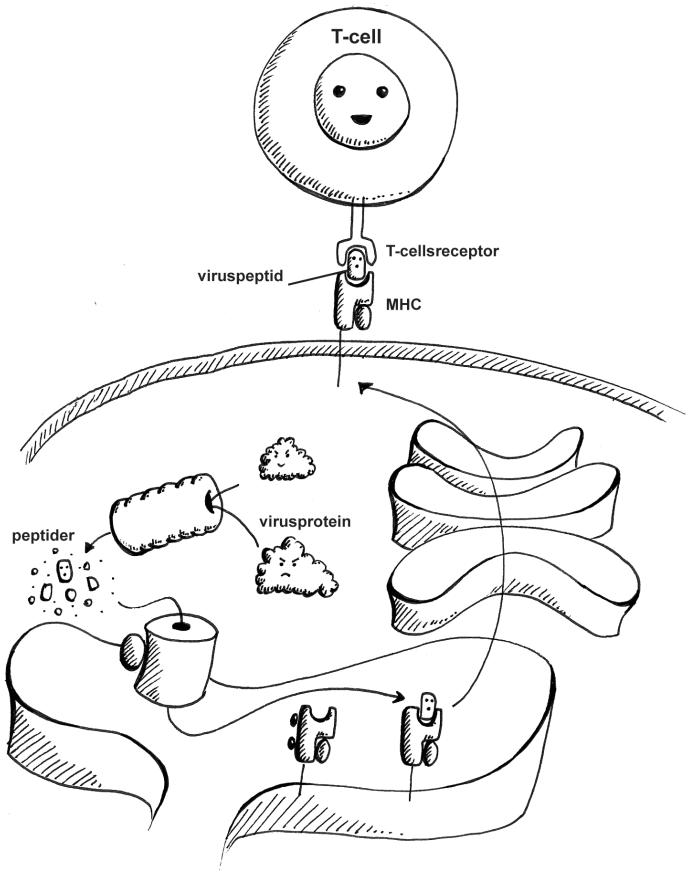
Nelson Mandela

1918-2013

POPULAR SCIENCE SUMMARY (Swedish)

T-celler ingår i vår kropps immunförsvar och har förmågan att detektera och döda virusinfekterade celler och en till viss mån cancerceller. De läser av markörer på cellytan; små delar av proteiner s.k. peptider, som kommer upp till cellytan inifrån cellen. Peptiderna sitter på proteinkomplex som heter MHC (Major Histocompatibility Complex). Om en cell är infekterad av ett virus kommer en del av peptiderna på MHC att komma från virusproteiner, vilka skiljer sig i utseende från våra egna proteiner. Cancer-associerade proteiner är i många fall likadana som proteiner hos friska personer, vilket gör det svårt för T-cellerna att särskilja cancerceller från friska celler, men de kan i vissa fall vara muterade eller presenterade i större mängd än vanligt. T-celler särskiljer på friska och sjuka celler genom att läsa av dessa peptider (antingen kroppsegna, virala eller cancer-relaterade) på MHC-komplex på cellytan genom en avläsningsmolekyl som kallas T-cellsreceptor. Virus är dock bra på att undkomma immunförsvaret genom att mutera sina proteiner, därigenom gömma sig för T-cellerna då de inte längre kan binda med sin T-cellsreceptor till peptiderna på MHC.

Målet med forskningen som ingår i den här avhandlingen är att designa och utveckla peptid-baserade vaccin som kan utbilda T-celler att känna igen muterade virus –eller cancerpeptider på ytan av infekterade och sjuka celler som annars inte skulle känts igen av immunförsvaret, och döda dessa celler. Vi har upptäckt och undersökt aminosyran prolin i peptiden (röd på omslagsbilden) som skulle kunna göra vaccin mer effektiva. Kunskapen från dessa studier kan förhoppningsvis i framtiden leda till förbättring och generell utveckling av cancervaccin såväl som vaccin mot virala sjukdomar.



ABSTRACT

T cells of the adaptive immune system recognize and kill infected cells by interacting with pathogenic peptides displayed on major histocompatibility complexes (MHC) through its T-cell receptor (TCR). TCR interactions with peptide/MHC complexes occur throughout the lifespan of T cells, from development to activation, with the origin of the peptide varying between self and foreign. The vast universe of foreign peptides that could appear on infected cells presented by polymorphic MHCs is by far outnumbering the size of the T-cell repertoire. Thus, a property that characterizes T cells is cross-reactivity which enables weak recognition of self-peptides and activation by foreign peptides. Despite being cross-reactive T cells are known to be highly specific for cognate antigens, which might seem like a paradox. Therefore, dual recognition of MHC and peptide with the need to be cross-reactive yet specific presents challenges to T cells and occurs through mechanisms that are not entirely understood.

The studies within this thesis made use of the P14 T-cell system to investigate how the TCR P14 can differentiate between peptides to learn more about the molecular basis dictating specificity, sensitivity and cross-reactivity. P14 is sensitive to the mutation Y4F at the main TCR-recognition site p4Y of the viral peptide gp33, which is utilized by the Lymphocytic choriomeningitis virus (LCMV) to escape P14 T cells. Surprisingly, alanine substitution at p4 (Y4A) of gp33 is weakly recognized by P14 through the use of a different thermodynamic signature.

At the center of these studies was a peptide modification where position 3 had been substituted to proline (p3P), enhancing both TCR affinity and peptide/MHC stability through independent mechanisms. Importantly, p3P did not alter the peptide conformation, which is important for T-cell cross-recognition. Weak interactions between p3P and the conserved H-2D^b residue Y159 accounted for the increased MHC stability, whereas enhanced TCR affinity seemed to arise from decreased binding entropy. The p3P modification was applied to the viral escape mutant Y4F, which reestablished P14 recognition. Co-crystal structures of P14 in complex with gp33/H-2D^b and the p3P-modifed gp33/H-2D^b visualized the structural basis for enhanced TCR affinity as well as the central role of p4Y of gp33 explaining its sensitivity to mutations.

Finally, P14 also weakly recognizes the self-peptide mDBM which under certain circumstances can lead to auto-reactivity. The p3P modification was also applied to mDBM, which increased TCR affinity and MHC stability, facilitating crystallization. mDBM was found to be a structural mimic of gp33, despite a moderate sequence homology. The ternary structure of P14 in complex with mDBM(3P)/H-2D^b demonstrated that flexibility in the CDR3β loop mediated cross-reactivity between gp33 and mDBM.

In conclusion, the results here visualize the different faces of TCRs; sensitivity, specificity and cross-reactivity on a molecular basis. Certain peptide substitutions are tolerated through TCR flexibility and peptide adjustments, whereas others are not. Finally, p3P substitution increased the immunogenicity of our H-2D^b-restricted peptides and could present a novel strategy for designing peptide vaccines with increased MHC binding properties.

PUBLICATIONS

I. **Unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics**
Allerbring EB, Duru AD, Uchtenhagen H, Madhurantakam C, Tomek MB, Grimm S, Mazumdar PA, Friemann R, Uhlin M, Sandalova T, Nygren PA, Achour A.
Eur J Immunol. 2012 Nov;42(11):2990-3000. doi: 10.1002/eji.201242588.

II. **Structural and thermodynamic basis underlying reestablishment of P14 T-cell recognition of a viral escape mutant**
Allerbring EB, Duru AD, Markov N, Uchtenhagen H, Popov A, Madhurantakam C, Sandalova T, Nygren PA and Achour A.
Manuscript

III. **Proline substitution independently enhances H-2D^{b}-complex stabilization and TCR recognition of melanoma-associated peptides**
Uchtenhagen H, Abualrous ET, Stahl E, **Allerbring EB**, Sluijter M, Zacharias M, Sandalova T, van Hall T, Springer S, Nygren PA, Achour A.
Eur J Immunol. 2013 Aug 13. doi: 10.1002/eji.201343456.

IV. **Structural basis for CD8^{+} T-cell auto-reactivity in LCMV infection**
Allerbring EB, Duru AD, Nygren PA, Sandalova T and Achour A.
Manuscript

Publication not included in this thesis

Melanocortin 1 Receptor-Derived Peptides Are Efficiently Recognized by Cytotoxic T Lymphocytes From Melanoma Patients
González F, Ramirez M, **Allerbring EB**, Fasching N, Lundqvist A, Poschke I, Achour A, and Salazar-Onfray F.
Immunobiology 2013 Oct 12. pii: S0171-2985(13)00177-0. doi:10.1016/j.imbio.2013.10.002.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
APL	Altered peptide ligand
β_2m	β_2-microglobulin
CD	Circular dichroism
CDR	Complementarity determining region
CTL	$CD8^+$ cytotoxic T-lymphocyte
DC	Dendritic cell
ΔC_p	Change in heat capacity
ΔG	Change in free energy
ΔH	Change in enthalpy
ΔS	Change in entropy
DN	Double negative
DP	Double positive
DRiP	Defective ribosomal product
ER	Endoplasmic reticulum
HLA	Human leukocyte antigen
IFNγ	Interferon-γ
ITAM	Immunoreceptor tyrosine-based activation motif
ITC	Isothermal titration calorimetry
K_a	Association rate
K_d	Dissociation rate
K_D	Binding affinity
LCMV	Lymphocytic choriomeningitis virus
MHC	Major histocompatibility complex
MHC-I	Major histocompatibility complex class I
MHC-II	Major histocompatibility complex class II
NK	Natural killer
pMHC	peptide/MHC complex
PTM	Posttranslational modification
p3P	Proline at peptide position 3
SPR	Surface Plasmon Resonance
TAA	Tumor-associated antigen
TAP	Transporter associated with antigen processing
TCR	T-cell receptor
TNF	Tumor necrosis factor
vdW	Van der Waal

1 INTRODUCTION

1.1 THE ADAPTIVE IMMUNE SYSTEM

The immune system is a complex ensemble of cells and macromolecules that has evolved in unity to provide protection for its host against invading pathogens. The adaptive immune system which is the second line of defense, following the initial innate arm, is characterized by high specificity, clonal selection [1] and memory. Lymphocytes of the adaptive immune system develop to be highly specific for a pathogen yet selected to tolerate self; two competing criteria that are not always compatible.

The adaptive immune system can be categorized into a cell-mediated and a humoral component. Lymphocytes of the cell-mediated branch are T and Natural Killer (NK) cells which target intracellularly infected cells. Communication occurs through interactions between antigen receptors on the lymphocytes and major histocompatibility complexes (MHC) presenting antigenic peptides on the surface of virtually all other cells. Humoral immunity is provided mainly by immunoglobulins produced by B lymphocytes to neutralize and kill extracellular pathogens, which may be surface bound or secreted.

Some features are shared between antigen receptors of B and T lymphocytes, such as the structures and genetic recombination processes during development. However, unlike immunoglobulins, which can recognize linear as well as nonlinear epitopes of seemingly endless chemical and structural variation, the ligands for TCRs consist of a composite surface of both self (MHC) and non-self (peptide) [2]. A second property that characterizes TCRs is their inherent cross-reactive nature, enabling recognition of multiple ligands in the context of MHC [3]. These two characteristics of TCRs, antigen recognition in an MHC-restricted fashion and cross-reactivity, are ever so fascinating and intriguing, though not fully understood. This thesis will focus on TCR/peptide/MHC interactions from a structural aspect with an emphasis on cross-reactivity and recognition enhancement through modulation of the presented peptides.

1.1.1 T Lymphocytes

T lymphocytes can be divided into two subsets, primarily defined by the co-receptor expressed, either CD4 or CD8. $CD8^+$ cytotoxic T lymphocytes (CTLs) have the ability to detect and kill infected cells by interacting with MHC class I (MHC-I) molecules presenting short antigenic peptides. $CD4^+$ T cells, mainly T helper and regulatory T cells, represent the second broad subtype of lymphocytes, recognizing MHC class II molecules (MHC-II) presenting longer peptides. The $CD4^+$ T cells bridge the two arms of adaptive immunity by interacting with and activating B cells. The biology of B cells fall outside the scope of this thesis and will not be discussed further. Instead, focus will be given to $CD8^+$ T cells and the interactions between their TCRs with pepetide/MHC-I molecules (pMHC).

1.1.1.1 Thymocyte development

T lymphocytes derive from hematopoietic stem cell precursors in the bone marrow and migrate to the cortex of the thymus where development and education take place. Initially, thymocytes do not express any co-receptors (CD4 or CD8), and are called double negative (DN). The assembly of the TCR begins at the DN stage with rearrangement of the TCR β-chain, which pairs with an invariable pre-α chain and concomitant expression of CD4 and CD8. The now double positive (DP) thymocytes will rearrange their TCR α-chain until an MHC-restricted receptor is generated. This can take days and is most frequently unsuccessful. Thymocytes unable to generate an MHC-restricted TCR will die by neglect before positive selection can occur [4].

Positive selection promotes survival of thymocytes with TCRs capable of recognizing self-MHC molecules presenting self-peptides on cortical thymic epithelial cells [5]. The specifics and number of the self-peptides that induce positive selection have been longstanding questions. Weak agonists have been shown to stimulate positive selection [6] but the ligands are usually unrelated to the pathogenic peptides recognized by these T cells later in their lifecycles [7]. Nevertheless, the affinity threshold is lower than for negative selection [8] and generally not stimulatory for mature T cells [4].

Negative selection, aiming at eliminating self-reactive T cells, is a process in which developing thymocytes with too high affinity for self-peptide/MHC complexes are deleted. This process may occur throughout the education and development, either at the DP or SP stage [9]. The outcome of the selection process provides self-tolerant T cells that maintain a low affinity towards self-MHC molecules.

1.1.2 Antigen processing and presentation of peptide/MHC-I

Antigen presentation on MHC-I molecules is a highly sophisticated communication system between T cells and essentially all other cells in higher organisms. Analogous to windows into the cell, pMHC molecules provide information about potential intracellular pathogens in forms of peptides displayed on the cell surface. The interaction with pMHC is crucial throughout the life cycle of a T cell, from the development stage [4] to homeostasis [10, 11] and activation of mature T cells, with the origin of the peptide varying from self to foreign.

Peptides presented by MHC-I are mainly derived from degradation of cytosolic proteins by the proteasome [12], a process tightly regulated to avoid destruction of essential self-proteins (Figure 1). The degradative pathways for MHC-I restricted peptides remain controversial, and the nature of endogenous antigens is poorly defined. Ideally, generation of pMHCs following infection should be rapid in order to mount an effective and early CTL response against quickly replicating pathogens [13]. A major source of peptides seems to originate from defective ribosomal products (DRiP), presenting a rapidly produced source for antigenic peptides [14]. The error prone nature of the ribosome will under infectious conditions increase generation of DRiPs, defined as *"prematurely terminated polypeptides and misfolded polypeptides produced from translation of bona fide mRNAs in the proper reading frame"* [14]. Ubiquitination of proteins is another important pathway for generating peptide antigens, a process in

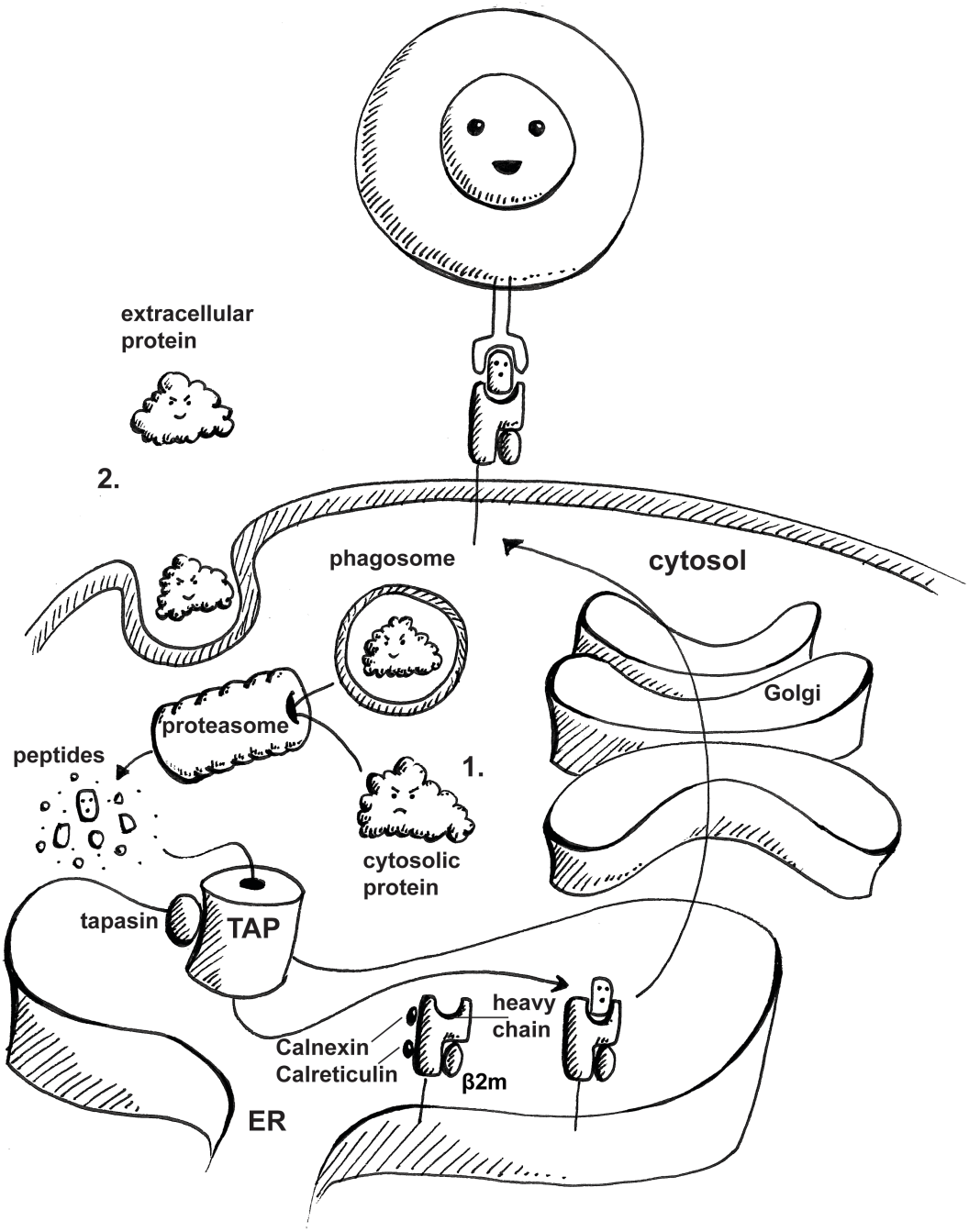


Figure 1. Antigen processing and pMHC presentation. ***1.*** *Peptides are mainly derived from degradation of cytosolic proteins or DRiPs by the proteasome. The peptides are thereafter transported from the cytosol into the ER by TAP. After association of β_2m with the MHC heavy chain, tapasin promotes binding of the peptide by bridging with TAP. The assembly of pMHCs is mediated by Calnexin and Calreticulin among other proteins. Peptide binding releases the pMHC molecule for transport to the cell surface through the Golgi apparatus.* ***2.*** *Antigen can also be taken up from the extracellular compartment through phagocytosis, to be presented on MHC-I. This phenomenon is known as MHC cross-presentation and occurs in professional APCs, mainly dendritic cells (DCs). Following internalization, extracellular proteins are exported to the cytosol for degradation by the proteasome and can thereafter either resume to the classical pathway by transportation into the ER, or get re-imported back to the phagosome for loading onto MHC. Alternatively the peptides can be degraded in the phagosome where loading on MHC-I also takes place.*

which ubiquitin molecules are coupled to cytosolic proteins subjecting them to proteasome degradation.

Following infection, enhanced expression of IFNγ and various cytokines consequently up-regulate proteasome activity. The advantage of the resulting immunoproteasome is increased quality and quantity of the generated peptides, which is attributed to structural changes in the catalytic site with an altered cleavage pattern [15]. Conclusively, proteasome-mediated degradation of proteins is an important step in generating peptide antigens ultimately influencing the epitope hierarchy in T-cell responses [16].

Peptides are transported from the cytosol across the endoplasmic reticulum (ER) membrane, where the assembly of pMHCs takes place. Translocation of peptides is carried out by the transporter associated with antigen processing (TAP) [17, 18]. The fact that TAP discriminates against peptide length as well as chemical properties of terminal residues contributes to the shaping of the peptides repertoire. The preference of TAP for peptides differs between species. In humans, the three N-terminal as well as the C-terminal residue have significant effects on TAP affinity [19]. Lengthwise, peptides of 8-13 residues are easily transported [20], however longer peptides will also be translocated [21] and subsequently trimmed to appropriate length by peptidases. There are also TAP-independent processing pathways, generating TEIPP *"T-cell epitopes associated with impaired peptide processing"* epitopes on MHC-I, which was discovered by immunizing mice with TAP-deficient tumor cells that elicited a CTL response towards TAP-deficient cells [22, 23].

In the ER the principal trimming of peptides occurs at the N-terminus by various aminopeptidases with different preferences for substrates also influencing the final pool of peptides [24]. The existence of C-terminal trimming proteases was long debated, however recent studies have shown the presence of carboxypeptidases both in the cytosol [25] as well as in the ER [26]. Still it is thought that the proteasome makes the final cut of the C-terminus [27]. Thus, the peptide selection for MHC-I molecules is not a random process, but rather carefully controlled by length and sequence [28].

The assembly of pMHCs is a multistep procedure involving several components. Briefly, among other proteins the chaperons Calnexin and Calreticulin mediate folding and binding of newly synthesized MHC heavy chains [29]. After association of β_2-microglobulin (β_2m), tapasin stabilizes the empty MHC molecule and promotes binding of the peptide by bridging with TAP, which delivers the peptide [30]. Peptide binding releases the pMHC molecule for transport to the cell surface through the Golgi apparatus, while lack of binding results in proteasome-mediated degradation.

All three components; MHC heavy-chain, β_2m and peptide are essential for successful pMHC formation. Cell-lines lacking β_2m fail to produce pMHCs on their cell surfaces [31, 32]. Similarly the peptide, *'acting like the keystone in an archway'*, is required for stable formation of pMHC, exemplified by the so far unsuccessful attempts to crystallize empty MHC-I molecules. The TAP-deficient cell line RMA-S, incapable of presenting endogenous peptides through the conventional TAP-mediated pathway, is characterized by poor expression of pMHC further indicating the essential role of the

peptide. Early experiments using RMA-S cells incubated at 26°C demonstrated that 'empty MHC molecules' would surface, although unstable in their state. Exogenous peptides added to the media would find their way into the clefts, stabilizing the resulting complexes [33]. Whether or not RMA-S cells express truly empty MHCs or perhaps harbor low-affinity peptides possibly derived from the surrounding media remains controversial. Regardless, the discovery by Ljunggren *et al.* evolved into a readily used method for assessing peptides´ abilities to bind and stabilize MHC [33].

1.1.2.1 MHC cross-presentation

Cross-presentation is an alternative route to the endogenous pathway for generating antigens for presentation on MHC-I (Figure 1). Naïve T cells require activation by professional antigen presenting cells (APCs) to become activated, see section *1.1.5*. When APCs are not directly infected, antigen can be acquired from the extracellular compartment through endo- or phagocytosis, a phenomenon mainly observed for dendritic cells (DCs) [34].

Following internalization of exogenous material, two main intracellular pathways for MHC cross-presentation have been described. In the cytosolic pathway antigen is exported to the cytosol for degradation by the proteasome and can thereafter either resume to the classical pathway by transportation into the ER, or alternatively get re-imported back to the phagosome for loading onto MHC. The second pathway, referred to as the vascular pathway, degrades peptides in the phagosome where loading on MHC-I also takes place. The mechanisms behind cross-presentation are not well understood, including the origin of MHC-I molecules [35]. In addition to cross-presentation, other means of deriving antigens for presentation by APCs exist whereby peptide-loaded MHC molecules can be picked up from neighboring cells in a process called '*cross-dressing'* [36].

1.1.2.2 The battle between a pathogen and its host in antigen presentation

There are numerous ways for a pathogen to interfere with the MHC presentation machinery in its attempts to escape immune surveillance. Survival of the fittest drives generation of viral mutants targeting just about every step in the highly intricate pathway of antigen presentation [37]. A few examples include amino-acid substitutions that inhibit proteasome-mediated degradation [38] and expression of microRNA targeting genes encoding for aminopeptidases [39]. Several viruses have also developed strategies to interfere with TAP [40]. In addition, mutations in viral epitopes can also affect the ability of the peptide to bind and stabilize the MHC complex [41-43].

1.1.3 The architecture of MHC-I molecules

Insights into the structure of peptide/MHC-I complexes have come from protein crystallization, beginning with the first reported crystal structure by Bjorkman *et al*. in 1987 where it was reported that the MHC contained *"a bound molecule of unknown origin that may be a peptide antigen"* [44]. Since then the structural database of pMHCs has considerably expanded, continuously advancing our understanding of the molecular basis underlying MHC-restriction and presentation of *'bound molecules'* which indeed proved to be peptides.

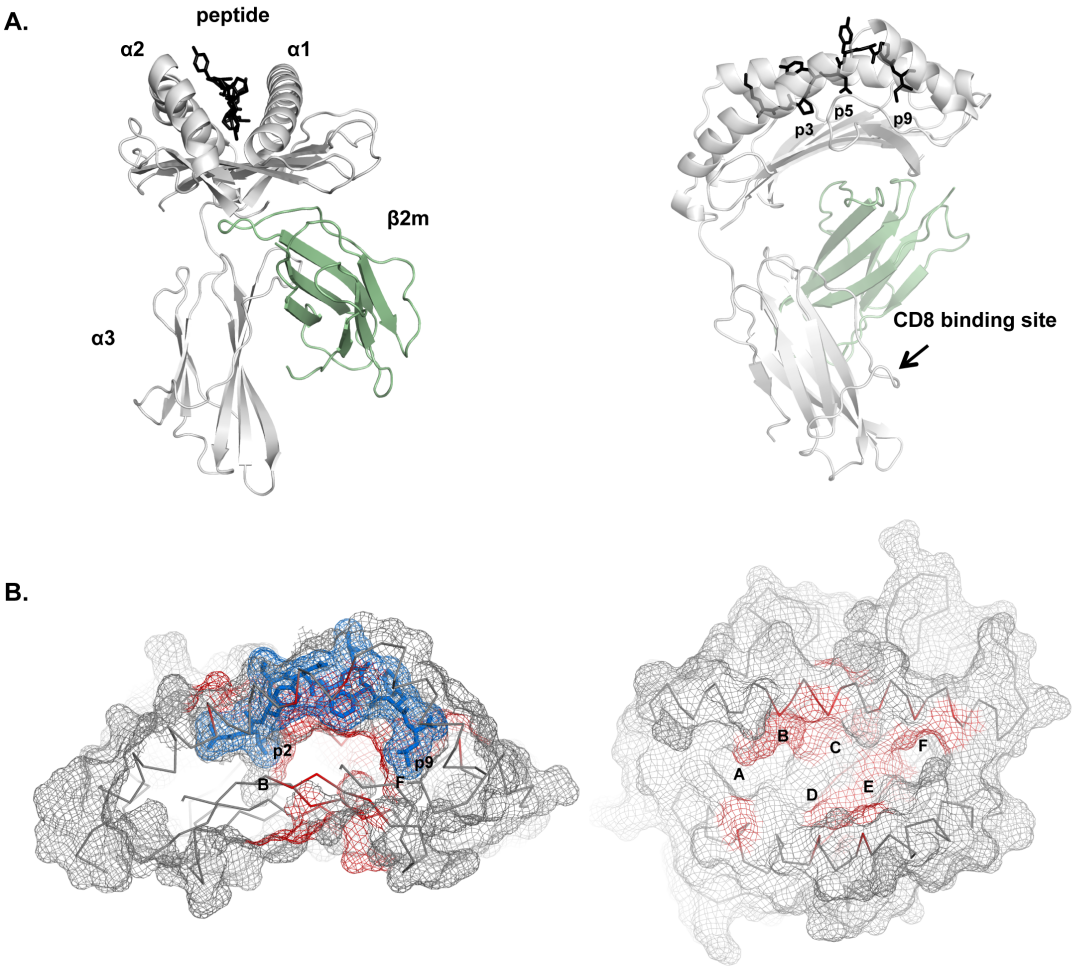


Figure 2: The architecture of a peptide/MHC-I molecule. A*. The MHC molecule, presented as cartoon, is made up by an MHC heavy chain (white) and a β_2m subunit (green). The peptide (black), presented as sticks, is accommodated in the binding groove between the α_1 and α_2-helices on a floor of a seven-stranded β-sheet (left panel). The molecule in the right panel is rotated 90 ° in relation to the molecule in the left panel. The peptide anchor residues, which in this case are p3, p5 and p9, are indicated, as well as the binding site for the CD8 co-receptor (right panel). The pMHC in Figure 2A is the mouse allele H-2D^b presenting the peptide mDBM(3P) from* ***Paper IV****.* ***B.*** *The MHC (grey) is presented as mesh and ribbon and the peptide (blue) is presented as sticks and mesh. The peptide is bound to the MHC molecule by burial of anchor residues in pockets in the peptide-binding groove (left panel). The most polymorphic sites of HLA class I (red) are located in and around the peptide-binding pockets (A-F) of the peptide-binding clef (right panel). The pMHC in Figure 2B is the human allele HLA-A2 presenting the tumor-associated antigen PRAME$_{300\text{-}309}$ (unpublished). The identities of the most polymorphic residues in HLA were kindly provided by Dr. Stephanie Gras.*

The MHC molecule is a heterodimer consisting of a heavy chain and β_2m subunit, organized in three domains. The two Ig-like domains, β_2m and the α3-domain of the heavy chain are non-covalently coupled. The α3-domain constitutes the binding-site for the co-receptor CD8 (Figure 2A, right panel) [45] . The third domain, constructed from elements of the heavy chain, accommodates the peptide on a floor of a seven-stranded β-sheet framed by two alpha helices (α_1 and α_2) [2] (Figure 2A). Generally, MHC-I molecules bind peptides with a length of 8-10 amino acids [46, 47] however longer peptides are also able to bind [48-50]. The length restriction is partly defined by the

binding pockets in the peptide groove harboring the terminal residues (Figure 2B) but also from the antigen processing described in the previous section. The typical nonameric peptide binds in an extended conformation, whereas longer peptides are more bulged due to locking of end terminal residues in binding pockets [51, 52].

Like the terminal residues, internal anchor residues are also buried within pockets in the peptide-binding groove, leaving side chains of other amino acids pointing upwards and available for TCR binding (Figure 2). However, amino acids other than the main anchor residues of the peptide are also important for binding [53].

Featureless peptides with few prominent up-facing side chains, commonly referred to as *"plain vanilla"*, can present challenges in the recognition and result in a biased TCR usage [54-56]. The opposite to vanilla in the world of peptides is represented by longer super-bulged *"hot chilli"* peptides, that can be up to 14 amino acids in length. Presenting more available peptide side chains, these interactions are characterized by a shifted focus from the MHC to the peptide [52]. Conclusively, the efficiency of antigen presentation is highly dependent on anchor residues of the peptide, both flanking and intra-epitope residues [57] but additional residues on secondary anchor positions are also important for a stable pMHC formation.

1.1.3.1 MHC-II

The overall structure of MHC-II is very similar to MHC-I, although there are a few differences [58]. The ends of the peptide-binding clefts are left open and can therefore accommodate significantly longer peptides than can those of MHC-I. The β_2m is replaced by a second heavy chain, the β-subunit, which together with the α-subunit assemble into a similar fold as in MHC-I, forming the peptide-binding groove [2]. MHC-II molecules are exclusive to professional APCs (B cells, DCs and macrophages) and constitute a binding site for the co-receptor CD4 instead of CD8.

1.1.3.2 MHC polymorphism

A central concept in adaptive immunity is the ability of MHCs to present peptides of yet unseen amino acid composition. Human MHC molecules, termed the human leukocyte antigen (HLA), are encoded by the HLA locus which represents the most polymorphic region within the human genome with more than 7000 distinct variants known [59]. Each individual expresses six different HLA class I molecules, two from each of the following loci; HLA-A, HLA-B and HLA-C, as well as six different HLA class II molecules [60]. The need to present a diverse array of peptides is the driving force in MHC evolution with a higher mutation rate located in the peptide-binding cleft (Figure 2B) [61]. A greater heterozygosity at the HLA locus is advantageous when encountering new pathogens. Similarly, pathogens and MHC alleles have co-evolved as evident from areas with endemic diseases where MHC polymorphism is driven by the need present antigen from a particular pathogen [62, 63].

Polymorphic sites in the peptide-binding groove impact the chemical and structural environment of the six peptide-binding pockets (Figure 2B), which define the peptide motif for each MHC allele [57, 64]. Closely related MHC-I alleles can be distinct from one another in as few as one amino acid. These so called micro-polymorphisms can

affect the conformation of the presented peptide and subsequently the mounted CTL response [51]. For example, micro-polymorphisms in HLA-B alleles are linked to the viral load in HIV infections. HLA-B3501 is a protective allele whereas HLA-B3502 and HLA-B3503, that differ in a few amino acids, are linked to disease progression [65]. Finally, MHC polymorphism can also influence the T-cell response without altering the peptide conformation, but instead affect TCR-contacting residues on the MHC [66].

1.1.3.3 Peptide design

Mapping conserved regions in binding pockets and preferred peptide anchor residues by different MHC alleles is the core concept when designing heteroclitic peptides with increased MHC binding properties [67-69]. The underlying theory is the link between increased peptide affinity for MHC, stability of the pMHC molecule and enhanced immunogenicity [70]. The application of modified peptides is mostly found in development of peptide vaccines and therapeutic treatments for cancer [71].

A number of tumor-associated antigens (TAA) presented by MHC-I on human cancer cells has been identified including peptides derived from MC1R [72], MART-1 [73], NY-ESO-1 [74] and gp100 [75] that are recognized by CTLs. However, TAAs generally possess low MHC affinity leading to poor immunogenicity. The preferred motif for HLA-A0201, the most common human MHC-I allele, is defined by p2L and pΩL/V (the last residue at the C-terminus of the peptide) [76], which is often not present in MHC-I restricted TAAs [77]. Moreover, TAAs are most often of self-origin, over-expressed or slightly modified versions of their parent peptides [78]. Negative selection will efficiently eliminate the majority of T cells recognizing TAAs in an attempt to maintain self-tolerance, thereby limiting the pool of TAA-reactive T cells. Anchor-modified peptides thus represent an attractive approach to overcome self-tolerance and increase MHC stability to target $CD8^+$ T cells for killing tumor cells. Modification of anchor residues results in better MHC stabilization capacity due to improved peptide binding which has been connected to increased TCR affinity, longer cell-surface presentation and increased T-cell activation [79-81]. Other than increasing MHC stability, modification of cysteine residues at anchor residues is also commonly used to prevent oxidation and dimerization [82-84] which also affects the immunogenicity of an antigen.

The assumption when modifying anchor residues is that the conformation the peptide takes in the MHC cleft is not altered which would ultimately interfere with the idea of eliciting cross-reactive TAA-specific T cells (Figure 3) [68, 71, 81, 82, 85]. The effects of anchor-modifications always have to be carefully evaluated since better anchoring in the MHC cleft and higher complex stability does not always correlate with improved T-cell activation [77]. The complexity of using anchor-modified TAAs is illustrated in for example studies of MART-1, where TCRs cross-reacted between the 9-mer and the anchor-modified 10-mer variant of MART-1 [86] but not with the anchor-modified 9-mer due to an altered conformation [87]. This particular example is further discussed in section *1.3.1* on T-cell cross-reactivity.

A study from our laboratory has previously demonstrated that the immunogenicity and

MHC-stabilization capacity of the TAA $gp100_{25\text{-}33}$ (EGSRNQDWL, abbreviated EGS) was dramatically improved following substitution of serine at position 3 to proline (p3P). The main anchor positions for peptides restricted by the mouse MHC-I allele H-$2D^b$ are defined by p5 and pΩ, with a preference for asparagine (N) at p5 and methionine (M) at pΩ [28], whereas p3 is a secondary anchor residue. Comparative structural analysis revealed that the conformation of the p3P-substituted EGP was similar to EGS, and that the stabilizing effect of p3P is accounted for by CH-π and van der Waals interactions with the H-$2D^b$ residue Y159, which is conserved among most known MHC-I alleles (Figure 3). Vaccination of C57BL/6 mice with EGP elicited high frequencies of EGS-specific CTLs from the endogenous repertoire that efficiently targeted natural wild-type EGS/H-$2D^b$ complexes on melanoma cells [80]. The interesting results from this study provided a promising methodology for also enhancing the MHC binding of other H-$2D^b$-restricted peptides, which we could demonstrate in **Papers II** and **IV**.

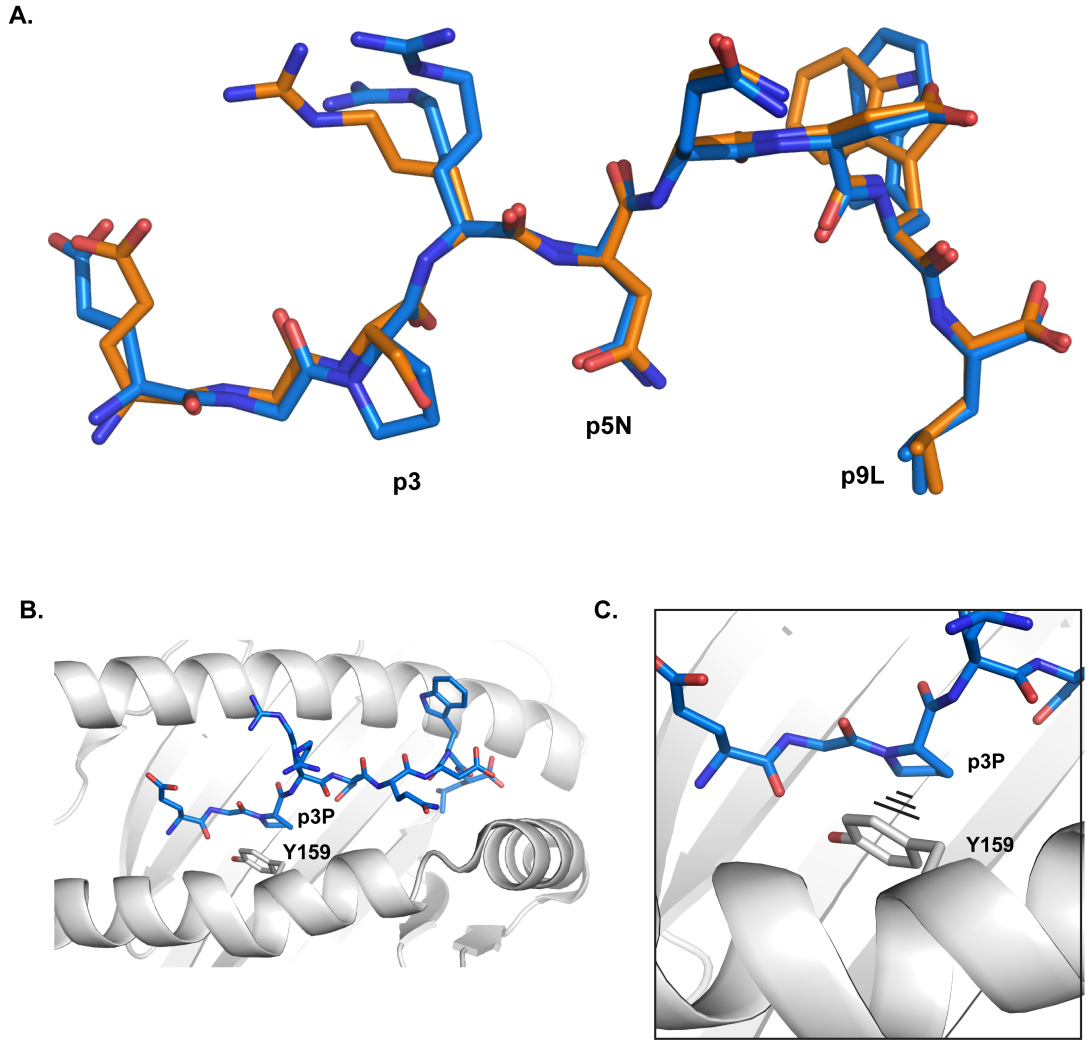


Figure 3. p3P modification of the TAA EGS ($gp100_{25\text{-}33}$) conserved the peptide conformation and enhanced H-$2D^b$-complex stability and immunogenicity. ***A.*** *Alignment of the wild-type peptide EGS (orange)(PDBID:3CCH) and the p3P-modified peptide EGP (blue) (PDBID:3CH1) demonstrated similar peptide conformations. The peptides are presented as sticks with their N-termini to the left. The main anchor residues p5N and p9L, as well as the secondary anchor residue p3, are indicated.* ***B.*** *Crystal structure of the p3P-modified peptide EGP bound in the cleft of H-$2D^b$.* ***C.*** *The increased pMHC stability is accounted for by van der Waals and CH-π interactions between Y159 of H-$2D^b$ and p3P of EGP.*

1.1.4 Structure and emergence of the TCR repertoire

The αβ TCR is the antigen receptor expressed on the cell surface of $CD8^+$ T cells. TCRs are heterodimers consisting of an α-chain and a β-chain (alternatively a γ and δ chain for γδ TCRs, which will not be addressed here) linked together by disulfide bridges. Each chain constitutes a variable and a constant Ig-like domain anchored with a trans-membrane domain followed by a short cytoplasmic tail [2]. Six complementarity determining region (CDR) loops create the antigen-binding site located on the variable domains of the α-chain (Vα) and β-chain (Vβ) (Figure 4).

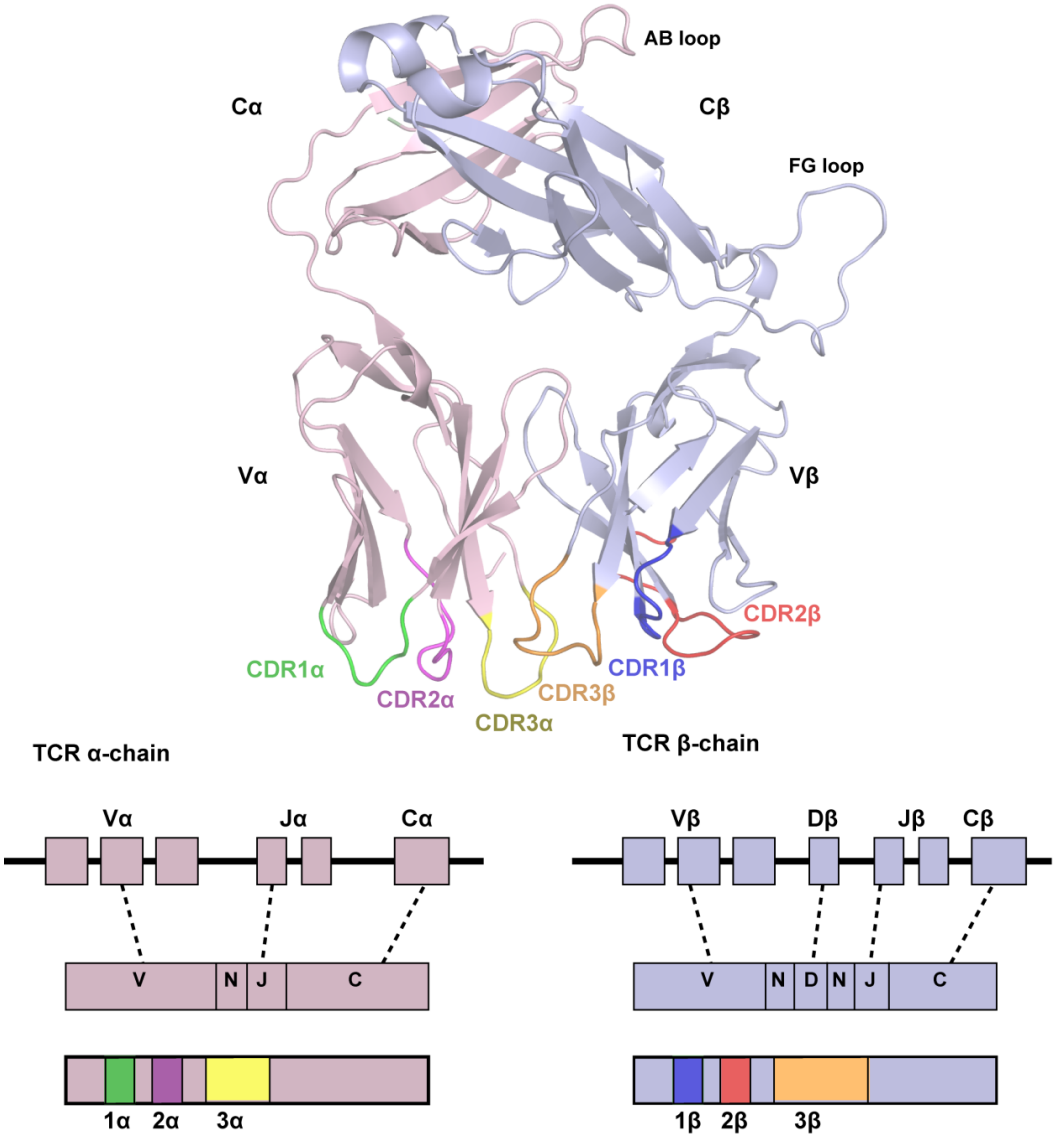


Figure 4. Recombination and structure of the αβ TCR. *The TCR, presented as cartoon, consists of an α-chain (pink) and a β-chain (light blue), each with a variable (V) and a constant (C) domain. The antigen-binding site, located on the V-domains, is created by the complementarity determining region (CDR) loops: CDR1α (green), CDR2α (pink), CDR3α (yellow), CDR1β (blue), CDR2β (red), and CDR3β (orange). The AB and FG loop are indicated, proposed as docking-sites for CD3 and involved in signal transduction upon pMHC binding (top panel). The TCR α-chain and β-chain are built from the C-V-(D)-J gene segments. The CDR1 and CDR2 loops are germline-encoded by the V-gene segments, whereas the CDR3 loops arise from recombination of V-(D)-J gene segments with addition of nucleotides (N) at the junctions (bottom panel).*

With far fewer TCR genes compared to MHC allele variants, the diversity of the TCR repertoire is manifested by multiple sets of similar gene segments that recombine to form functional genes during thymic development (see section *1.1.1*). The number of variable germline-encoded segments differs between species and even individuals (http://www.imgt.org). The joining of segments is mediated by two recombination activation gene proteins (RAG-1 and RAG-2) [88]. The β-chain is encoded by variable (V), diversity (D), joining (J) and constant (C) gene segments located at the TCRβ locus. The α-chain is similarly encoded by additional sets of V, J and C segments located at the TCRα locus [89]. The CDR1 and CDR2 loops are germline-encoded by the TRAV (Vα) or the TRAB (Vβ) genes, respectively [90]. The CDR3 loops confer the greatest variability, created by somatic recombination of the gene segments V-(D)-J. Additional diversification arises from end trimming of the junctions through addition or deletion of nucleotides (N) [91] (Figure 4). Finally, allelic polymorphism in the TCR loci adds to the diversity of the T-cell repertoire [92].

1.1.5 T-cell activation

T-cell activation begins with the interaction between cognate pMHCs on a professional APC and TCRs (Figure 5A). Although the TCR provides the specific activation signal when bound to an antigenic pMHC, activation also involves participation of the co-receptor CD8, which serves to recruit the Src-family kinase Lck to the cytoplasmic tails of the forming TCR/pMHC/CD8 complex. Further, the role of CD8 is to stabilize the TCR/pMHC interaction as well as enhance antigen sensitivity and response of activated cytotoxic T cells to pMHCs. CD8 exists in different isoforms; homo- or heterodimer of an α-chain and/or a β-chain, where CTLs and αβTCR thymocytes express the CD8αβ isoform [93]. Apart from CD8 in the TCR/pMHC interaction an additional signal is also required which can only be delivered by professional APCs. The co-stimulatory molecules CD80 or CD86 on APCs, which interact with CD28 on T cells provide the second signal (Figure 5A). Without co-stimulation from an APC the T cell becomes anergic, as a mechanism to protect against autoimmunity. The interaction between the T cell and the APC is mediated by adhesion molecules, which is further increased as microclusters of hundreds of TCRs form. The TCR clustering is thought to sensitize the T cell, where a small number of agonist pMHCs can trigger several closely located TCRs [94].

T-cell activation, where naïve T cells differentiate into CTLs, is characterized by clonal expansion and expression of cytokines such as interferon-γ (IFNγ) and tumor necrosis factor (TNF) used by T cells to inhibit viral replication and kill infected cells (Figure 5A, B). One of the earliest events in T-cell activation is cell surface down-regulation of TCRs [95]. These activation markers occur to varying extents and are often measured to evaluate the level of T-cell activation.

The cytoplasmic tails of the TCR α-chain and β-chain are very short, instead signal transduction to the cell interior is mediated by the membrane spanning complex CD3 [96] (Figure 5C). CD3 plays a central role in T-cell activation as evident from stimulation with anti-CD3 antibody, which can alone trigger T-cell activation [97]. The three signaling dimers of CD3: CD3δε, CD3γε, and CD3ζζ, form pairwise ionic

interactions between basic residues in the trans-membrane domains of the TCR and acidic residues located in each of the three CD3 dimers [98]. TCR stimulation results in phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) of the cytoplasmic portions of CD3 by Lck. This event initiates a downstream phosphorylation cascade involving ZAP-70 among others amplifying the signal which results in an altered gene expression that induces differentiation into a cytotoxic T cell [99].

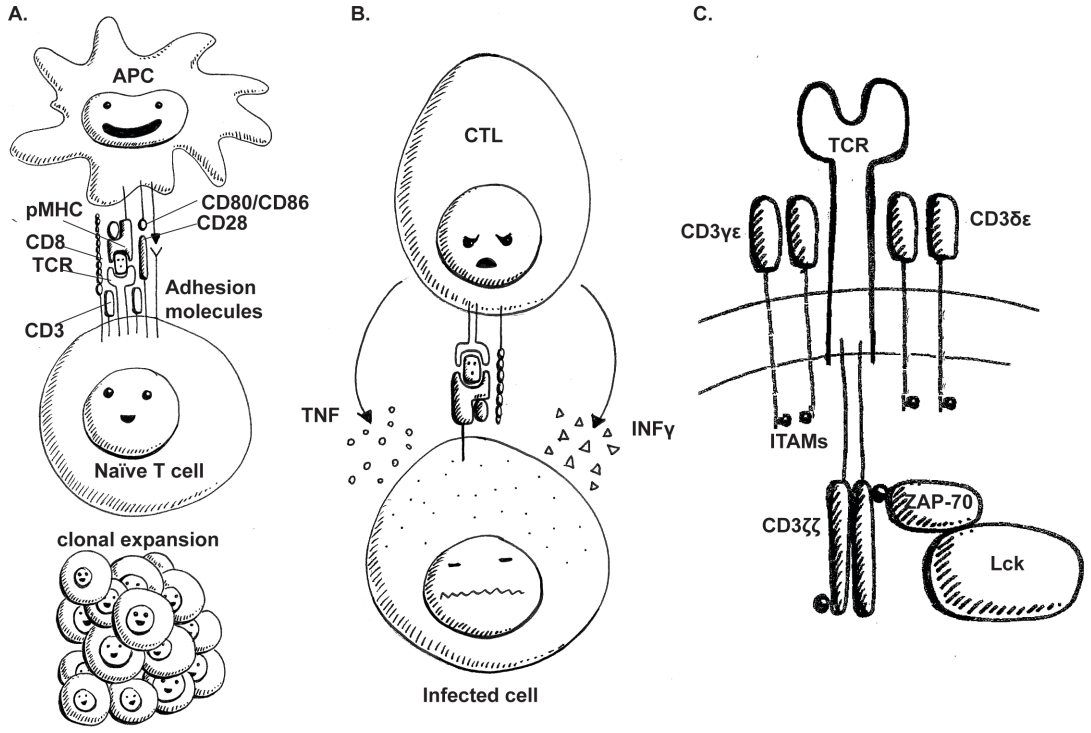


Figure 5. T-cell activation. A. *Naïve T cells are activated by professional APCs. The TCR provides the specific activation signal when bound to an antigenic pMHC. The interaction also involves the co-receptor CD8, which binds to the pMHC. Co-stimulation is also required for activation, which can only be delivered by professional APCs, through CD80 or CD86 interacting with CD28 on the T cell. The interaction between the T cell and the APC is enhanced by adhesion molecules. Following activation the T cell goes through clonal expansion* ***B.*** *Activated CTLs express cytokines such as IFNγ and TNF used to inhibit viral replication and kill infected cells* ***C.*** *The cytoplasmic tails of the TCR are very short; instead signal transduction to the cell interior is mediated by the three signaling dimers CD3δε, CD3γε, and CD3ζζ. Binding of CD8 to the pMHC serves to recruit the kinase Lck to the cytoplasmic tails of the TCR/CD3 complex which phosphorylates the ITAMs of the cytoplasmic portions of CD3. This event initiates a downstream phosphorylation cascade involving ZAP-70 among others amplifying the signal which results in differentiation into a CTL.*

Although it is clear that CD3 transduces signals upon TCR interaction with a pMHC, exactly how ligation translates into the first signal remains controversial [100]. Several non-excluding models exist trying to explain signaling transduction upon TCR triggering, including TCR clustering and conformational changes in the TCR [99]. An interesting study by Hawse *et al.* demonstrated that the TCR globally rigidified upon pMHC ligation, in particular the FG loop (Figure 4) on the TCR β-chain that has been proposed as a docking site for CD3εγ [101]. Another study by Beddoe *et al.* observed a conformational change in the AB loop located within the Cα domain of the TCR upon

ligation to pMHC [102] (Figure 4). These two studies provide insights into initiation of signal transduction to the CD3 complex upon T-cell triggering, however many details around CD3 in TCR activation remain elusive, including the stoichiometry between the TCR and CD3 [96].

1.1.5.1 Altered peptide ligands

T-cell activation is not digital, rather various levels of activation can be reached depending on the peptide´s ability to stimulate a T cell. Altered peptide ligands (APLs) have been defined as synthetic peptide analogues of immunogenic peptides where TCR contact residues have been substituted [103]. They can elicit completely different T-cell responses compared to their wild-type counterparts, although they might differ in only a single amino acid.

APLs are categorized according to the immune response elicited; *agonists* have the capacity to trigger a full range of T-cell activation events; *weak agonists* induce a subset of activation events; *null peptides* have no effect; and *antagonists* inhibit immune responses by the agonist in a competitive and dose-dependent manner [104]. Most commonly APLs evoke a weaker response than the wild-type peptide does [103]. However, super-agonist APLs with greater T-cell activation potencies also exist, exemplified by the H-2K^{b}-restricted SIYR peptide which represents a 10^{5}-fold more potent version than the self-peptide dEV8 to 2C T cells [105]. The picture gets more complicated as it has been demonstrated that weak agonists can also act as partial antagonists [106] so the concept of agonists and antagonists is not straightforward. In summary, the wide range of activation potencies elicited with different APLs is useful when studying mechanisms underlying T-cell activation.

It has so far not been possible to explain T-cell activation potencies and the different responses induced by agonists, weak agonist or antagonists from a structural perspective [107, 108]. However, reduced surface complementary at the binding interface in complexes with weak agonists and antagonists compared to agonists [105, 107], as well as deviating binding topologies, have been observed [109]. Thus, the different functional outcome could be manifested by very subtle structural differences at the binding interface somehow, which is transduced to the CD3 signaling complex [102]. A correlation between functional T-cell phenotypes and altered phosphorylation patterns of the CD3 ζζ-dimer has been described [103]. Incomplete phosphorylation of CD3 from interaction with APLs would dictate subsequent downstream signaling in the T cell ultimately leading to different biological responses [110].

1.1.5.2 Different theories explaining T-cell activation

T cells are proposed to be highly sensitive to cognate pMHC ligands, where the number of ligands required to initiate TCR signaling and achieve full activation ranges from one to a few hundreds pMHCs per APC [111-113]. It has been suggested that endogenous or null pMHC ligands are involved in activating T cells by enhancing the interactions with agonist pMHCs. The pseudo dimer-model for CD4^{+} T-cell activation proposed by Davis *et al.* suggests that an agonist TCR/pMHC complex forms a stable pseudo dimer with a *'co-agonist'* endogenous pMHC [114]. The contribution of

endogenous pMHCs has similarly been shown to play a role in $CD8^+$ T-cell activation [115-117].

Different models exist to explain how various factors, such as affinity, kinetics and complex half-life may influence T-cell activation [99]. The kinetic proofreading-model explains the observed differences in activation levels as a consequence of the duration or half-life ($t_{1/2}$) of the TCR/pMHC interaction [118]. Weak interactions will not endure long enough for the intracellular signaling cascade to be completed resulting in inefficient or no activation. This model is supported by reports of agonists having slower dissociation rates compared to semi-agonists or null ligands [119].

Serial triggering is another model used to explain T-cell activation [120], which suggests that an optimal dwell-time (or confinement time) between the TCR and the pMHC leads to the most efficient T-cell activation. It was hypothesized that the number of triggered TCRs decides whether or not a T cell gets activated [120, 121]. This theory argues that a minimal $t_{1/2}$ is required in order to complete the intracellular signaling cascade, but that too long of an interaction would impair the serial triggering resulting in insufficient activation [122, 123]. Further, interactions with fast association rates (k_{on}) will result in more efficient activation due the phenomenon of rebinding between TCR and pMHC. Results from other studies appreciate that the density of antigenic pMHC on the surface of infected cells is the most crucial parameter, which finds support in the serial triggering model [124]. Finally, the affinity model correlates affinity alone with T-cell activation without taking in consideration the influence of kinetic parameters [125]. This model states that high-affinity pMHC ligands require fewer TCRs on the T cell to induce activation [126]. However, none of these models can explain all available experimental data, so the underlying basis for T-cell activation remains elusive.

It is in many cases difficult to simulate *in vivo* environments and measure biological parameters correctly *in vitro*. More recently developed techniques for measuring kinetic parameters between membrane-bound TCRs and pMHCs contradict results obtained from 3D measurements using soluble receptors. Kinetics parameters measured in 2D were substantially faster, and the dissociation rate showed an inverted relationship with the T-cell activation potency [127, 128]. It is also difficult to mimic the natural antigen presentation *in vitro*, where the distribution of MHC molecules in clusters on the cell surface [129] are thought to sensitize T cells to antigen [130]. Cell-based assays to measure target killing by T cells often use exogenous loading of peptides, which does not generate MHC clustering on the cell surface thereby requiring higher levels to reach the same T-cell activation [131]. In conclusion, the complexity of *in vivo* environments, with the many factors that influence T-cell interactions might reflect the difficulty in finding a model or parameter that correlate with T-cell activation in all cases.

1.1.5.3 T-cell activation studied with the P14 TCR system

A few well-characterized model systems have significantly contributed to our understanding of T-cell specificity, cross- and auto-reactivity highlighting the subtle differences that translate into a whole spectrum of functional outcomes. It is not coincidental that the two, perhaps most exploited, TCRs are the H-$2K^b$-restricted 2C

[132-134] and the HLA-A2-restricted A6 [107, 135, 136], which were the first ternary complexes to be reported [132, 135]. For decades both have been used in structural, thermodynamic, kinetic and functional studies providing an extensive base for understanding the molecular interactions between TCRs and MHCs. The human A6 TCR is not only specific for the Tax peptide from the human T-lymphotropic virus 1 (HTLV-1), from which a whole set of APLs have been designed [107, 136, 137], but also cross-recognizes the yeast peptide Tel1p [138] as well as the self-antigen HuD [139]. The catalogue of solved structures involving A6 [107, 135, 137-140] in various state with and without its ligands is rather complete, which is an important reason for its prominent role as a model TCR. Similarly, the 2C TCR, recognizing the self-antigen dEV8, has been used extensively to study for example cross- and allo-reactivity [134] with different MHC-I and peptide ligands and has also been crystallized in several forms [105].

The studies included in this thesis are built on the P14 TCR system. **Paper I, II** and **IV** all utilized P14 to investigate various aspects of T-cell activation including sensitivity, specificity and cross-reactivity of different pMHC ligands. The P14 T-cell clone was isolated from mice, infected with the Lymphocytic Choriomeningitis Virus (LCVM) by Pircher *et al.* [141] with subsequent cloning of the TCR P14 by the same investigators [142]. P14 T cells and TCRs have ever since the discovery and isolation in 1987 been used in numerous studies resulting in a well-established model system for T-cell activation [95, 125, 143-146].

The P14 TCR exhibits high specificity for its cognate antigen, the immunodominant peptide gp33 [147] (KAVYNFATC, residues 33-41 of the LCMV glycoprotein, however we are using the C9M-variant for stability reasons in all of our studies similar to previous studies [83]) presented by the mouse MHC-I allele H-2D^b. LCMV is an RNA virus with a naturally high mutation rate, facilitating together with a selective CTL pressure, the emergence of viral mutations in the gp33-epitope, which can escape P14 T-cell surveillance [148]. Crystal structures of gp33/H-2D^b revealed the side chains of p4Y and p6F to project out of the peptide binding cleft likely acting as TCR contact sites [149-152] rendering them attractive mutation targets for LCMV. Indeed, escape variants have been isolated harboring mutations at these positions, of which p4Y mutated to a phenylalanine (Y4F) [148] is in focus in **Papers I** and **II**. The Y4F mutation in gp33 abolished P14 T-cell mediated immune responses against LCMV-infected cells [125, 146, 148]. In **Paper II** we present the crystal structure of P14 in complex with gp33/H-2D^b as well as an APL, which demonstrates the prominent role of p4Y in the interaction.

Beyond the viral escape variant Y4F, a hierarchical set of APLs ranging from a full agonist, weak/partial agonists to antagonists has also been defined [95, 106, 146, 153] carrying various mutations at p4 and p6. Substitution of p4Y to a serine renders Y4S a P14 antagonist [6, 95]. In contrast, substitution to alanine (Y4A) was still weakly recognized by P14 [106, 125] and able to induce positive selection [6] although it carried the most significant structural alteration when compared to both Y4F and Y4S [154].

Finally, the P14 system is also suitable for studying auto-reactivity. The mDBM peptide (KALYDYAPI) was together with other rodent peptides identified by their sequence homology to gp33 and ability to bind H-2D^b [145]. mDBM is derived from the enzyme dopamine-mono-oxygenase and found in the adrenal medulla [155]. LCMV infection of P14 TCR transgenic mice gives rise to T-cell infiltration in the adrenal medulla along with altered dopamine levels. Molecular mimicry between gp33 and mDBM was suggested to cause the auto-reactivity [145]. The processing and presentation of mDBM on H-2D^b was confirmed as well as recognition by the P14 TCR [145]. In addition, mDBM has been shown to mediate positive selection of P14 T cells [156]. Subsequent structure determination of DBM in rat (rDBM, KALYNYAPI) in complex with H-2D^b revealed a striking structural similarity to gp33 [157]. Collectively, these findings were interesting and form the basis for **Paper IV**, where we further investigate the structural basis for cross-reactivity between gp33 and mDBM.

1.1.6 Mechanisms of viral escape

In addition to interfering with the intricate MHC presentation system, viruses have evolved to escape T-cell recognition by directly targeting TCR-interacting residues on the peptide [43, 158]. T-cell responses in viral infections are typically directed towards a limited number of viral epitopes termed immunodominant, which is largely determined by peptide binding affinity to MHC [159]. The resulting selective pressure imposed by the adaptive immune system often leads to the emergence of viral populations with a limited number of escape mutations [148, 150, 154], since a majority of escape mutations results in a significant reduction of viral fitness [65]. Previous studies have correlated viral escape variants with disease progression [160, 161] and thus represent a major hurdle for disease control. Attempts to induce cross-reactive T-cell responses against viral escape mutants have thus far been unsuccessful [162, 163]. The ability to design viral vaccines targeting escape variants is therefore desirable and in greater detail investigated in **Paper II**.

1.2 TCR RECOGNITION OF PEPTIDE/MHCS AT THE MOLECULAR LEVEL

The simultaneous recognition of antigenic peptides and MHC molecules by TCRs lies at the heart of adaptive immunity. The Nobel-prize winning discovery by Zinkernagel and Doherty demonstrating that T cells recognize *"altered self"* [164] began unraveling the basis for T-cell mediated immunity, which was visualized decades later with the first crystal structures of a TCR/pMHC complex [132, 135]. Ever since then the number of determined co-crystal structures of TCR/pMHC complexes has slowly grown, outlining some general rules of engagement. Although the accumulation of TCR/pMHC structures has answered a lot questions in cellular immunity, many remain unanswered for example how TCRs can distinguish agonists from antagonists [107].

1.2.1 Structural overview of TCR interactions with pMHC

Typically, the TCR sits atop diagonally over the pMHC making contact with both peptide and the two α-helices of the MHC using the six CDR loops (Figure 6A). This docking angle, which normally falls within 22-70° degrees for TCR/peptide/MHC-I,

leaves Vα positioned over the α_2-helix of the MHC, and Vβ over the α_1-helix [2]. The four germline-encoded CDR1 and CDR2 loops contact mainly the MHC, whereas the hyper-variable CDR3 loops interact more extensively with the peptide [109, 139, 165] (Figure 6AB). This is however not a strict observation, rather a common feature with several exceptions reported. Structures involving the A6 TCR highlight the exception from these rules where HLA-A2 is not contacted by CDR1β and CDR2, but instead can contact the peptide, and the CDR3 loops make contacts with the MHC [166, 167].

Crystallographic studies of free and bound pMHCs and TCRs have demonstrated that conformational adjustments in all three components [138], including peptide [51, 136, 168], MHC [51, 169] and TCR [170], commonly occur. A closer look reveals that the surface complementarity between TCR and pMHC is often imperfect, which requires compensation and rearrangements to enable binding. This is sometimes referred to as *"conformational melding"* [139] and is most commonly conferred by the CDR loops, exhibiting great flexibility in the unliganded state as evident from crystal structures that to various degrees lack electron density around the CDR loops [140].

1.2.2 TCR/pMHC docking mode

The underlying basis for the seemingly conserved TCR/pMHC binding mode remains a debated issue. Two competing, although not mutually exclusive, hypotheses exist trying to explain the roughly diagonal binding mode.

Some evidence suggests that TCRs have been evolutionary selected to bind MHC molecules [171]. This hypothesis, originally put forward by Niels Jerne, states that TCRs and MHCs may have evolved together [1], as evident from conserved interactions and patterns occurring in several structures between the germline-encoded CDR1 and CDR2 loops and MHC residues.

Alignment and analysis of the structural database have found common TCR residues involved in MHC binding, this is however more evident for binding to MHC-II than MHC-I [171, 172]. Two tyrosine residues in the CDR2β loop, Y46 and Y48, have been identified as conserved and are shared even across distantly related species [171]. Likewise, certain MHC residues are readily contacted in the majority of solved structures, although not necessarily by the same TCR residues. The *"restriction triad"* comprising positions 155, 65 and 69 [173], is described as a crucial interaction site as well as K66 [174] that, together with R65 and R69 forms a TCR footprint on HLA-A2 [167, 175] (Figure 6C). Proving the model correct is however not trivial as T cells without MHC-reactivity are not positively selected during thymic development, at the same time as negative selection consequently deletes TCRs with strongest preference for MHC. One also has to bear in mind the bias in the structural TCR database that has arisen towards the mouse Vβ8 family, or the equivalent Vβ13 in humans, due to higher protein expression levels in *E. coli* and more successful protein folding. It makes more sense to compare TCRs belonging to the same Vβ family binding to the same MHC allele, since variations in both TCR and MHC genes would lead to different patterns of conserved interactions for each combination of alleles and families. Thus, conclusions regarding a co-evolution between TCRs and MHCs with conserved interactions are mostly derived from one Vβ family.

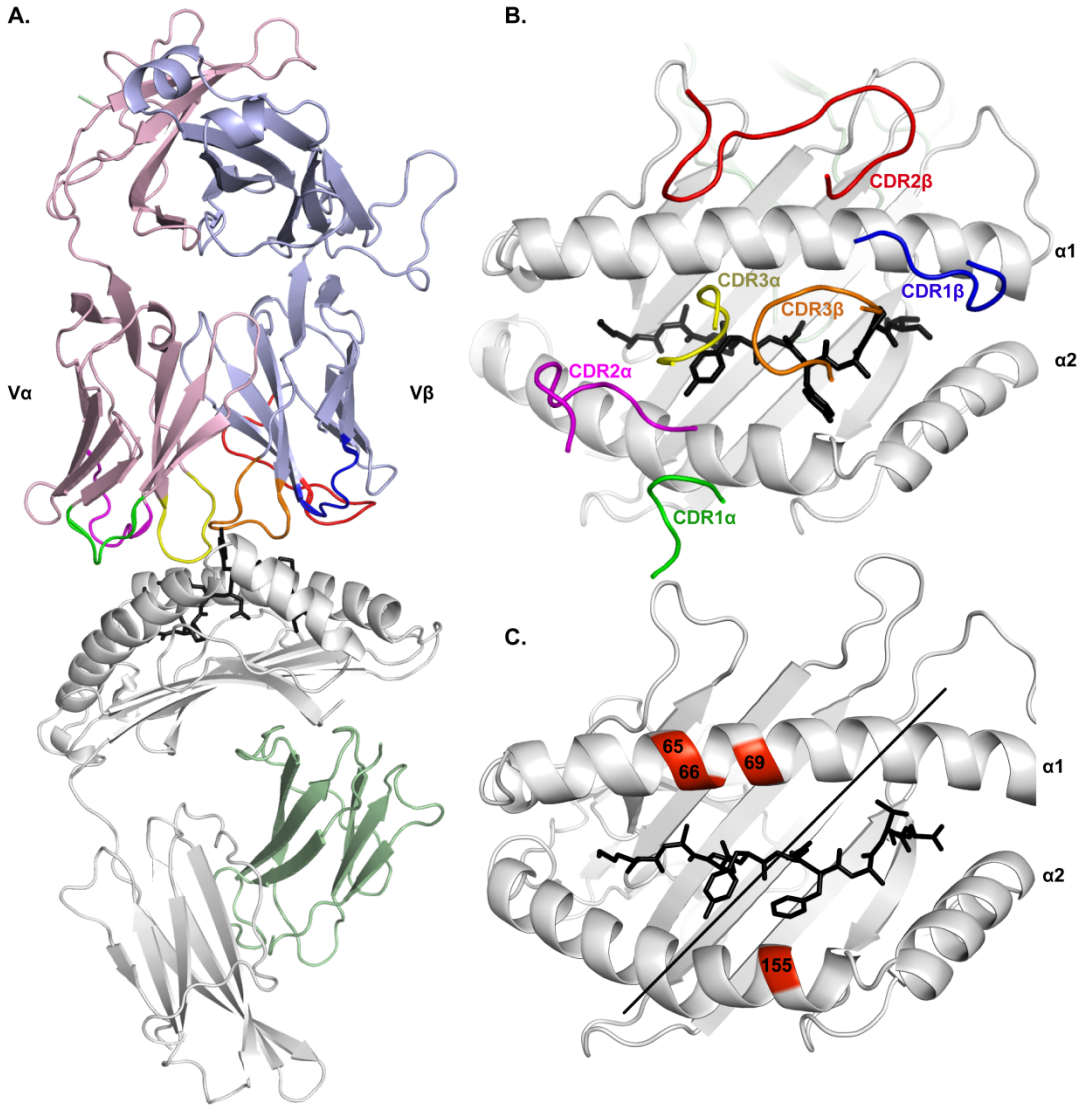


Figure 6. The TCR/pMHC interaction. *The TCR and MHC are presented as cartoons and the peptide is presented as sticks. The TCR α-chain (pink), β-chain (light blue), CDR1α (green), CDR2α (pink), CDR3α (yellow), CDR1β (purple), CDR2β (red), and CDR3β (orange).* ***A.*** *The TCR binds diagonally over the pMHC, with Vα over the α_2-helix of the MHC and Vβ positioned over the α_1-helix.* ***B.*** *The pMHC surface seen from the TCR´s perspective. The CDR1 and CDR2 loops are mainly interacting with the MHC, whereas the CDR3 loops binds over the peptide.* ***C.*** *The TCR binds to the pMHC using a diagonal docking angle (black line). The MHC residues 65, 66, 69 and 155 (red) are frequently contacted by TCRs.*

The conserved TCR/pMHC docking topology could also be a consequence of co-receptor steric influence during thymic selection [172, 176, 177]. CD8 binds to a non-polymorphic site in the α3 domain of the MHC (Figure 2A) [45] and to conserved residues of the TCR thereby possibly constraining the docking angles. It is not entirely clear whether the docking angle needs to fall within a certain range in order to generate a productive intracellular signaling or is merely a result of the physical restrain imposed by formation of the TCR signaling complex. Interestingly, a skewed docking angle has been connected to impaired T-cell signaling and antagonism [109]. In other cases cross-

reactive TCRs able to bind APLs with varying T-cell activation potencies have shown similar docking modes [178], and in many cases share key TCR contact residues [107, 178, 179] showing no correlation between docking angle and quality of intracellular signaling. In support of thymic selection and co-receptors as determinants for MHC-restriction, mice lacking MHC and co-receptors have TCRs that are not biased towards pMHCs, but instead recognize ligands in an MHC-independent manner similar to antibodies [180].

1.2.3 Thermodynamics

While X-ray crystallography can give us mainly static snapshots, thermodynamic analysis complements structural biology in an insightful way providing dynamics and energetics to the picture of TCR/pMHC interactions. Methods for predicting thermodynamic parameters in protein-protein interactions from available structures exist and are largely based on changes in polar and non-polar surface area [181]. Some investigator have reported an apparent lack in correlation between change in binding enthalpy/entropy and structural features at the binding interface for TCR/pMHCs interactions [175, 182]. However, in many studies thermodynamic measurements have nevertheless proven useful for understanding the impact of specific alterations in CDR loops, modification in MHC residues [174] or peptide [183, 184] on the resulting interactions.

The first reported thermodynamic measurements of TCR/pMHC interactions were characterized by enthalpically favorable and entropically unfavorable thermodynamic signatures [182, 185]. The simple explanation for the unfavorable entropy was provided by structural studies of free and bound TCRs where ordering of the flexible CDR loops upon binding was observed [186]. This thermodynamic trend was questioned with the report of the A6 TCR binding the Tax-peptide in complex with HLA-A2 through a favorable entropic interaction [175]. More recent studies also add to the notion that recognition of pMHCs by TCRs can be entropically driven [178, 187-189] thus overthrowing the idea of a general thermodynamic signature. The early view stating that it was the consequence of ordering CDR loops that would lead to unfavorable entropy of TCR/pMHC interactions was clearly over-simplified. Multiple factors come into play influencing the energetic profile of any given protein-protein interaction, including conformational changes, peptide mobility, desolvation and overall reduced flexibility but also bond formation and hydrophobic interactions among others.

The great variation in the composition of TCRs, peptides and MHC molecules generates interfaces with widely different chemical properties and features. Although the interpretation that ordering of CDR loops upon binding occurs at an entropic cost seems correct this effect might be cancelled out by e.g. release of water molecules [187]. High-resolution crystal structures allow for accurate modeling of water molecules that can influence protein-protein interactions in favorable ways by filling cavities, thereby increasing surface complementary, as well as mediating hydrogen bonds [51, 190, 191]. However, ordering of waters in hydrophobic cavities on the other hand is not thermodynamically favorable, and associated with an entropic cost [192]. In contrast, release of coordinated water molecules at the interface will both increase hydrophobic interactions and result in a favorable entropic contribution through a gain

in freedom for the released water molecules [187]. Dehydration using osmolytic agents was tested on two different TCRs and pMHCs with opposite effects, weakening one while enhancing the other [193]. In conclusion, the role of water molecules in TCR/pMHC interactions has various impact factors depending on the interaction but is many times an important player.

A favorable change in enthalpy is generally connected to a net increase in hydrogen bonds and van der Waals interactions [191]. Importantly, internal contacts in the free state have to be taken into account, not just the ones formed at the TCR/pMHC interface [187]. This is why it is difficult to connect the number of intermolecular contacts formed to the change in enthalpy [182].

So far, change in heat capacity (ΔCp) has always been found to be negative for TCR/pMHC interactions, indicating an overall net increase in burial of hydrophobic surfaces area (BSA) upon complex formation [181, 194]. Commonly, |ΔCp| is larger than predicted [184] which supports TCR binding through induced fit-mechanisms consistent with reduced conformational entropy [194, 195].

What can be concluded from thermodynamic data gathered from many TCR/pMHC interactions is that the ΔG term always appears within a narrow window whereas the spread in the contributing enthalpy/entropy terms is much greater [182]. The variation in ΔS and ΔH are interconnected in a compensatory manner, which can be interpreted in terms of protein-protein interactions as the balance between flexibility and formation of bonds. Whether this is a valid conclusion for TCR/pMHC interactions remains controversial. Speaking against, is an apparent lack of correlation between number of contacts formed in TCR/pMHCs interaction and the value of ΔH [182]. Unfortunately, structural data is often missing for all participants, before and after binding, making analyses of this kind impossible in many cases. It should also be said that the conformational entropy and energetic state of free receptors might vary widely. NMR spectroscopy studies can provide information regarding the dynamics of CDR loops in the unliganded state, as in the case of the D10 scTCR where great mobility on the picosecond timescale was recorded [196]. However this study is the only one of its kind and equivalent NMR studies of other free TCRs are necessary to draw general conclusions.

1.2.3.1 Measurement techniques – SPR vs. ITC

Two techniques are routinely used for assessing thermodynamics and binding constants in protein-protein interactions with different advantages and disadvantages.

Van´t Hoff analysis has traditionally been the most used technique for thermodynamic analyses [182], where measurements are typically performed on a Surface Plasmon Resonance (SPR) instrument. SPR is an extensively used method to characterize protein-protein interactions and retrieve kinetic and equilibrium data. It is an easy-to-use method suitable for comparative studies where low-affinity interactions and protein available in limited amounts can be analyzed since ligand immobilization on sensor chips requires very little protein. Kinetic studies of TCR/pMHC interactions with SPR have indicated that the association rate is slow to medium, typically $<10^5$ $M^{-1} \cdot s^{-1}$ [197],

supporting conformational adjustments upon binding. However, results from recent 2D methodologies have measured much faster on-rates [127, 128].

The affinity can be calculated either from dividing the dissociation rate (K_d) with the association rate (K_a) or from the steady state equilibrium plotted against analyte concentrations. For thermodynamic analyses the affinity is detected over a range of temperatures. ΔG is directly obtained from the binding affinity (K_D):

$$\Delta G=RT\ln(K_D)$$

Where R is the universal gas constant (8.314 $J\cdot K^{-1}\cdot mol^{-1}$) and T is the temperature in Kelvin. ΔH and ΔS are calculated from the equation of ΔG as a function of temperature:

$$\Delta G=\Delta H-T\Delta S$$

In the case of protein-protein interactions ΔG usually varies non-linearly with the temperatures, so the equation can be rewritten with the incorporation of a change in heat capacity, (ΔCp):

$\Delta G = \Delta H_{T0}-T\Delta S_{T0} + \Delta Cp(T-T_0) – T\Delta Cp\ \ln(T-T_0)$, where $T_0 = 298.15K$.

The indirect way of deriving ΔH and ΔS presents the main disadvantage with this method since even small inaccuracies in the binding affinity will have a profound effect on the thermodynamic parameters calculated from the van´t Hoff equation [175]. Soluble TCRs and pMHCs are often difficult to produce due to low protein stability [198] resulting in low yields that can affect the quality and precision of data acquisition. However, the possibility to measure interactions between proteins using only trace amounts is a clear advantage with SPR. Although, relatively high concentrations of the analyte are normally required to reliably determine TCR/pMHC interactions, which are typically of low to medium affinity (1-100μM) [64].

In contrast to SPR, isothermal titration calorimetry (ITC) has been described as the more reliable and therefore preferred technique to use for thermodynamic analysis [199]. However, several studies report similar thermodynamics values between van´t Hoff analysis derived from SPR measurements and ITC [184, 185, 187]. In ITC, the heat absorbed or released during an interaction is directly measured and corresponds to the change in the reaction enthalpy ΔH. Additionally, the stoichiometry and change in free energy ΔG can also be obtained from fitting data to the appropriate equation. Unlike SPR, in which multiple measurements over a spectrum of temperatures is required in order to obtain all thermodynamic parameters, ITC will return ΔG, ΔH and ΔS in one experiment. If the stoichiometry of an interaction is known, as in the case for TCRs that bind pMHCs in a 1:1 ratio, uncertainties in concentrations or proportion of active protein can be corrected for. Although superior to SPR, ITC has not been used nearly as much for assessing thermodynamics in TCR/pMHC interactions owing to the requirement for higher protein concentration, which is a constant limitation in TCR/pMHC studies [199].

1.3 THE BALANCE BETWEEN IMMUNITY AND SELF-TOLERANCE

The early clonal selection hypothesis, put forward by Jerne [1] and later developed by Burnet [200], proposed that each T cell is specific for a single pMHC molecule. Based on a *"one-clonotype-one-specificity"* assumption, this hypothesis requires $>10^{15}$ TCRs to be generated in order to cover the vast universe of potential foreign peptides that can bind MHC [60]. However, the number of possible TCR variants humans has been estimated to 10^{12} with typically less than 10^8 present in the naïve repertoire [201] which means that T cells must be able to recognize multiple pMHCs to cover all potential foreign antigens. To meet the two criteria; self-tolerance and protection against all possible pathogens, antigen specificity is ultimately compromised leading to cross-reactivity.

1.3.1 T-cell cross-reactivity at the structural level

It has been estimated that any given T cell is capable of recognizing approximately 10^6 different pMHC ligands [202]. T-cell cross-reactivity is described in numerous studies [86, 203-205] most commonly explaining the ability to recognize multiple pMHCs by molecular flexibility and dynamics of the CDR loops [185, 186]. A large enough number of TCR structures is now available in both free and liganded states [54, 105, 132, 137, 140, 169, 170, 188, 206-209] to allow for general conclusions regarding conformational changes that occur upon binding. Although one should keep in mind that limitations in crystallizing free TCRs with high degree of disorder could present a bias in the analysis.

Overall, it appears not to be a difference between MHC-I and MHC-II restricted TCRs, instead all structures of TCRs pre *versus* post pMHC ligation indicate alterations in one if not more of the CDR loops upon binding [186]. Some TCRs do not adjust as much as others [81]. A feature that promotes flexibility is the presence of glycine residues, which is common in CDR3 loops, and thought to facilitate rearrangements at a lower energetic cost [107, 210]. The loop movements observed going from unbound to bound are confined to specific adjustments that can be broadly categorized into three types: loop remodeling, hinge-bending motions and rigid-body shifts. Thus, the notion by Garcia and Adams that CDR loops are unlikely to be "*easily accommodating limp noodles"* [211] fits well with this analysis [186]. Typically, the largest shifts are observed in the CDR3 loops with greater movements in the α-chain than the β-chain of the TCR [186]. Thus, it seems safe to assume that conformational changes occur routinely in TCRs upon binding, mediating cross-reactivity.

Induced fitting of CDR loops could be carried out in a two-step mechanism, in which the TCR first adjusts to the MHC and thereafter scans and adjusts to the peptide proposed by Davis *et al*. [212]. Alternatively, the existence of a series of compatible TCR conformations in equilibrium allows for cross-reactivity between multiple ligands according to the *"conformer"* model [213].

The A6 TCR bound to HLA-A2 presenting the Tax peptide is one of the first two reported ternary TCR structures [135] and has since then been crystallized together with six APLs of Tax [107, 137, 214] and recently in the free state [140] which makes for an excellent case to investigate TCR cross-reactivity. In summary, what can be said

from the analysis of these structures is that the CDR3β loop shows a greater variability in its conformations – ranging from similar to binding of the wild-type Tax, to more significant alterations depending on the APL. In contrast, the conformations of CDR3α as well as the germ-line encoded loops are seemingly conserved. However, both CDR3 loops occupied different conformations before and after pMHC binding. Similarly, the Tax-peptides did also adjust upon TCR binding. Other examples of residues in the peptide [136, 168] or MHC adjusting upon TCR binding have been observed, sometimes referred to as *"conformational melding"* [139] meaning that all three components can be repositioned to facilitate binding.

Another interesting case of T-cell cross-reactivity has been observed between the nonameric (residues 27–35, AAGIGILTV) and decameric (residues 26–35, EAAGIGILTV) variants of the melanoma antigen MART-1. TCR cross-recognition in this case is of clinical importance since the nonamer is the biologically relevant form whereas the anchor-modified 10-mer is more stable when bound to HLA-A2. In the unbound form the 9-mer assumes an extended conformation in the groove of HLA-A2 whereas the 10-mer is more bulged [87]. Interestingly, upon TCR binding the nonamer adopts the bulged conformation similar to the 10-mer [86]. This adaptation is prevented if the 9-mer is anchor-modified with a leucine at p2 hence preventing cross-reactivity to this variant. An interesting and different strategy was used by the TCR DMF4 when cross-recognizing the nonameric form of the MART-1, originally selected to react towards the decamer. The docking angle over the pMHC was rotated by 15° resulting in alterations in all of the CDR loops *except for* the CDR3β loop which remained over the C-terminal part of the peptide. Thus, this case is quite the opposite compared to cross-recognition by A6 and other TCRs, where the CDR3β loop was showing the highest variability [190].

What can we learn from these examples in terms of TCR strategies for cross-recognition? Really just that it can occur in so many ways. Each TCR/pMHC interaction is unique and with today´s technologies impossible to predict before determination of the ternary structure.

1.3.2 T-cell auto-reactivity

The need to be cross-reactive also comes with disadvantages, accounting for rejection of organs after transplantation and autoimmune pathologies. Central tolerance mechanisms in T-cell development as well as checkpoints during homeostatis of naïve T cells are in place to protect against autoimmunity. Although negative selection is not always a complete process, most self-reactive T cells are eliminated [215]. Thymic selection relies on TCR interactions with self-MHC complexes to be of low affinity [9]. Nevertheless, low-affinity TCR/pMHC interactions passing negative selection might still be of sufficient strength to activate T cells in the periphery accentuated by locally high antigen densities compared to thymic levels [216]. Further, expression of tissue-specific antigens in the thymus can also be dysregulated [217]. Another explanation for escape from negative selection could be the poor stability of certain self-peptide/MHC complexes [139].

Autoimmunity requires activation of auto-reactive T cells, which occurs through distinct mechanisms including bacterial super-antigens [218], auto-antigen release during inflammation [219] or molecular mimicry with pathogen-derived antigens [139, 220-222]. Genomic mapping also indicates that certain MHC alleles can predispose individuals to autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis and diabetes [223-226]. A contributing factor might also be infection during which levels of co-stimulatory molecules are up-regulated on APCs increasing the ability of weak agonist peptides to stimulate auto-reactive T cells [227]. Thus, autoimmunity can have many origins with multiple factors that interplay leading to the onset of disease [223, 228].

The current database of auto-reactive TCRs in complex with pMHCs is still very limited with eight reported structures to date [138, 139, 229-234]. Structural determination of a substantial number of auto-reactive TCRs is necessary to define recognition properties at a repertoire level and how escape from elimination is achieved. However, these eight structures can give some insights into features of auto-reactive TCRs as well as hints of mechanisms to escape from negative selection [228].

Three reported structures display an unconventional TCR docking topology relative to pathogen-specific TCRs [230, 232, 234]. A different docking topology could affect the higher order assembly of TCR/pMHC/CD3/co-receptor and thereby influence intracellular signaling [228]. Thus, it was reasoned that altered binding properties might mediate escape from negative selection but still allowed them to engage their self-pMHCs in the periphery with sufficient strength to get activated.

In the case of the TCR Ob.1A12, MBP/HLA-DR2b was bound asymmetrically by Ob.1A12 with a focus on the N-terminal part of the peptide [230], as also observed for another MPB-specific TCR 3A6 [232]. Further, the TCR Ob.1A12 docking angle of 110° [230] was by far outside the range of 45-80° reported for other TCR/pMHC interactions [228]. Finally, the TCR Hy.1B11 TCR bound MBP/HLA-DQ1 with a high tilt towards the α_1-helix of the MHC making very little contact with the β_1-helix, with only one germline-encoded CDR loop engaging HLA-DQ1 [234]. Another deviating structural feature connected to auto-reactive complexes was observed in the MHC-II complex MBP_{1-11}/I-Au where the peptide-binding groove was only partially occupied by the peptide [235], although TCRs bound in a traditional docking mode to this complex [208, 233]. Collectively, these examples point to the fact that auto-reactivity could be manifested by atypical structural features of various kinds.

These are exciting observations that could lead us to think that a rule to the structural basis for auto-reactive TCRs is beginning to take form, if it would not be for other reported auto-reactive TCRs that do not deviate structurally [139, 229]. These two TCRs were however restricted to MHC-I so it is possible that different rules apply to these two classes. It is notable that most of the reported structures of auto-reactive TCRs are indeed MHC-II-restricted and specific for myelin basic protein (MBP) [208, 230-234], an auto-antigen in multiple sclerosis. Structural determination of MHC-I restricted auto-reactive TCRs is lagging behind with only two structures reported [139, 229]. MHC-I specific auto-reactive TCRs are discussed further in **Paper IV** where the structure of P14 bound to the modified self-antigen mDBM(3P)/H-$2D^b$ is described.

A common property of auto-reactive TCRs is a relatively low affinity for their target pMHCs [139, 229, 236], compared to pathogen-specific TCRs [237]. This supports the idea that auto-reactive TCRs acquire an affinity slightly below the threshold of negative selection but still high enough for activation [236]. There are of course exceptions to this "rule"; Yin *et al.* reported the structure of the auto-reactive TCR MS2-3C8 that bound with an exceptionally high affinity of 5.5 μM to MBP/HLA-DR4, and proposed that MS2-3C8 T cells instead escaped negative thymic selection as a result of weak peptide MHC affinity [231].

1.3.2.1 Molecular mimicry as a mechanism of autoimmunity

The cross-reactive nature of T cells can lead to activation by foreign antigens sharing structural features with self-peptides, a phenomenon known as molecular mimicry [220, 221, 238]. Molecular mimicry between pathogen-derived and self-antigens is a potential cause for autoimmunity and has been implicated in several autoimmune pathologies such as type-1 diabetes and multiple sclerosis [139, 221, 236, 239-241] as well as in allo-reactivity [179].

The A6 TCR cross-reacts with the self-peptide HuD in complex with HLA-A2, sharing key structural features with the Tax peptide [139]. The HuD peptide could not be classified as a mimotope of Tax in the unliganded state, however several side chains in HuD were altered upon A6 binding reducing the deviations between the two peptides considerably, similarly to that observed in another study [236].

Whether the concept of atypical binding mode for auto-reactive TCRs fits with the idea of molecular mimicry as the underlying mechanism is not clear. It might seem contradictory to have differential recognition of two mimotopes of self and foreign origin by the same TCR. However, binding of the TCR Ob.1A12 to an *E.coli*-derived peptide demonstrated the same unusual docking mode as to the self-peptide MBP/HLA-DR2b despite clear structural homology between the two peptides [236]. This was similarly shown for the TCR Hy.1B11 TCR which bound MBP/HLA-DQ1 and two viral peptides in complex with HLA-DQ1 using the same tilted binding mode [242].

Conclusively, a substantial amount of work shows that molecular mimicry between foreign and self-antigens is important in T-cell mediated autoimmunity. However, structural mechanisms of cross-reactivity underlying autoimmune diseases are not well known and more solved structures are necessary to draw any general conclusion.

1.3.2.2 Alterations in self-peptides that trigger T-cell responses

Alterations in self-peptides, such as mutations or posttranslational modifications (PTMs) can render self-epitopes visible to T cells, thereby providing yet another way to break tolerance. Many PTMs are up-regulated at inflammatory sites creating neo-epitopes that alter the peptide repertoire and influence T-cell recognition [243]. This might be a desirable effect, as in the case for tumor-reactive T cells recognizing mutated epitopes with higher affinity [169], or an unwanted event contributing to autoimmune disease [244]. Some PTMs, including citrullination and deamidation, have

been shown to increase MHC binding affinity [245, 246] thereby activate neo-antigen specific T cells.

In conclusion, the inherent cross-reactivity of T cells enables recognition of multiple ligands which gives a broader protection against the universe of pathogenic antigens that the host might encounter. Moreover, one of the selection criteria during thymic development of T cells is weak binding to self-antigens. However cross-reactivity comes at a price: the weak affinity towards self-ligands could pose problems in terms of auto-reactivity, why central and peripheral tolerance mechanisms delete most T cells that recognize self-antigens too strongly. The affinity-threshold inducing negative selection is usually a magnitude lower than that leading to T-cell activation in the periphery [247], thereby providing a safety window. However, several factors can contribute to the activation of self-reactive T cells, for example foreign antigens mimicking self-antigens, infection, structural deviations including TCR-docking angle and PTMs of self-peptides are all mechanisms that can render self-antigens visible to T cells.

2 AIMS OF THIS THESIS

The overall aim of this thesis was to study how modifications in MHC- I-restricted peptides can affect T-cell recognition. Of particular interest were peptide modifications that could enhance the immunogenicity of pMHCs in a general ways, which could be used in rational design of future peptide vaccines.

The specific aims were as follows:

- Investigate the molecular basis for enhanced T-cell recognition with p3P-modified H-2D^{b}-restricted peptides

- Structurally characterize the TCR P14

- Gain insights into TCR/pMHC interactions through the use of thermodynamics.

3 RESULTS AND DISCUSSION

3.1 THE FINE BALANCE BETWEEN T-CELL SPECIFICITY, SENSITIVITY AND CROSS-REACTIVITY

We made use of the P14 T-cell activation system in **Paper I, II** and **VI** to better understand how a TCR can differentiate between peptides and the basis of discrimination that dictates specificity and sensitivity or in other cases lead to cross-reactivity. In the center of these studies was the peptide modification p3P that enhanced TCR affinity and improved MHC-stabilization. **Paper III** dissects the nature and contribution of the interaction between p3P and the conserved MHC residue Y159.

By investigating the structural, functional and biochemical properties of the same TCR interacting with several different pMHC ligands we learned more about the rules, or perhaps lack of rules, governing T-cell activation. How can certain substitutions in a T-cell epitope be tolerated when others, naturally occurring in viral infections, efficiently abort T-cell recognition? How can we reestablish T-cell recognition of a viral escape mutant by simply optimizing the binding between the peptide and the MHC molecule? Further, what is the explanation at the molecular and structural level? Finally what is the basis of auto-reactivity for our TCR, seemingly specific for its viral epitope?

Paper I '*Unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics'* focuses on TCR specificity and how relatively conserved mutations at TCR interacting residues on the peptide disrupt binding when a more drastic substitution retains some T-cell activation capacity. P14 T cells demonstrate high sensitivity to a naturally occurring mutation at position 4 of the viral epitope gp33, tyrosine to phenylalanine (Y4F), which escapes P14 T-cell recognition [148]. The APL gp33(Y4S), contains the hydroxyl group at p4, similarly to tyrosine, however the substitution renders Y4S an antagonist [6, 95]. Surprisingly, modification of p4Y to alanine (Y4A) maintained partial agonistic properties [95], despite being more radical a change than phenylalanine or serine. The aim of **Paper I** was to provide an explanation as to how P14, with high sensitivity to mutations at p4Y in gp33/H-2D^b, could still be activated by Y4A/H-2D^b through comparative structural, functional and biochemical analyses.

The functional hierarchy of gp33 and its´ APLs has previously been defined as gp33>Y4A>Y4S [95, 248] and the viral escape mutant Y4F has been shown to not be recognized by P14 T cells [148] displaying an almost undetectable affinity for P14 TCR [143]. In **Paper I** these four ligands were compared and analyzed in parallel for their abilities to bind and stimulate P14 T cells, in the search for differences explaining the observed immunological hierarchy (**Paper I**, Figure 1). The TCR affinities for the different pMHC ligands corresponded well with their T-cell activation potencies also shown in previous studies [125, 143, 144]. As increased MHC stability is linked to enhanced immunogenicity in many [79-82], but not all cases [66], cell-based and biochemical stability assays were performed to confirm all four APLs´ similar H-2D^b

binding and stabilization properties (**Paper I**, Supporting Information Figure 1 and Figure 2), and the results were well in line with previous notions [95].

Structure determination of Y4A/H-2D^b and Y4S/H-2D^b allowed for comparison with previously solved crystal structures of gp33/H-2D^b and Y4F/H-2D^b. The binding interfaces were highly similar, providing no clear clue to the discrepancy in T-cell activation. Alignment of the peptides revealed small but non-excluding differences; the conformations of the flexible side chain of lysine at p1 as well as p6F were not the same in all four peptides (**Paper I**, Figure 4). However, crystal structures provide a snapshot of a favorable conformation the ligands occupy before ligation of the TCR. It is important to remember that proteins are dynamic *in vivo* and structural rearrangements are common upon binding [140, 178, 249]. In addition, water molecules and allosteric forces can extrinsically influence TCR interactions with pMHC [101, 187], adding a level of complexity to the equation. In an attempt to understand the dynamic and energetic forces involved that could provide an explanation as to how P14 recognizes Y4A a thermodynamic analysis was undertaken. While recognition of gp33/H-2D^b was entirely enthalpy-driven, recognition of Y4A/H-2D^b was instead entropy-driven with a large reduction in the favorable enthalpy term. The reduced ΔH term for the P14/Y4A/H-2D^b interaction suggested lost intermolecular contacts following mutation of position 4 to alanine, resulting in a smaller entropic cost associated with ordering of CDR loops upon binding. Water coordination around polar groups at the binding interface, such as hydroxyl groups of tyrosines or serines, could reduce desolvation effects and thereby negatively affect the entropy of the system [250, 251]. It is possible that more water molecules were excluded from the binding interface of Y4A, compared to Y4S or gp33 contributing to an entropic pathway of recognition.

It is the sum of all energy-releasing events in an interaction that decides the outcome when a TCR engages a pMHC molecule. Whether the energy is derived from enthalpic or entropic contributions does not seem to matter, rather it seems to be the strength that determines the quality and level of T-cell activation [182]. However, thermodynamics will tell us something about the nature of the interaction and the components involved, taking us one step closer to understanding. In this study we learned that the same TCR may respond to an agonist and a semi-agonist ligand through the use of diametrically opposed thermodynamic signatures. TCRs have previously been shown to be flexible and adjustable, and this study confirms that TCR interactions with pMHCs are as specific as they can be promiscuous.

3.2 A PEPTIDE MODIFICATION THAT ENHANCES MHC STABILIZATION, TCR AFFINITY AND T-CELL FUNCTION

In **Paper II**, *'Structural and thermodynamic basis underlying reestablishment of P14 T-cell recognition of a viral escape mutant'* we turned our attention to the viral escape mutant Y4F that ducks under the radar of P14 T cells by simple means of mutating a TCR contact residue. This is an example of exquisite TCR specificity so sensitive that removal of a single hydroxyl group decimated the affinity and failed to activate P14 T cells [148]. In this study we attempted to reestablish the T-cell recognition through an unconventional peptide modification.

Paper II and **III** are both based on the novel and interesting findings in the study from van Stipdonk *et al.* 2009 [80], in which position 3 of the melanoma-associated antigen EGS from gp100 was modified to proline. The fact that both EGS and gp33 were restricted by H-2D^b made the p3P modification promising in the way p3P interacts with the conserved H-2D^b residue Y159.

In **Paper II** the viral epitope gp33 and the two variants, Y4F and Y4A, were subjected to the p3P modification (the p3P-modified peptides are hereafter referred to as V3P, PF and PA, respectively) and analyzed for increased immunogenicity. The capacity to activate P14 T cells with the p3P-modifed peptides was assessed in a series of functional experiments using both peptide vaccination as well as LCMV infection of mice, analyzing TCR down-regulation, proliferation, expression of TNF, IFNγ and CD107a, and finally lysis of target cells. The p3P-modified peptides demonstrated in all cases superior activation potencies of P14 T cells over the unmodified wild-type peptides. Particularly notable were the effects upon p3P-modifying the viral escape variant and null ligand Y4F, which reestablished P14 T-cell recognition to the p3P-modified PF (**Paper II**, Figure 1).

The PF peptide was tested for its ability to stimulate P14 T cells as well as endogenous T cells after LCMV infection of C57BL/6 mice adoptively transferred with P14 T cells. P14 T cells stimulated with PF produced nearly as much IFNγ as stimulation with gp33 and significantly more than Y4F (**Paper II**, Figure 2). Further, endogenous T cells produced more IFNγ upon simulation with PF compared to Y4F. Finally, approximately two thirds of P14 T cells were also stained with PF tetramer. However, Y4F tetramer did barely stain P14 T cells.

An additional experiment not included in any of the four papers utilized Influenza A virus to express gp33-epitopes on the surface of infected cells, allowing us to study cross-reactivity with the p3P modification. The PF and Y4F epitopes were engineered into the stalk-region of neuraminidase of the Influenza A strain HKx31 (H3N2), as previously described [252], and used to infect C57BL/6 mice. The spleens were harvested on day 10 post infection and stimulated with PF, Y4F or gp33 peptide to study the cross-reactivity between PF and Y4F. A pilot experiment indicated that infection with HKx31(PF) gave a better response upon stimulation with Y4F compared to infection with HKx31(Y4F) measured by IFNγ and TNF expression. The responses were compared to infection with LCMV resulting in presentation of gp33 on infected cells. These preliminary results need to be repeated with flu(gp33) as a proper control but indicate that infection with flu(PF) might give a better T-cell response towards Y4F than infection with flu(Y4F) or LCMV(gp33) (Figure 7).

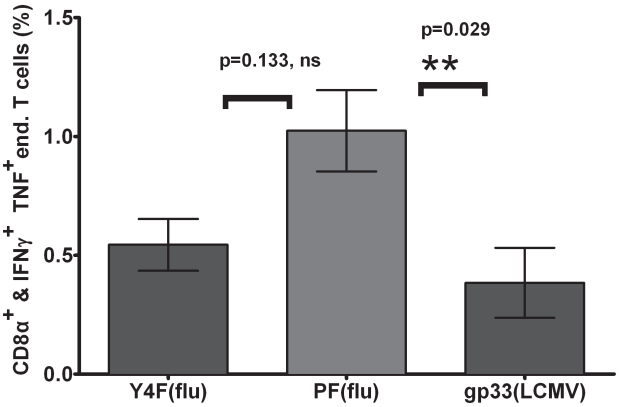


Figure 7: Infection with flu(PF)results in higher expression of IFNγ and TNF by T cells upon stimulation with Y4F peptide. *T-cell response upon stimulation with Y4F peptide after infection with the indicated Influenza-variants carrying Y4F or PF epitopes compared to LCMV (containing the wild-type gp33 epitope) on the x-axis is measured by IFNγ and TNF expression, as indicated on the y-axis.*

The thermostability of the p3P-modified pMHCs was assessed with CD and compared to their unmodified counterparts. The three peptides already possessed the preferred H-2D^b anchor residues, p5N and pΩM [28], as opposed to the TAA EGS which poorly stabilized H-2D^b [80]. The thermostability of the p3P-modified pMHCs was therefore only slightly, however consistently improved compared to the unmodified counterparts, with melting temperatures (T_m values) increasing by 3.2°C on average (**Paper II,** Figure 7, Table II). CD measurements have been used in other studies as well to assess MHC stability [43, 82]. The P14 TCR affinity was also improved with the p3P-modification, (**Paper II**, Figure 7, Table II). Most noteworthy was the >10-fold increase in P14 TCR affinity for PF. The affinity of P14 TCR for Y4F/H-2D^b was in our hands not reliably measureable, but indicated a very weak interaction slightly above the negative control.

An important feature following a modification is conserved conformation of the peptide as it is presented in the MHC cleft in order to enhance the T-cell response towards the wild-type epitope. The p3P modification had previously proven to not alter the peptide backbone conformation in the study by van Stipdonk *et al.* 2009 [80]. We solved the crystal structures of V3P, PF and PA in complex with H-2D^b and compared these to the previously reported crystal structures of the unmodified pMHCs (**Paper II**, Figure 4). The backbones of each p3P-modified and parent peptide-couple superimposed very well upon aligning residues 1-176 of H-2D^b, however different conformations of the side chains of p6F and p1K were observed. It is well known from previous structural studies that binding-induced conformational alterations, including in the peptide, can occur in TCR/pMHC interactions [136, 168], so the more relevant question is conserved conformation post TCR binding. To address this we solved the ternary structures of P14/gp33/H-2D^b and the p3P-modified variant P14/V3P/H-2D^b and made a thorough comparison of the structures. Several changes occurred in both peptides and MHC-residues upon binding of P14, of which the vast majority was shared between the two ternary complexes. In fact the two structures were virtually identical with regards to the conformation of P14 and H-2D^b, with the same docking angle (**Paper II,** Figure 3D). The key TCR-interaction residue p4Y was altered in both structures by approximately 4Å upon TCR binding (**Paper II**, Figure 5). The hydroxyl group of p4Y was centrally located in a network of hydrogen bonds and vdWs interactions (**Paper II**, Figure 3C).

Removal of the hydroxyl group in Y4F would disrupt the network of contacts surrounding p4Y as well as increasing the hydrophobicity of the peptide. Since the CDR loops of P14 are relatively polar and charged, a hydrophobic surface could repel the TCR and prevent binding. Interestingly, a similar substitution in the Tax-Y5F peptide did not result in nearly as reduced affinity of the A6 and B7 TCRs, despite disrupting a hydrogen bond [175]. The antagonist Y4S still contains the OH-group at p4, but alignment of Y4S/H-2D^b and P14/gp33/H-2D^b showed that the position of the OH-group of serine is out of range to form a similar hydrogen bond network as tyrosine, even if the conformation of the side chain is altered. Perhaps the presence of an OH-group in the incorrect position disturbs the recognition and causes Y4S to antagonize P14. It is still puzzling how P14 can bind to Y4A, which lacks the OH-group as well. The P14 affinity is indeed lower towards Y4A. Perhaps the contacts with the rest of the peptide are enough to partially activate P14 T cells, or a completely different binding mode is used. Only the co-crystal structure determination of P14/Y4A/H-2D^b will tell.

Upon binding of P14 the side chains of p1K and p6F was altered in gp33 to adopt the same conformation as in V3P. The movement in p6F was correlated with H155 on the α_2-helix, which was reoriented to avoid steric clashes and subsequently contacted by the P14 residue R97β (**Paper II**, Figure 6). It thus seems likely that the other APLs might also undergo a similar conformational change in these peptide side chains and H155. Notably, we observed the same conformations of residues p1K, p6F and H155 in the crystal structures of all three p3P-modified peptides as well as the semi-agonist Y4A as we do in the TCR-bound states of V3P and gp33 (**Paper II**, Figure 4). This could imply that the side chain conformations of the p3P-modified peptides are already compatible with TCR binding and do not have to be altered upon binding. However, it is unclear to us how the proline modification at p3 could propagate and influence the side chain conformation at distant sites in the peptides. This is an observation, difficult to explain, as no close crystallographic contacts could influence the side chain conformations. However, it has previously been observed that anchor modifications of MHC-I restricted peptides have altered side chains of residues other than the direct neighboring ones [87, 139, 253, 254].

One of the most interesting observations involved the conformation and rearrangement around p3 upon P14 binding. Valine at p3 in gp33 shifted downwards by approximately 1.2Å into the peptide-binding groove of H-2D^b along with a side chain rotation. Proline at p3 in V3P, on the other hand, stayed in the same conformation as before TCR ligation. (**Paper II**, Figure 5). The distance and angle between p3P and Y159 were unchanged, thereby not disrupting the CH-π and van der Waals interactions. A better anchoring of the peptide in H-2D^b and increased peptide rigidity could account for the observed increase in immunogenicity and thermostability.

To characterize and compare the two P14 TCR interactions a thermodynamic study with ITC was performed. The analysis revealed that binding of P14 to V3P/H-2D^b was entirely driven by enthalpy (ΔH) with almost no change in entropy (ΔS), whereas the interaction with gp33/H-2D^b was entropically unfavorable (**Paper II**, Table II). The thermodynamics of the interaction between P14 and gp33/H-2D^b, previously assessed in **Paper I** (Figure 3) with SPR revealed some discrepancies compared to the results in **Paper II.** ITC has been described as the more reliable methods for thermodynamic

analyses [199], however limitations in protein amounts and concentrations prevented us from using ITC in **Paper I**. Although we measured different values for ΔH and ΔS with SPR compared to ITC (ΔH = -15.8 vs. -10.2 and ΔS -9.0 vs. -3.1), the overall pattern remains the same. Direct measurement of ΔH is of course preferable as in **Paper II**. Despite, the results in **Paper I** remain true. Since the two thermodynamic signatures are assessed using the same technique they are suitable for comparison. Several other studies of TCR/pMHC interactions comprise thermodynamic analyses using SPR [178, 189, 194, 205, 210].

It has previously been stated that unfavorable ΔS in TCR/MHC interactions arise from ordering of the CDR loops [186], however loop alterations cannot explain the more unfavorable entropy observed for P14 binding to gp33 since the crystal structures show virtually identical conformations. The ΔH in the two interactions was of the same magnitude, in agreement with similar number of contacts formed in both crystal structures between the TCR and pMHC (**Paper II**, Table SI). Thus, it seems that the p3P modification increased the affinity by decreasing the entropic cost upon TCR binding. This might be a result of better anchoring and increased rigidity of the peptide.

The results in **Paper II** were intriguing since they demonstrated that the p3P modification could increase the immunogenicity not only towards a TAA but also in a viral escape variant, implying that the p3P modification could work for other H-2D^b-restricted peptides as well.

Paper III, *'Proline substitution independently enhances H-2D^b complex stabilization and TCR recognition of melanoma-associated peptides',* provides a more in-depth biochemical analysis of the CH-π interaction between the conserved MHC-I residue Y159 and p3P, and its role in increasing both MHC-stability and TCR affinity. We used the melanoma-associated antigen EGS from gp100$_{25\text{-}33}$ and the TCR pMel, specific for EGS/H-2D^b to investigate the molecular basis for enhanced immunogenicity with the p3P modification. The hydroxyl group of Y159 forms a hydrogen bond with the carbonyl group of the p1 residue as well as a CH-π interaction with the pyrrolidine ring of p3P (**Paper III**, Figure 1). The stabilizing interactions between Y159 and the peptide were stepwise disrupted by mutating Y159 to phenylalanine (F), leucine (L) and alanine (A) in an attempt to answer whether the interactions between Y159 and p3P accounted for the observed increased TCR affinity and MHC stability. The Y159F-mutation disrupts the hydrogen bond with the carbonyl group of p1, but maintains the CH-π interaction between the aromatic ring of F159 and the side chain of p3P. The Y159L and Y159A mutations break the CH-π interaction by progressively decreasing the size of the side chain.

CD measurement of the thermostability of EGS/EGP in complex with the different Y159-variants of H-2D^b showed that the p3P modification greatly improved MHC stability (**Paper III**, Figure 2). This was in line with the results in **Paper II** (Table II, Figure 7) where the p3P modification increased the T_m values in all gp33-variants. One important difference between the two studies was that EGS poorly stabilized H-2D^b whereas gp33 displayed much better stabilization capacity with a higher T_m value, rendering the effects of p3P at first less noticeable. As expected, the superior stability of EGP/H-2D^b was reduced with the Y159F-mutation and finally decimated with Y159A

or Y159L, to reach the same level as EGS/H-2D^b (**Paper III**, Figure 2C). The fact that EGP/H-2D^b(Y159F) still conferred a stability advantage over EGS/H-2D^b, although the hydrogen bond with p1 was broken, strongly indicated that the CH-π interaction with p3P was responsible.

The affinity of the pMel TCR for EGP/H-2D^b was higher compared to for EGS/H-2D^b.Unexpectedly the TCR affinity did not follow the pattern of MHC stability upon mutating Y159 (**Paper III**, Figure 4). Instead, the p3P modification worked seemingly independently in increasing the TCR-affinity with a consistently lower K_D value for EGP/H-2D^b compared to EGS/H-2D^b upon mutating Y159 to F or A. In **Paper II** we measured a remarkable increase in TCR affinity upon p3P-modifying the viral escape mutant Y4F, however just a slight improvement in MHC stability. In **Paper III** we observed the reverse effects: the poor stability of EGS/H-2D^b was greatly improved, but the affinity, already being of intermediate strength, was only slightly increased. In **Paper III** we showed these two properties of p3P, increased TCR affinity and MHC stability, to not be interconnected. It thus seems as though the improvement of stability is the crucial effect in **Paper III**, whereas the increase in TCR affinity was the determining factor in **Paper II** to improve T-cell activation. In conclusion, these studies demonstrated that both TCR affinity and stability of MHC are crucial parameters in T-cell activation. T-cell activation correlates well with TCR affinity in our studies, but if the pMHC complex is too unstable the pMHC fail to sufficiently activate T cells, which might be due to a short half-life on the cell surface.

It is important to investigate the molecular basis underlying enhanced immunogenicity of anchor-modified peptides, which may lead to better understanding of T-cell based vaccine design. The effects of proline on protein characteristics are well studied. Proline in a polypeptide chain imposes a restriction in the possible Φ and Ψ angles (backbone Φ ±65°) [255], at the proline itself as well as of the preceding residue, rigidifying the peptide [256]. The rigidifying properties are believed to account for observed favorable entropic effects by reducing protein flexibility [256-258]. Proline is also known to increase the thermal stability of various other proteins and enzymes [255], similar to in **Paper II** and **III**. Further, aromatic residues are frequently found in the active site of enzymes that bind proline-rich motifs in their substrates [258]. The role of proline in peptides to enhance binding to MHC has been less appreciated. Proline is indeed common at position 3 of peptides eluted from H-2D^b, and described as a preferable auxiliary p3-residue along with other aliphatic residues that could interact favorably with Y159 [57, 259]. However, it has not been used specifically to optimize binding to H-2D^b before the study by van Stipdonk *et al. 2009* [80].

There is also the possibility that the p3P modification could affect the overall rigidity of the MHC molecule and not just the peptide, something that could facilitate TCR binding through decreased conformational entropy. These speculations find support in a study by Baker and colleagues, presenting the interesting observation that both TCR and pMHC rigidify upon binding, facilitating CD3 complex formation as well as CD8 docking [101]. However speculative, it is interesting to consider the possibility that proline could allosterically influence TCR binding by lowering the entropic cost of binding.

3.3 MOLECULAR MIMICRY AS MECHANISM FOR CROSS-REACTIVITY BETWEEN VIRAL AND SELF-PEPTIDES

In **Paper IV**, *'Structural basis for CD8$^+$ T-cell auto-reactivity in LCMV infection'* we investigated the basis for cross-reactivity by P14 TCR between the self-antigen mDBM and the viral epitope gp33 triggered by LCMV infection of P14 transgenic mice. Although sensitive to some mutations P14 can still cross-react with the self-peptide mDBM sharing only 44% sequence similarity. **Paper IV** aimed at exploring the basis of auto-reactivity from a structural perspective where molecular mimicry has been suggested as the underlying basis.

The mDBM peptide exhibits poor binding and stabilization capacities of H-2D^b (**Paper IV**, Figure 1 and Table I) most probably due to a suboptimal anchor residue at position 5, aspartic acid (D) [28]. Further, the affinity of P14 TCR for mDBM/H-2D^b was relatively low (**Paper IV**, Table I). These two properties, low pMHC stabilization capacity and poor TCR affinity, are typical for self-peptides [80, 231], and can present challenges in crystallography. We used the p3P modification to enhance the affinity and stability in an attempt to solve the crystal structure mDBM(3P)/H-2D^b in complex with the P14 TCR. The p3P modification of mDBM/H-2D^b increased P14 TCR affinity by >10-fold to reach almost the same level as for gp33, well in line with the results obtained for PF in **Paper II**, Table I. However, unlike EGS/H-2D^b (**Paper III**, Figure 2) with an equally poor H-2D^b stabilization capacity as mDBM, the stability was only increased by approximately 4°C (**Paper IV**, Table I). This relatively modest stability improvement with p3P can be explained by the suboptimal anchor residue D at p5 [28], which is still present in mDBM(3P) and destabilizes the pMHC complex. We show in **Paper III** that the effect of the p3P modification is dual, independently increasing the affinity as well as the stability. Interestingly, the stability of mDBM(3P)/H-2D^b should still be considered as rather low with a Tm value of 41°C (**Paper IV**, Table I), but the increase in TCR affinity is tremendous and reaches almost as high levels as for gp33. Thus, the low affinity towards the unmodified mDBM cannot be explained by poor MHC stability alone since mDBM(3P)/H-2D^b is not nearly as stable as gp33/H-2D^b but still exhibits a high TCR affinity. Finally, P14 T cells killed target cells loaded with mDBM(3P) to a higher degree than with mDBM indicating that increased TCR affinity resulted in higher immunogenicity despite a relatively low pMHC stability.

The p3P modification allowed for crystallization of both mDBM(3P)/H-2D^b and P14/mDBM(3P)/H-2D^b. Structure determination of the unmodified mDBM/H-2D^b has thus far been unsuccessful (personal communication, A. Achour and A.D. Duru). Therefore, we used the previously reported structure of rDBM/H-2D^b to confirm that the p3P modification did not alter the peptide confirmation [157]. rDBM is the equivalent epitope in rat and differ at p5 with the preferred residue N instead of D, making the pMHC more stable and thus easier to crystallize. The structures of mDBM(3P)/H-2D^b and rDBM/H-2D^b were both determined at the same resolution, 2.7Å, but in different space groups. The two peptides occupied nearly identical conformations in the binary complexes, which led us to believe that the p3P modification did not alter the conformation of mDBM (**Paper IV**, Figure 2B). This is consistent with results from p3P modification of EGS [80] and gp33-variants

demonstrating that the conformation is not altered with p3P. Subsequently, comparing gp33/H-2D^b with mDBM(3P)/H-2D^b in the unliganded states demonstrated molecular mimicry between the two peptides (**Paper IV**, Figure 2A).

In the next step we compared the two ternary crystal structures of P14 binding mDBM(3P)/H-2D^b and gp33/H-2D^b to establish the basis for the observed TCR cross-recognition The structure of P14/mDBM(3P)/H-2D^b was solved at a higher resolution than P14/gp33/H-2D^b (1.9Å and 3.2Å, respectively) which was kept in mind when comparing and analyzing the two structures. The high resolution of P14/mDBM(3P)/H-2D^b allowed for modelling of water molecules, some of which were important in the formation of hydrogen bonds between the TCR and peptide.

P14 bound to mDBM(3P)/H-2D^b in a similar fashion as gp33/H-2D^b and gp33(3P)/H-2D^b with the same docking angle and similar buried surface area (Figure 3). Thus, unlike some other auto-reactive TCRs [230, 232, 234], P14 recognized mDBM(3P) using a traditional docking mode also observed for the 1E6 and A6 TCRs [139, 229]. p4Y remained the major TCR-contacting residue, with the same contacts formed (**Paper IV**, Figure 3, Supplementary Table IA) and similar conformational change induced upon P14 binding as observed in gp33 and gp33(3P) (**Paper II**, Figure 5). The differences between mDBM and gp33 were located to the C-terminal parts of the peptides, and especially p6. The side chain of p6 in mDBM did not fold down upon TCR ligation as in gp33, but instead remained projecting out of the binding cleft (**Paper IV**, Figure 4) probably due to the additional hydroxyl group which was stabilized through a water-mediated hydrogen bond with N98β on the CDR3β loop. This structural difference induced remodeling of the CDR3β loop (**Paper IV**, Figure 5) affecting the contacts made with p6, with a net loss of two VdW interactions (Table SI). A network of contacts involving water molecules was located at the N-terminal corner around p1K (**Paper IV**, Figure 6). It is possible that some or all of these water-mediated hydrogen bonds observed in mDBM(3P) are also formed when P14 binds gp33 and its APLs. Mutational studies of gp33 have shown that p1K substitution affects the P14 TCR affinity [152] as well as the T-cell activation potency measured by TCR down-regulation (**Paper I**, Figure 5).

Conclusively, the overall pattern of residues used to form the ternary complexes was similar for gp33 and mDBM(3P). The modified self-peptide mDBM(3P) mimicked the viral peptide gp33, apart from the conformation of p6F, which was tolerated through remodeling of the flexible CDR3β loop, thereby attributing the cross-recognition by P14 TCR.

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The focus of this thesis is on TCR interactions with peptides presented by MHC-I and more specifically how alterations in the peptide can influence T-cell responses. We could show that the p3P modification significantly enhanced TCR binding to several H-2D^b-restricted epitopes including a self-antigen and a viral escape mutant. Co-crystal structures of P14 TCR in complex with different pMHCs provided important structural insights into molecular events underlying viral escape, enhanced recognition with p3P as well as cross-reactivity in this system. Finally, the P14 structures will provide an important contribution to the limited pool of ternary TCR/pMHC structures especially with regards to auto-reactive MHC-I restricted TCRs.

In **Paper I** we demonstrated that P14 recognizes the semi-agonist Y4A and the agonist gp33 using different thermodynamic signatures. It still remains puzzling to us why the antagonist Y4S cannot activate P14. The crystal structure of Y4A/H-2D^b would tell us whether P14 uses a different binding mode to compensate for the mutation at position 4. The importance of water molecules at the binding interfaces could be investigated using osmolytic agents for all gp33-variants.

Paper II demonstrated that the p3P modification could enhance P14 T-cell recognition and reestablish binding to a viral escape mutant. The increased TCR affinity came from decreased binding entropy which was connected to optimized MHC binding and reduced mobility and movements of the peptide. Completing the infections with Influenza carrying the different gp33-epitopes would tell us if PF can indeed induce a cross-reactive T-cell response to Y4F.

In **Paper III** we investigate the molecular basis for enhanced immunogenicity with the p3P modification. We showed that increased MHC stability is accounted for by interactions between p3P and the MHC-residue Y159, whereas increased TCR affinity is an independent mechanism. A nice complement to this study would be a thermodynamic analysis of P14/gp33(3P)/H-2D^b(Y159A) which would tell us if the decreased binding entropy with p3P is connected to the interaction with Y159 or not.

In **Paper IV** we show that the underlying mechanism of P14 cross-reactivity between the self-peptide mDBM and the viral peptide gp33 is molecular mimicry. A difference at position 6 in mDBM is tolerated through remodeling of the flexible CDR3β loop. Several other self-peptides were identified as potential cross-reactive ligands for P14 in the study by Ohteki *et al.* 1999 [145]. It would be interesting to see how P14 TCR is able to bind these H-2D^b-restricted peptides by determining the ternary crystal structures. The p3P modification could be used again to facilitate the crystallization.

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