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MHC-CLASS I RESTRICTED PEPTIDE BASED IMMUNOMODULATION OF CD8$^+$ T AND NK CELLS

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To Orhan Hilmi Cosan (1953-2003)

“Morality is herd instinct in the individual.”

Friedrich Wilhelm Nietzsche

Die fröhliche Wissenschaft, 1882

ABSTRACT

The main aims of the immune system are to protect the host from potential threats by distinguishing self from non-self and altered-self. T cells and NK cells play a key role in the identification and elimination of threats by scanning a repertoire of peptide epitopes presented by major histocompatibility complex (MHC) molecules. Thus, MHC molecules play a pivotal role in the initiation and/or modulation of both T and NK cell effector functions, acting as 'windows' of the cells presenting their inner condition. This thesis focuses on the molecular interactions between T cell receptors (TCRs), NK cell receptors (NKR) and MHC class I molecules (MHC-I). The presented results demonstrate that it is possible to efficiently manipulate T and NK cell responses through MHC-restricted epitopes and altered peptide ligands (APL).

Primarily, our investigations of the potential impact of post-translationally modified (PTM) peptides on immunosurveillance revealed the first structural and biochemical evidence for how nitrotyrosinated neoantigens may enable viral escape from immune recognition, as well as break immune tolerance by either impairing MHC/peptide complex (pMHC) stability and/or altering interactions with the TCR surface.

Moreover, structural alterations can change the biochemistry of TCR-pMHC interactions, which may affect the immunogenicity of altered peptide ligands (APLs). We demonstrated that a TCR specific for an immunodominant epitope makes use of a different thermodynamic strategy to cross-react with a weak agonist APL in order to adapt to structural modifications in the pMHC. Thus, understanding the molecular constraints of TCR interactions with MHC-restricted epitopes and APLs is essential to develop novel approaches to modulate T cell responses and to achieve "T cell cross-reactivity", which is the main objective of designing APLs targeting viral- and tumor associated antigens.

Additionally, we have demonstrated that it is possible to modulate T cell responses through the use of an unconventional peptide modification strategy that systematically targets evolutionarily conserved residues of the MHC in order to improve pMHC stability and thus immunogenicity. Importantly, introduced modifications efficiently improved the immunogenicity of a viral escape epitope and immunization with the modified epitope generated distinct and focused cross-reactive T cell populations against the original peptide. Thus, targeting evolutionarily conserved residues of MHC provides a novel approach to optimize MHC-I restricted epitopes for future anti-viral or -tumor vaccines.

Finally, our findings suggest that, in interactions between NK cells and normal cells, MHC-I is in most cases expressed in excess ensuring self-tolerance and preventing autoimmunity. More interestingly, we demonstrated that NK cell activation can be modulated through the use of MHC-I restricted peptides. This may have future implications in attempts to sensitize the immune system against previously inert targets, which stands out as an important outcome in the frame of this thesis.

LIST OF PUBLICATIONS

I. **Inflammation-associated nitrotyrosination affects TCR recognition through reduced stability and alteration of the molecular surface of the MHC complex**

   Madhurantakam C*, **DURU AD***, Sandalova T, Webb JR, Achour A.

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II. **The unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics**

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III. **Induction of efficient CTL responses against a viral escape mutant through an unconventional peptide optimization**

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IV. **Natural killer cell tolerance persists despite significant reduction of self MHC class I on normal target cells in mice**

   Brodin P, Lakshmikanth T, Mehr R, Johansson MH, **DURU AD,** Achour A, Salmon-Divon M, Kärre K, Höglund P, Johansson S.

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LIST OF ABBREVIATIONS

APC Antigen presenting cell
APL Altered peptide ligands
ATP Adenosine triphosphate
BCR B cell receptor
CD Circular dichroism
CDR Complementarity determining region
CLIP Class II associated invariant chain peptide
cSMAC Central supramolecular activation cluster
cTEC Thymic cortical epithelial cell
CTL Cytotoxic T lymphocytes
DC Dendritic cell
DP Double positive
DRiP Defective ribosomal product
ER Endoplasmic reticulum
ERM Ezrin-Radixin-Moesin
FR Framework region
FRET Fluorescence resonance energy transfer
H-2 Histocompatibility 2
HCV Hepatitis C virus
HIV Human immunodeficiency virus
HLA Human leukocyte antigen
IFN Interferon
IS Immunological synapse
ITAM Immunoreceptor tyrosine-based activation motif
ITIM Immunoreceptor tyrosine-based inhibitor motif
KIR Killer-cell immunoglobulin-like receptor
LCMV Lymphocytic choriomeningitis virus
LFA-1 Lymphocyte function-associated antigen 1
LMP Large multifunctional peptidase
MC1R Melanocortin 1 receptor
MELC1 multicatalytic endopeptidase-like-complex-1
MHC Major histocompatibility complex
MHC-I Major histocompatibility complex Class I
MHC-II Major histocompatibility complex Class II
NCR Natural cytotoxicity receptors
NK Natural killer
NKG2 NK group 2
NKT Natural killer T cell
PKC Protein kinase C
PLC Peptide loading complex
pMHC MHC/peptide complex
pSMAC Peripheral supramolecular activation cluster
PTM Post translational modification
PV Pichinde virus
RAG Recombination activating gene
SHP SH2-contatining protein tyrosine phosphatase
SIV Simian immunodeficiency virus
SP Single positive
SPR Surface plasmon resonance
TAA Tumor-associated antigen
TAP Transporter associated protein
TCR T cell receptor
TEIPP T cell epitope associated with impaired peptide processing
Th $CD4^+$ T helper cells
Treg regulatory T cell

1 INTRODUCTION

The immune system is a sophisticated ensemble of biological structures and mechanisms aiming to protect the host from potential threat, which has evolutionarily developed in order to distinguish self from non-self and, ultimately, maintain sexual compatibility and sustain survival. Throughout evolution, the immune system developed several different sensors and mechanisms to detect and eliminate invasions. Components of the primordial innate immune system can be traced back to unicellular organisms such as the amoebae as well as to multicellular organisms such as sponges and invertebrates. Several innate-like immune structures (i.e. scavenger receptor cysteine-rich domains, Toll-like receptors, cytokine-like molecules) are used to recognize and eliminate potential threats[1-3]. The basis of this ancestral innate immune system has been largely preserved through the evolutionary transition from invertebrate to vertebrate and was later complemented by the development of the adaptive branch of immunity[4].

The innate immune system of higher vertebrates comprises natural killer (NK) cells, macrophages, neutrophils, dendritic cells (DC), epithelial barriers and the complement system. In contrast, the eclectic antigen-specific adaptive immune system, which includes T- and B-cells, could only be traced to vertebrates (jawed fish)[5,6]. When or how exactly the adaptive immune system evolved is still largely unknown. It is however believed that it is a consequence of a long stepwise accumulation of alterations in cells, molecules and organs, through gene duplications, chromosomal re-organization and inter-domain exchanges of pre-existing structures that are found in eukaryotes or early vertebrates that do not have functional adaptive immune systems[7]. Adaptive immunity enables specific recognition of target molecules through sophisticated structures such as the B cell receptor (BCR) and/or the major histocompatibility complex (MHC)-restricted T cell receptor (TCR). Importantly, these recognition steps establish specific B and T cell memory to the encountered pathogen. The complex cooperative organization of the immune system is achieved in higher vertebrates through a combination of the innate and adaptive branches. Overall, the immune system works in a well-orchestrated manner, and every molecule and cell type has essential and complementary roles in the protection of the host from potential threats.

For example, T and NK cells need to communicate with other cells in the organism in order to coordinate the identification of self, non-self or altered self. Direct communication between T cells and antigen presenting cells (APC) is achieved through the interactions of specialized cell surface receptor molecules, such as major histocompatibility complex class I and II molecules (MHC-I and MHC-II, respectively), with TCR. Thus, MHC molecules serve as windows into the cells, displaying at their surface internally processed peptides and providing a basis for T cells to distinguish self from non-self. On the other hand, NK cells, instead of using highly specialized TCRs, use

a battery of activating and inhibitory receptors in order to distinguish self from non-self. The balance in between signals transmitted through activating and inhibitory receptors defines the fate of NK cells. Interestingly, MHC, which is an essential molecule for T cell based immune defense, also plays a pivotal role in the modulation of NK cell biology. MHC-I molecules are scanned by T and NK cell receptors, triggering effector responses if the cells are infected and/or altered (i.e. cancerous). Identification and elimination of infected and/or altered cells should be very rapid and tightly controlled so that infected or cancerous cells do not evade detection, while healthy cells are not eliminated. The significance of MHC and the use of MHC-I-restricted peptides in the regulation of T and NK cell effector functions is the main focus of my studies, which will be described and discussed in detail later in this thesis.

1.1 MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

MHC genes are located on chromosomes 6 and 17, in human and mouse, respectively. The MHC locus is polygenic, encoding the MHC-I genes, human leukocyte antigen (HLA)-A, -B and -C, as well as the MHC-II genes, HLA-DP, DQ, and DR in humans. The corresponding MHC-I genes in the mouse are (H-2) K, -L, -D, and the MHC-II I-A and I-E. In addition to these so-called classical MHC molecules, several proteins involved in antigen processing and presentation, including the transporter associated with antigen processing (TAP), non-classical MHC genes, several components of the complement system and certain cytokines (such as TNF) are also encoded in the MHC locus. The MHC genes are highly polymorphic and most of the polymorphisms are located in the peptide-binding cleft of the MHC molecules. For example, in 2003 more than 200, 500 and 100 variants of HLA-A, HLA-B, and HLA-C genes, respectively, had already been identified[8,9]. As a consequence, polymorphism in the peptide-binding groove allows for unique peptide-binding properties and requirements for each MHC allele. Moreover, the polymorphic MHC structure enables the host to increase the possibility to generate T cell responses against a wide array of antigens since each individual carries two alleles of each MHC gene (one paternal and one maternal).

1.2 T LYMPHOCYTES

T lymphocytes (T cells) are a crucial part of the adaptive immunity. The central role of T cells is due to their ability to recognize a very large number of antigens that are presented in complex with MHC molecules on the surface of APCs[10]. Recognition of MHC-I or MHC-II-restricted antigenic peptides is required for the generation and maintenance of the T cell repertoire as well as to mount a coordinated immune response. T cells scan the MHC/peptide (pMHC) population displayed on the cell surface of APCs through their extremely diverse TCRs that form a multi-subunit complex together with the CD3 signal transduction complex, that includes the CD3γ, CD3δ, CD3ε and TCRζ molecules. All CD3 subunits contain immunoreceptor tyrosine-based activation motifs (ITAM) in their cytoplasmic domains, which upon recognition

of cognate pMHC get phosphorylated. This leads to the activation of various signaling and gene expression pathways resulting in T cell activation and the initiation of effector functions such as TCR downregulation, cytokine secretion, proliferation, cytotoxicity, phenotypic differentiation, initiation of B cell help and/or apoptosis. Following antigen clearance, the majority of the antigen-specific T cells undergo apoptosis, while a small fraction of the cells survive to establish long-lived memory T cells. Upon re-challenge with the same antigen, memory T cells provide extremely fast and efficient antigen-specific immune responses compared to naïve T cells. Therefore, the generation of a large population of memory T cells is an appealing goal for modern T cell-based vaccination strategies against a variety of human diseases[11].

T cells can be classified into several different subgroups according to their TCR composition, MHC specificity and effector functions. Major T cell subgroups comprise the cytotoxic T lymphocytes (CTLs), T helper cells, regulatory T cells (Tregs), Natural Killer-T (NKT) cells and $\gamma\delta$ T cells. The different T cell lineages occur during T cell differentiation, development and thymic education.

1.2.1 T cell development

T cells primarily originate from hematopoietic stem cells in the bone marrow. T cell progenitors differentiate in the thymus and go through several selection processes before becoming mature T cells. T cells must first produce a TCR by somatic recombination of the variable (V), diversity (D), and joining (J) segments of the Tcrb locus and V and J segments of the Tcra locus. According to the type of TCR expressed, T cell lineages diverge either into $\alpha\beta$ or $\gamma\delta$ T cells. T cells that successfully produce $\alpha\beta$ TCR and express both co-receptors CD4 and CD8, are thus called double positive (DP) thymocytes. DP thymocytes go through additional selection processes in the thymus, called positive and negative selection, which maintain central immunological tolerance and generate a T cell population potentially reactive against non-self antigens.

Positive selection occurs in the cortex of the thymus and allows for the maturation of DP thymocytes bearing TCRs that recognize self-MHC in complex with self-peptides. In other words, this process enriches the population of T cells specific to self-pMHCs. At this stage of the selection the fate of T cells is defined according to their preferences towards either pMHC-I or -II and as a consequence DP thymocytes separate into single positive (SP) thymocytes. Thymocytes reacting with self-MHC-I are directed towards the cytotoxic T cell lineage and express the CD8 co-receptor on their cell surfaces. Conversely, thymocytes specific to self-MHC-II preferentially express CD4 and diverge to the T helper lineage. At this stage thymocytes that do not have enough affinity to self-pMHC will be eliminated through "death by neglect".

Negative selection is the following step of thymic education, which occurs in the medulla of the thymus and results in the deletion of all SP thymocytes with high reactivity to self-pMHCs, thus playing an essential role in prevention of autoimmunity.

The majority of DP thymocytes (98%) are eliminated during positive and negative selection and the cells that successfully pass these two selection stages become mature naïve T cells. The latter are released to the periphery with embedded properties of low self- and potentially high foreign-antigen reactivity, thus establishing simultaneously central tolerance and protection from pathogens and tumors.

The final selection of T cells encompasses peripheral T cell tolerance. Indeed, T cells educated in the thymus can still escape central tolerance and become auto-reactive in peripheral tissues. These potentially dangerous auto-reactive T cells can be eliminated through interactions with dendritic cells (DC) and other T cells, in order to guarantee self-tolerance in combination with continuous control by suppressive Tregs.

1.2.2 T cell triggering

Under steady state conditions self-pMHC/TCR interactions occur routinely where naïve T cells survey the surface of DCs in the periphery. This provides a tonic TCR signaling that maintains the T cell pool and enhances responsiveness towards foreign antigens. This process also increases the probability of triggering very rare antigen-specific naive T cells through interactions with few DCs that present their cognate antigen in complex with MHC molecules during the initial course of an infection.

T cell activation followed by clonal expansion and acquisition of effector functions starts when a TCR encounters a cognate peptide in complex with self-MHC presented by APCs. Complete activation of T cells depends on at least two signals. Signal 1 is derived from the interaction between the pMHC/TCR complex, which is the prerequisite for T cell triggering and following downstream events. Signal 2 is provided by interactions with co-stimulatory molecules and amplifies the signaling events resulting in essential gene transcription and ultimately, T cell activation. Signal 3 is mediated by cytokines produced by APCs and drives $CD4^+$ and $CD8^+$ T cell differentiation into effector cells.

Engagement of the pMHC/TCR complex leads to phosphorylation of its cytoplasmic ITAMs by the tyrosine kinase LcK, allowing recruitment of the Syk family kinase ZAP-70, which phosphorylates a number of downstream substrates. In addition, a number of accessory molecules, including the co-receptors CD4 or CD8, the co-stimulatory molecule CD28 and the adhesion molecules CD2 and LFA-1, are engaged by their specific ligands during antigen recognition (**Figure 1A**). Additional signaling by these receptors can modulate TCR signals, resulting in a finely calibrated biochemical relay that encodes the strength of pMHC binding, thus allowing the T cell to make appropriate context-specific responses.

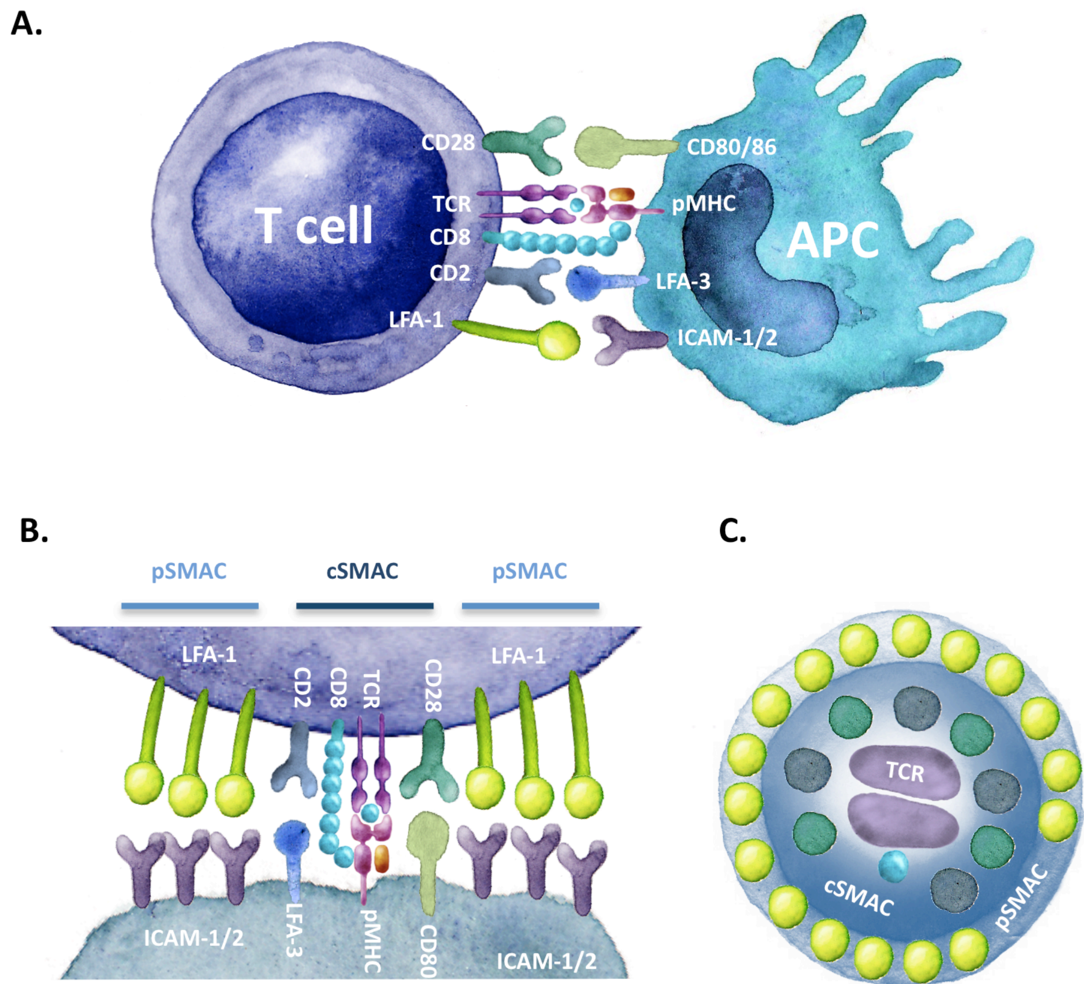

Figure 1. The central role of the TCR/pMHC complex in T cell recognition and the formation of the immunological synapse (IS). An illustration of a T cell and an antigen presenting cell (APC) interaction. **A.** Additional interactions provided by the CD8 co-receptor and co-stimulatory molecules CD2, LFA,1 and CD28 improve the T cell-APC interaction in order to achieve an effective immune response. **B.** Side view of "bull's eye shaped" T cell-APC IS displaying cSMAC in the central zone and pSMAC in the surrounding peripheral zone. **C.** Top view of the "Bull's eye shaped" IS looking down on the T cell.

The pMHC/TCR interaction and the following co-stimulatory signaling, trigger key events for T cell activation, inducing a change in cell membrane topology and the rearrangement of cell surface molecules, assembling into a unique molecular structure at the T cell-APC contact interface termed the immunological synapse (IS)[12,13]. The formation of IS promotes stability of the intercellular contact and allows for a more orchestrated T cell signaling that results in optimal activation and effector functions[12,13].

IS consists of two concentric rings of molecules, known as the central supramolecular activation cluster (cSMAC) and the peripheral supramolecular activation cluster (pSMAC), which form the bull's eye shaped IS (**Figure 1A and C**). The central zone of the IS (cSMAC), enriched with TCR and CD3 complexes, also consists of CD28, CD4 or CD8 and the protein kinase C (PKC) molecules. Since many T cell activating receptors

and signaling molecules are present in the cSMAC, and since its formation strongly correlates with the efficiency of T cell stimulation, this supramolecular domain was initially thought to mediate T cell activation[12,13]. However, recent studies suggested additional alternative roles for cSMAC such as trapping the target pMHC and enhancing serial engagement of TCRs, allowing termination of TCR signals, as well as downregulation, absorption and degradation of TCR/pMHC complexes. This possibly leads to “homotypic T cell-T cell interactions” and induces cross killing to limit the size of clonal expansion or to facilitate directional $CD4^+$ T cell help required for optimal $CD8^+$ T cell activation[14]. Finally, integrin-rich outer ring of the IS (pSMAC) can serve as a sealing ring to prevent the diffusion of perforins and granzymes, which can be important for CTL activity in the frame of focused target cell killing[12,13].

In resting naïve T cells, the TCR complex, the activating and the inhibitory receptors group in membrane structures called nanoclusters[15]. Initial T cell signaling starts at these pre-existing nanoclusters upon antigen recognition and induces formation of microclusters – also called protein islands - where 20 or more TCR complexes come together resulting in enhanced and focused T cell signaling. Additionally, emerging biological and biochemical evidence suggests that these pre-clustered TCRs may exist on the cell surface particularly in previously activated T cells enabling a faster “ready-to-go” response[16-18]. Generation of the bull’s eye shaped IS follows the formation of TCR microclusters. This two-concentric ring shaped structure is not observed during interactions between DCs and T cells, as DCs are excellent APCs with the ability to induce strong T cell activation. Instead, T cells and DCs establish multifocal ISs where TCRs cluster at multiple sites at the T cell-DC interface[12,13]. The clustering of several TCRs on the cell surface can also provide an additional explanation for major T cell triggering hypotheses, such as kinetic-proof reading[19] and serial triggering[20].

1.3 NATURAL KILLER (NK) CELLS

Natural Killer (NK) cells are members of the innate immune system with the ability to kill virus-infected cells and tumor cells in the absence of prior stimulation or immunization[21,22]. Thus, they stand out as promising actors in the treatment of infectious diseases and for cancer immunotherapy[23-25]. Even though NK cells make use of different modes of target recognition compared to T cells, both NK cells and T cells use MHC-I molecules for the regulation of their effector functions[26].

NK cells interact with their potential target cells through various transmembrane inhibitory and activating receptors. The balance between these inhibitory and activating signals defines the action of the NK cells[27,28]. Despite structural differences between the inhibitory and activating receptors, signaling pathways seem largely conserved[29-32]. Activating and inhibitory receptors make use of ITAMs and immunoreceptor tyrosine-based inhibition motifs (ITIMs), respectively, which are

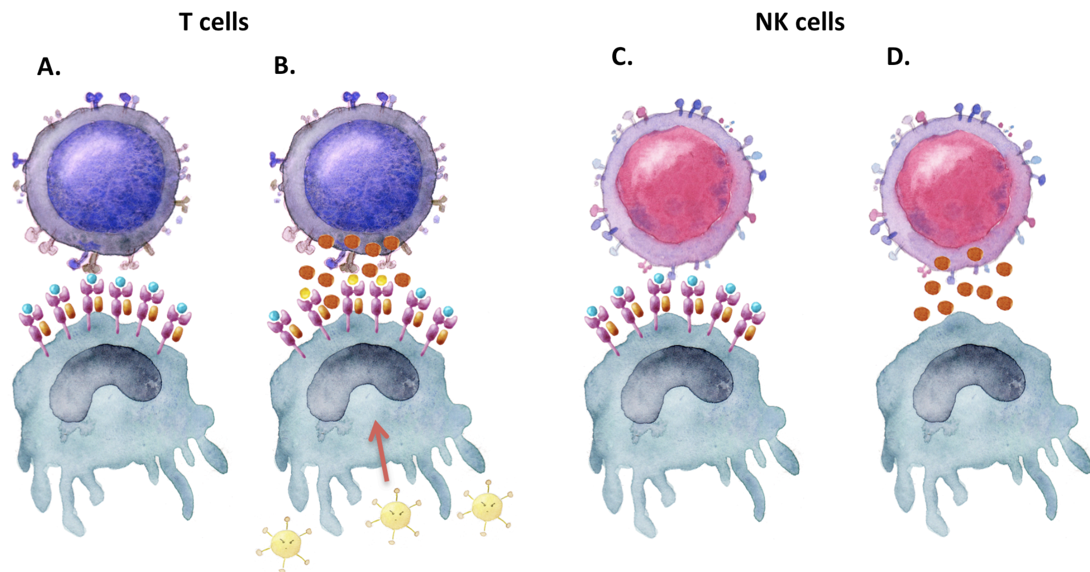

Figure 2. The pivotal role of MHC-I in T and NK cell responses. While T cells respond to non-self peptides presented by APCs, NK cells are sensitive to the loss/absence of MHC **A.** Antigen presenting cell displaying self-epitopes (blue) to T cells through MHC-I. **B.** Infected APC presenting viral peptides (yellow) through MHC-I, resulting in T cell response. **C.** NK cells scanning the surface of a healthy APC. **D.** NK cells responding due to the loss of MHC-I "missing self" resulting in target cell killing.

located on adaptor molecules that interact with the cytoplasmic tails of the receptors. While major activating receptors comprise the natural cytotoxicity receptors, NCRs (NKp30, NKp44 and NKp46)[33-36] and NK group 2D (NKG2D)[37], the major inhibitory receptors include NKG2A, the inhibitory killer cell immunoglobulin-like receptors (KIRs) in human and the highly polymorphic C-type lectin-like Ly49 family of receptors in mice[29] .

Inhibitory receptors tightly control NK cell activity by negatively regulating NK cell activation against healthy cells expressing normal amounts of self MHC-I on their surface. This is possible through the action of KIRs and Ly49 receptors, which are mainly specific for MHC-I, enabling recognition of target cells with down-regulated surface MHC-I expression[38-40], a phenomenon referred to as "missing-self"[41] (**Figure 2**). A common characteristic of MHC-I-specific inhibitory receptors is the presence of ITIMs in their cytoplasmic tail, which enables them to recruit and activate SHP1 and SHP2 phosphatases[29,42] in order to switch off the activating signaling. Interestingly, the importance of negative signaling has also been recently demonstrated in T cells[43].

Both KIRs and Ly49 receptors are clonally expressed on NK cells in variable frequencies and are also found on NKT cells as well as on some T cells. NK cells can express one or multiple receptors, which are specific for one or a few MHC-I alleles, allowing for a wide and complex repertoire of NK cells that is able to respond to the loss of a single MHC allele[44-47]. Taken together, this information highlights the importance of MHC molecules as a fundamental regulator of NK cell biology. Additionally, NK cells undergo a process of education where the ability to be inhibited by self MHC-I confers full functionality[44]. This topic will be expanded in section 1.3.1.

In contrast to T and B cells, NK cells lack clonally distributed receptors for activation. Instead, NK cell activation is maintained through a complex network of various activating receptors[30]. Upon recognition of ligands on the surface of target cells by NK activating receptors, various intracellular signaling pathways drive NK cells towards cytotoxic action, ultimately resulting in target cell killing[48]. The activating receptors are associated with ITAM-bearing signaling adaptors in their cytoplasmic tails such as CD3ζ[49] or DAP12[50], which further highlights the common characteristics of NK and T cell signaling. The ITAM motif was initially recognized as a common sequence in the cytoplasmic tails of the signaling chains associated with the TCR[51]. In T cells, the CD3ζ part of the CD3 complex that is non-covalently associated with the TCR complex, plays a central role in the transmission of signals via sequential tyrosine phosphorylation of its ITAM region. Interestingly, it has also been demonstrated that T cells (which generally lack DAP12), gene-engineered with DAP12, display NK-like effector functions[52], suggesting the significance of DAP12 in both NK and T cell signaling.

In summary, NK cell activation may occur depending on the net result of activating and inhibitory signaling triggered by cell surface receptors. The balance between these signals decides the fate of the interactions between NK cells and their targets. Just as downregulation of MHC-I can diminish the inhibitory signaling, it has also been demonstrated that, even in the presence of normal MHC-I levels on the target cells, NK cells can still be triggered if a higher amount of activating signal is achieved as a result of stress-induced upregulation of activating ligands, a phenomenon referred to as "induced-self recognition"[53-55].

1.3.1 NK cell development

NK cells develop primarily in the bone marrow. Unlike T cells, NK cells do not go through positive and negative selection in the thymus and exist in normal numbers in athymic nude mice[56-58]. However, it is also possible that some NK cells can originate from the thymus[59], liver[60] and lymph nodes[61].

During development NK cells go through several stages of differentiation that have been defined based on the sequential acquisition of specific markers. The last stages of NK cell development include acquisition of functional capabilities, such as cytokine secretion and cytotoxicity[62]. Unlike B and T cells, individual NK cells lack antigen-specific receptors. Furthermore, NK receptors do not go through recombination activation gene (RAG)-dependent rearrangements, although during NK cell development low frequency of V(D)J recombination and transient expression of RAG have been observed[63-67]. Upon leaving the bone marrow NK cells can reside in the blood, spleen, liver, lung and various other organs[68], where they stand ready to rapidly respond against pathogens. As for T cells, NK cells continue to maturate in the periphery following exit from the thymus and the bone marrow, respectively[69-71].

1.3.2 NK cell triggering

Similarly to T cells, functional activation of NK cells requires a number of activating stimuli for efficient effector functions. Engagement of a single or several activating receptors (Signal 1) can promote NK cell activation and clonal proliferation. On the other hand CD28, which plays an essential role in T cell co-stimulation (Signal 2), is expressed at lower levels in NK cells compared to naive T cells. While stimulation of CD28 in NK cells is not required for cytotoxicity, it seems to be central for optimal cytokine secretion and proliferation[72]. Other similarities between the triggering of T and NK cells comprise the use of pro-inflammatory cytokines IL-12 and type-I IFNs provided by DCs upon sensing viral infection (Signal 3)[73-76]. It should also be noted that even in the absence of signals 1 and 2, signal 3 alone could promote an innate-like ability in memory CTLs to produce IFNγ and proliferate[77,78].

In conclusion, the mechanisms of integrating activation, co-stimulation and cytokine signaling seem to be common to both NK and T cells.

1.4 ROLE OF MHC IN DEVELOPMENT AND REGULATION OF T AND NK CELLS

MHC is a key molecule in several different stages of T cell biology. The importance of MHC in thymic education, peripheral tolerance, T cell activation and effector functions results in a bias of the TCR towards MHC. Non-self and self-peptide antigens are continuously displayed on MHC to $\alpha\beta$ T cells acting as a window that exhibits the inner status of the cell. In other words, MHC molecules form a platform for the docking and scanning of various different TCRs during their quest to discover cognate peptide antigens and in order to keep T cell-based adaptive immune responses alert and active. Although their gene loci are localized on different chromosomes, TCR and MHC have co-evolved since the creation of adaptive immune systems in vertebrates. It has been recently hypothesized and partially proven that TCRs make use of germline encoded evolutionarily conserved residues localized on the complementarity determining regions, CDR1 and CDR2, to interact and recognize MHC molecules[79,80]. Despite the emergence of recent challenging findings[81,82,83], it is generally considered that $\alpha\beta$ TCRs cannot recognize antigens that are not presented by MHC. This is one of the major indications for the essential role of MHC molecules in T cell recognition. Furthermore, T cells that are only reactive to self-pMHC molecules are allowed to maturate during thymic education. T cells educated in the absence of self-pMHC can still recognize MHC molecules, although most frequently allogeneic[84].

Finally, presence of MHCs and their ability to present antigenic peptides is one of the requirements to initiate the signals that are necessary to trigger T cells. For example, formation of the immunological synapse during T-cell and APC interactions is mediated through MHC and TCR. Thus, in order to acquire a better understanding of the relationship between MHC and TCR, and possibly develop novel T cell-based

vaccination strategies, it is essential to enhance our understanding of their interactions, and thus determine the three dimensional structure, the chemistry and the biology of both MHC and TCR.

Similarly to T cells that undergo positive and negative selection in the thymus, developing NK cells are educated or selected in the bone marrow through engagement of their inhibitory receptors with various MHC-I ligands, which is an essential step for establishing efficient missing-self recognition[39,71,85]. Engagement of these receptors with cognate MHC-I leads to the generation of functional NK cells in the periphery, whereas failure to engage inhibitory receptors during development results in NK cell hypo-responsiveness[86-90]. Unlike "death by neglect" seen in T cell selection, NK cells that do not get stimulated by inhibitory ligands during development are still exported to the periphery but remain anergic. This process where effector functions are conferred upon those NK cells that have the ability to sense the presence of MHC-I is termed licensing[91].

However, upon adoptive transfer, mature NK cells can undergo a re-education process whereby functional competence is reset according to the new environment[92,93]. An example of such a phenomenon is the acquisition of effector functions by previously anergic NK cells transferred into an MHC-I sufficient environment, suggesting that continuous engagement of inhibitory receptors with MHC-I is required for NK cell responsiveness. Notably, NK cells that lack an inhibitory receptor for autologous MHC-I can still respond normally in inflammatory settings[88,94,95] and more robustly against viral infections and leukemia than their counterparts that express self-specific inhibitory receptors for MHC-I[96-98].

Additionally, ligation of developing NK cells' activating receptors with cognate viral or self-ligands leads to anergy as well as a partial repertoire deletion resembling the negative selection of developing thymocytes[99-102].

1.5 THREE-DIMENSIONAL STRUCTURE OF MHC MOLECULES

MHC molecules are mainly represented by two distinct classes, I and II, in higher vertebrates. MHC-I molecules, which form the main recognition platform for CTLs, present on the cell surface endogenous antigens that result from the processing of cytosolic proteins. In contrast to MHC-I that are expressed by all nucleated cells, MHC-II are only expressed by professional APCs such as DCs, macrophages and B cells. Antigens derived from engulfed exogenous proteins are processed through an endocytic pathway and presented on the cell surface of APCs on MHC-II, ultimately targeting recognition by $CD4^+$ T helper cells. Although MHC-I and -II are triggering distinct subgroups of T lymphocytes, both molecule classes have similar overall architectures **(Figure 3)**. MHC can generally be described as heterodimeric proteins that consist of a membrane spanning tail followed by two extracellular Ig-like domains

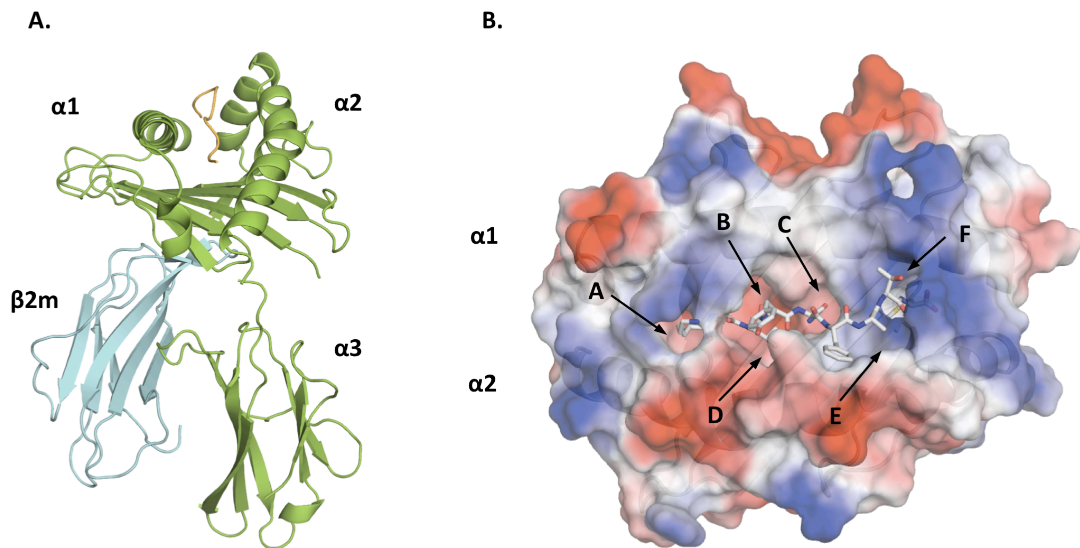

Figure 3. Overall structure of the MHC-I molecule and peptide-binding cleft. A. Crystal structure of MHC-I (PDB ID: 1S7U). MHC-I heavy chain in green, β_2m in cyan, side view **B.** Electrostatic representation of MHC-I, displaying the peptide binding cleft and six major pockets (A-F), top view.

(the latter important for CD8/4 engagement and/or recognition by specific NK receptors such as Ly49), and a membrane-distal highly polymorphic super-domain that forms the peptide binding groove. The peptide-binding cleft is formed by two anti-parallel broken α-helices that are diagonally located over eight anti-parallel β-sheets establishing a restrictive sandwich-like landscape for peptide binding and presentation. The main differences between the two MHC classes result from alterations in domain organizations that allow both classes to achieve discrete characteristics in three-dimensional structures and thus presentation of peptide repertoire[103,104].

More specifically, MHC-I is comprised of a single membrane spanning heavy chain composed of three α-domains, which also establishes the peptide-binding groove. The heavy chain binds non-covalently to a soluble Ig-like molecule called β_2-microglobulin (β_2m) (**Figure 3A**). In contrast, MHC-II is composed of two similarly sized heavy chains called α and β, which contribute equally to the peptide-binding groove. MHC-I and -II present peptides of differing sizes and properties as a result of differentially built architectures of their peptide binding grooves[104-106]. It is generally considered that MHC-I can accommodate 8-10 amino acid-long peptides since the peptide-binding cleft is closed at both ends[103,104]. Conversely, longer peptides can be presented by MHC-II since the ends of the peptide-binding cleft are open, allowing longer peptides to extend out from both sides of the groove[103,104]. Another consequence of these structural differences is that peptides presented by MHC-II are held in long extended conformations and reside slightly shallower in the peptide-binding groove, compared to MHC-I restricted peptides. Thus, peptides accommodated by MHC-I are *de facto* restricted to a relatively smaller peptide binding groove and most often bulge out more or less in the middle depending on the size and the chemistry of the presented peptides[103,104]. More prominent conformations enable peptides to be more accessible

for TCR scanning in the case of MHC-I-restricted epitopes. Additionally, longer peptides can also bind to MHC-I with either an even more bulged structure or by extending out and upwards from the peptide-binding groove at the N- or the C-terminus[107,108]. Finally it should be noted that several very long viral peptides and tumor-associated antigens (TAAs) that bulge out from the peptide-binding groove, have been three-dimensionally established and thoroughly functionally studied[109-113].

1.5.1 Structural insights into peptide-MHC binding and restriction

MHC molecules make use of their peptide-binding sites in order to present antigens to T cells. Pamela Björkman and Don Wiley provided the first insights into the structural and biochemical features of MHC/peptide restriction when they determined the crystal structure of HLA-A2[114,115]. Since then several structural and biochemical studies have provided and still provide additional important information, adding constantly to our understanding of the molecular basis underlying MHC/peptide restriction.

Although an oversimplification, it is considered that the peptide-binding cleft of MHC-I consists of six major pockets that accommodate the presented peptides **(Figure 3B)**. Each of these pockets, named A to F, plays an important role in peptide binding. Unidirectional binding of peptides to MHC is one of the important features of MHC peptide-binding grooves. This directional binding is achieved through the unique chemical environment created by conserved amino acids in the vicinity of these pockets, orientating the N- and C-termini of the peptide towards the A- and the F-pockets, respectively. In the case of MHC-I, the majority of the amino acids in the A- and F-pockets are bulky aromatic residues that seal-off the ends of the peptide-binding groove.

Additionally, the remaining four pockets (B-E) are located mainly in the most polymorphic regions of MHC class I, defining one of the key characteristics of the peptide binding-clefts, which is peptide specificity. This specificity is individually designed through the polymorphic residues residing in the peptide-binding groove. Indeed so-called binding motifs are characterized by the requirements of certain pockets that strongly select for certain amino acids at specific peptide positions. It is generally agreed today that most of the peptide binding to a specific MHC allele is achieved through the use of these preferred residues called anchor residues. As each MHC allele has individual preferred MHC anchor residues, each allele has the potential to present a different peptide repertoire, which is mainly tuned by the highly polymorphic nature of the MHC molecules. For example, the human HLA-A2 allele mainly makes use of position 2 (p2) and the C terminus (pω) of the epitopes as main anchoring peptide residues, generally preferring residues such as leucine (L) and methionine (M) at p2, and valine (V), L and isoleucine (I) at pω. In comparison, mouse MHC-I allele H-2D^b prefers p2, p5 and pω as main anchoring residues, with alanine (A), M, serine (S) at p2, asparagine (N) at p5 and I, L and M at pω of the peptides [116]. The

identification and characterization of the binding properties of each MHC molecule is very important in order to design more immunogenic altered peptide ligands (APLs). It should be noted that the remaining pockets, which are not occupied by main anchoring residues, have so far been believed to play no or a minor role in both affinity and specificity of the presented peptides to the MHC binding-cleft. However, they have also been proven as vital for peptide binding since additional interactions between the presented peptides and MHC residues that compose these pockets/regions may serve as secondary anchors, providing significant additional overall binding strength and MHC/peptide complex stability[117-120]. The systematic targeting of such residues represents an alternative approach for the design of APLs with higher immunogenicity[117,118,121] as described in paper IV. This topic represents one of the main aims of my PhD thesis.

1.5.2 Antigen processing resulting in conventional and alternative peptide repertoires

Antigen processing is one of the fundamental features of the T cell-dependent adaptive immune system. The main role of antigen processing is to convert antigenic proteins from the extracellular space or the cytosol to appropriate sizes that enables MHC-restricted cell surface presentation of peptide antigens.

1.5.2.1 Generation of MHC class II-restricted epitopes

The main role of the MHC-II-restricted peptide presentation pathway is to recruit and direct CD4$^+$ T cells against extracellular antigens that are internalized by professional APCs such as DCs, B cells, monocytes and macrophages. The internalized proteins are localized in endosomes and phagosomes that can later be fused to lysosomes. The acidic environment in these vesicles enables acidic proteases, such as cathepsins, to degrade captured proteins to peptide antigens that are 10 to 30 amino acids long. The peptide-binding grooves of MHC-II, which is synthesized in the endoplasmic reticulum (ER), are occupied by the invariant chains (Ii) throughout their transport to the intracellular vesicles. In order to load the adequate repertoire of peptides in the peptide-binding groove of MHC-II, Ii must be removed from the peptide-binding cleft. This is achieved by the combined action of proteolytic enzymes and the MHC-II-like non-polymorphic molecule HLA-DM. Cathepsin S cleaves and removes Ii, leaving the 24 amino acid-long class II-associated invariant chain peptide (CLIP) in the peptide-binding cleft. Finally, following the HLA-DM-regulated exchange of the CLIP peptide with an appropriate antigen peptide, stable pMHC-II complexes are transported to the cell surface in order to present the bound peptide and communicate with CD4$^+$ T cells.

1.5.2.2 Antigen processing pathways for MHC class I-restricted epitopes

Unlike MHC-II, MHC-I is expressed by all nucleated cells with a potential to process and present antigens to CD8$^+$ T cells. MHC-I-restricted antigenic peptides are generally derived from cytosolic proteins and processed in the cytosol by the cylindrical (barrel-

like) multi-subunit 26S proteasome. This enzyme complex, which plays a central role in the ubiquitin proteasome system, generates 8-10 amino acid long peptides by fragmenting ubiquitinated proteins. The 26S complex is formed by the main catalytic 20S proteasome and two 19S regulator complexes that first both bind and unfold ubiquitinated proteins, and thereafter activate the catalytic 20S core.

The catalytic 20S proteasome is composed of two inner and two outer rings containing seven α-subunits and seven β-subunits, respectively. Peptide fragmentation occurs in the inner β-rings. The subunits β1, β2 and β5 are mainly responsible for the generation of MHC-I restricted epitopes. The selective fragmentation of the epitopes can be divided into three major classes; chymotrypsin like activity (β5), trypsin like activity (β2) and peptidylglutamyl peptide hydrolyzing activity (β1) with preferred cleavage sites after hydrophobic residues, basic residues and acidic residues, respectively[122].

Proteasome-generated peptides are thereafter transported to the ER by an adenosine triphosphate (ATP)-dependent pathway through the protein complex called transporter associated with antigen processing (TAP). The ER membrane-bound TAP is formed by two subunits called TAPI and TAPII. It is frequently thought that hydrolysis of ATP molecules leads to conformational changes of the TAP complex allowing for the transfer of processed peptides to the ER. TAP molecules are capable of transporting 8-30 amino acid long peptides into the ER with a preference for basic or hydrophobic residues at the carboxyl termini of the peptides. This preference is one of the features that modulate the MHC-I-restricted peptide repertoire. Peptides that are transported into the ER are directed to the peptide-loading complex (PLC) where they are loaded on MHC-I with the assistance of several aminopeptidases as well as the molecules tapasin, ERp57 and calreticulin[123,124]. While aminopeptidases additionally trim the N-termini of peptides to appropriate sizes for efficient MHC-I binding, tapasin, ERp57 and calreticulin mediate successful formation of pMHC complexes[124].

The process through which MHC-I are expressed, folded and loaded with antigenic peptides in the ER is tightly modulated by several chaperone proteins. First, the MHC-I heavy chain binds to calnexin that stabilizes the newly synthesized heavy chain and enables the engagement of the heavy chain with the β_2m subunit. Upon binding to β_2m, calnexin dissociates from the heavy chain and is rapidly replaced by the PLC proteins calreticulin and tapasin. Tapasin binds to both MHC-I and TAP bringing them together and thus enabling efficient peptide loading[125]. Calreticulin binds to the MHC-I heavy chain and controls the quality of the formed pMHC complex. MHC-I successfully loaded with peptide is finally released by the PLC in order to be transported to the cell surface through the Golgi apparatus.

1.5.2.3 Immunoproteasome

Mammalian proteasomes can be constructed by a variable ensemble of subunits, resulting in altered regulator affinities, substrate specificity and cleavage preferences

[126-128]. While standard proteasomes are constitutively expressed in all cells of an organism, immunoproteasomes are specifically expressed in immune relevant cells and can be induced by IFN-γ in all other cells. Both standard proteasomes and immunoproteasomes can cleave ubiquitinated proteins and produce MHC-I-restricted epitopes. However substrate specificity, cleavage preferences, cleavage efficiency and eventually peptide repertoire can differ dramatically as a result of substitutions in the catalytic β-subunits in the conventional proteasome with the β2i (MELC1) and the MHC class II gene loci-encoded β1i (LMP2) and β5i (LMP7) subunits[129,130]. The immunoproteasome cleaves antigens into 6 to 30 amino acids long peptides and displays an enhanced level of antigen turnover with a selective preference for basic or hydrophobic amino acids at the carboxyl terminal of the produced peptides. For example, while antigen processing of the lymphocytic choriomeningitis virus (LCMV)-derived immunodominant mouse MHC-I H-2D^{b}-restricted epitope gp33 is regulated by the immunoproteasome subunit β5i (LMP7), antigen processing of the subdominant H-2D^{b}-restricted LCMV epitope gp276 is negatively affected by the presence of the immunoproteasome[131]. Similar differential antigen processing patterns can also be detected for MHC-I-restricted TAAs. While antigen processing of the HLA-B40-restricted MAGE-3-derived epitope is principally mediated by the immunoproteasome[132], the HLA-A2-restricted Melan-A/Mart-1 epitope (Melan-$A_{26\text{-}35}$) is poorly cleaved by the immunoproteasome but successfully processed by the proteasome[133]. Thus, the observed differential antigen processing and presentation of MHC-I-restricted epitopes by different types of proteasomes underlines the importance of epitope selection for future peptide vaccination strategies since absence or presence of certain inflammatory cues, such as IFN-γ, can strongly influence the efficiency of the treatment.

1.5.2.4 Thymoproteasome

The recent discovery of the thymus-specific proteasomes unveiled that the β5 subunit of the 20S proteasome is replaced by the thymus-specific subunit, β5t, which in combination with βi1 and βi2 forms the thymoproteasome. Expression of β5t is specific to thymic cortical epithelial cells (cTECs). In contrast to other proteasomes, the β5t-proteasome exhibits low chymotrypsin-like activity[134], with a decreased preference for cleavage after hydrophobic residues, resulting in the generation of an altered repertoire of MHC class I-restricted peptides. β5t-dependent changes in the peptide repertoire have an essential impact on optimal positive selection, and are required for the generation of an immunocompetent CD8^{+} T cell repertoire since CD8^{+} T cells generated in the absence of β5t displayed impaired alloreactivity and anti-viral responses[135]. Additionally, it is also very interesting to see that T cells that are specific to a certain peptide repertoire shaped by β5t and self-MHC, can successfully proceed through the positive selection[136].

1.5.2.5 Antigen cross-presentation

The majority of antigens presented by MHC-I are generated through proteasome-dependent cleavage of ubiquitinated polypeptides originating from cytosolic proteins, defective ribosomal products (DRiPs)[137], alternative reading frames and untranscripted regions of genes, providing a variable range of antigens to $CD8^+$ T cells[138]. However, it should also be noted that an alternative phenomenon known as antigen cross-presentation can take place where exogenous antigens, in addition to constituting the main source for the MHC-II pathway, are presented by MHC-I. Transfer of exogenous peptide antigens to the cytosol allows such fragments to be transferred to the ER by TAP. Aminopeptidases can then trim these exogenous epitopes to the appropriate MHC-I binding sizes so that they can finally be presented on the cell surface by MHC-I and induce $CD8^+$ T cell responses. Antigen cross-presentation mediated by professional APCs, such as DCs, B cells and macrophages, plays an essential role in both the rapid initiation of antigen-specific T cell responses and the control of central and peripheral tolerance, a phenomenon known as 'cross-tolerance'. Thus, antigen cross-presentation plays a very important role in maintaining immunological defense against viruses, bacteria and tumors. Some of the cross-presented antigens can also bind directly to MHC-I in endosomes and skip the TAP-dependent cytosolic pathway. This feature allows the host to generate and present an alternative repertoire of MHC-I-restricted epitopes, overcoming immune evasion strategies used by certain viruses that inhibit or suppress classical antigen processing[139-142].

1.5.2.6 TAP-independent processing of MHC-I-restricted epitopes

The classical pathway of MHC-I-restricted antigen presentation comprises cleavage of cytosolic proteins by proteasomes followed by TAP complex-mediated transport of peptide antigens to the ER where peptides are loaded on MHC-I at the PLC. However, TAP-independent pathways for processing and presentation of MHC-I-restricted peptides also exist. Antigen cross-presentation, described above, represents one possible gateway for TAP-independent expression of exogenous epitopes that are directly loaded to MHC-I residing in endosomes[143,144]. Moreover, MHC-I-restricted peptides can also be processed and presented independently from antigen cross-presentation even though TAP levels are suppressed. Signal peptides derived from membrane or secreted proteins can be cleaved by ER-located signal peptidases and signal peptide peptidases that enable direct loading of peptides on MHC-I through direct release into the ER[145-147]. For example, the HLA-A2-restricted TAA (MLLAVLYCL) derived from the signal sequence of tyrosinase is presented on melanoma cells through such a TAP-independent pathway[148].

Interestingly, a unique category of CTLs that can target alternative MHC-I-restricted repertoires of self-peptide epitopes presented by cells with impaired TAP, tapasin or proteasome functions has been recently identified[149-151]. T cell epitope associated with impaired peptide processing (TEIPP) constitutes a group of immunogenic neo-antigens

that are not presented by normal cells. Moreover, it has been clearly demonstrated that TEIPP-specific CTL responses result in selective eradication of TAP-deficient tumors *in vivo*. The existence of TEIPP and TAP-independent MHC-I-restricted epitope repertoires highlights the existence of alternative defense mechanisms towards pathogens and tumors, especially when TAP-mediated antigen processing is diminished or inhibited. This repertoire of TAP-independent MHC-I-restricted peptides may represent an excellent target for the design of novel altered peptides for peptide-based therapeutic approaches.

1.5.3 Post-translational modifications

Post-translational modifications (PTMs) may result in the production of MHC-restricted neo-antigens that can trigger novel/different T cell responses and can avoid the constraints of immunological self-tolerance. Infection, inflammation, cellular transformation, cell death or altered signaling pathways may affect the frequency of PTMs that may result in changes in peptide repertoire presented by APCs[152-156]. Additionally, PTMs can also be used by bacterial and viral pathogens to compromise critical immune responses and host factors against infection[157,158]. It has been demonstrated that several kinds of PTMs such as deamidation[159], cysteinylation[160,161], glycosylation[162,163], phosphorylation[164,165] or nitrotyrosination[166] may affect T cell immunoreactivity, resulting in immune escape and/or initiation of autoimmunity[152,167,168]. For example, Jason A. Tye-Din and Robert P. Anderson recently analyzed 16.000 potentially toxic peptides derived from wheat, barley and rye in order to identify immunogenic epitopes that may break tolerance and cause celiac disease. This study demonstrated that most of the T cells were specific for only three of the investigated peptides, providing a clear example for immunodominance and the limited diversity of immunogenic epitopes. Additionally, Hardy *et al* recently demonstrated that conversion of the tyrosine residue (p4Y) of LCMV-derived immunodominant H-2D^b-restricted epitope gp33 (KAV<u>Y</u>NFATC) to a nitrotyrosine significantly affected recognition of H-2D^b/gp33 specific T cells[166]. We provide in PAPER I the underlying biochemical and structural basis for these observed functional effects. Similarly to TAP-independent and TEIPP epitopes, PTM epitopes can also play a key role in immune surveillance[152,168]. Since PTM epitopes may constitute a novel repertoire of antigens, it might be possible to use these as novel candidates for future attempts to target infected or altered cells.

1.6 MHC-I RESTRICTED EPITOPES

1.6.1 Epitope discovery

Initially MHC-I restricted epitopes were identified through peptide elution followed by sequencing using mass spectrometric methods, leading to the identification of allele-specific binding motifs for MHC-I restricted antigens[169]. Based on this information,

sequence-based computational peptide prediction algorithms emerged allowing for the discovery of several additional MHC allele-specific peptide epitopes[116,170-172]. However, although very successful in identifying large amounts of novel epitopes[116], this approach also carries intrinsic biases since it only allows for the identification of epitopes with optimal anchor residues. For example, murine MHC-I molecule H-2D^b-restricted epitopes generally make use of certain amino acids at positions 2, 5 and at the C-terminal part of the peptide for optimal peptide binding. This profile of H-2D^b restricted epitopes leads the computational peptide prediction algorithms to search for certain amino acids at specific positions such asparagine (N) at peptide position 5 (p5). As a result of this, such algorithms predict higher peptide binding scores for peptide fragments containing conventional peptide binding motifs while the identification of unconventional epitopes may be shadowed. Moreover, the presence of this motif does not always guarantee efficient binding of the identified peptides to MHC or their processing. Indeed a large range of the identified epitopes, predicted to bind with high affinity to their cognate MHC, do not bind at all and/or are not processed naturally. In line with this, our research group has demonstrated that the melanoma-associated H-2D^b-restricted epitope gp100$_{25-33}$ (EGSRNQDWL) binds very weakly to H-2D^b despite the fact that it contains all the preferred anchor residues[118]. Taken together, these data indicate that identification of epitopes represents a multi-faceted challenge that needs to take into account the whole range of potential interactions between the peptide and the MHC. Moreover it is essential to extensively make use of peptide elution and, more sensitive mass spectrometry approaches in order to identify novel epitopes more precisely and to improve the efficiency of the currently available peptide prediction tools.

1.6.2 Viral epitopes

Several virus proteins expressed during infections are processed by proteasomes, immunoproteasomes and other alternative processing pathways, resulting in surface presentation of MHC-I-restricted peptides with variable affinities. Virus-associated MHC-I restricted immunogenic epitopes can display a relatively high affinity to their cognate MHC molecules, efficiently stabilizing pMHCs. As these antigens are theoretically not presented in the thymus and as T cells specific to MHC/viral peptide complexes are not negatively selected during T cell development, it is highly possible that a significant amount of T cells with high specificity to these foreign antigens are available and can be efficiently activated. However, despite the intrinsic potential to create a very broad T cell repertoire with the capacity to recognize a wide variety of epitopes, large parts of the immune responses are particularly focused on the so-called immunodominant epitopes, which usually represent only a very small fraction of the entire epitope spectrum[173-175]. This process gives rise to T cells clones that are highly specific for MHC alleles in complex with immunodominant peptides.

Immunodominant epitopes are generally able to elicit an entire range of cytotoxic T lymphocyte (CTL) responses including cytokine production, proliferation and cytotoxicity and are thus classified as **agonist epitopes**[117]. However, selective T cell pressure on these few agonist epitopes can result in the emergence of viral escape mutations that abrogate CTL recognition and lead to escape from immune surveillance[176]. Viral escape mutations can be classified as **weak-agonists** if the intensity of T cell responses is reduced. Most importantly, if the mutations lead to complete abrogation of all CTL responses, the mutated peptides are classified as **viral escape epitopes** and if they interfere and diminish T cell responses to the agonist peptide, they are classified as **antagonist epitopes**[43]. Additionally, some viral escape mutations can completely diminish the antigenicity of the epitope and convert them to ghost peptides (**null epitopes**) where T cells specific to immunodominant pMHC can no longer detect the existence of the mutated epitope[177]. Successful CTL escape variants both in immune surveillance and viral fitness that correlate with disease progression have been reported in various infection models including human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV)[178], hepatitis C virus (HCV)[179], Influenza[180] and LCMV[181]. Importantly it should be noted that, up to now, immunizations with antagonist or viral escape epitopes have failed to improve the efficiency of CTL responses towards to the escape mutations[43,180,182,183]. Therefore, developing a better way to engineer altered peptide ligands may have a very crucial role in the design of future vaccine strategies. In line with this we used in PAPER III an unconventional peptide modification in order to modulate the immunogenicity of the LCMV-derived immunodominant peptide gp33 (KAVYNFATM) and several gp33-derived APLs.

1.6.3 Tumor-associated antigens (TAAs)

In contrast to immunogenic viral epitopes, which most often bind with high affinity to MHC-I, TAAs represent a peptide repertoire that is much more variable in terms of proteomic diversity and MHC binding affinity. As TAAs are mainly derived from self-proteins, it is likely that high affinity T cell populations specific to very stable MHC/TAA complexes are either negatively selected during thymus development or eliminated through peripheral tolerance by professional APCs[184,185]. However, it should also be noted that it is still possible to identify high affinity T cells depending on the antigen source and on the existence of high affinity T cells that escaped negative selection[186-188]. Indeed, this is one of the features that broadens the MHC affinity of the peptide repertoire since self-derived peptides with weak MHC affinity can be still immunogenic, challenging the general features of immunodominance derived from viral infection models.

TAAs can be classified into at least four conventional classes that comprise cancer testis antigens[189,190], differentiation antigens[191-193], mutated antigens[194] and antigens that are over-expressed in tumors[195,196].

Cancer-testis antigens are a category of TAAs which are normally expressed on male germline cells, and whose expression is upregulated due to dysregulation of gene-programs in a variety of tumors. Since male germline cells do not display MHC-I molecules on their surface[197], the antigenic peptides derived from these cancer-germline genes are strictly tumor-specific and are therefore targets of choice for cancer immunotherapy. Examples of cancer-testis antigens are NY-ESO-I and MAGE-A[198-200].

Despite the fact that **tissue/cell type specific differentiation antigens** are not exclusively expressed on tumors, but also on the corresponding normal tissues from which the tumors arose, these epitopes can still be immunogenic and provide attractive targets for T cell-based immunotherapy. For example, the melanoma-associated antigen MC1R and the multiple myeloma-associated Framework region derived antigen FR20 are also expressed in their respective non-malignant tissues or cell types. Additional identified differentiation antigens include peptides derived from gp100/pmel17[201-203], Melan-A/MART-1[192,193] and TRP2[204].

TAAs can also arise from **point mutations** in genes controlling natural cellular processes such as cell division and proliferation. In the incidence of such a mutation the role of the affected gene can be altered and a tumor promoting function may be conferred. For example, a mutation identified in the β-catenin gene[205] that is potentially involved in melanoma progression, has been shown to stabilize the protein, forming constitutive complexes between β-catenin and the transcription factor Lef-Tcf that results in persistent transactivation[206].

Finally, regarding one of the most well established examples of antigenic peptides identified from an **over-expressed gene** is an epitope derived from the protooncogene HER-2/Neu[207], which is often overexpressed in breast and ovarian cancers[208,209].

1.7 αβ T CELL RECEPTOR AND MHC/PEPTIDE INTERACTIONS

αβ T cell receptors are cell surface heterodimeric proteins that enable MHC-restricted antigen-specific T cell responses. The overall structure of the TCR is similar to antibody Fab fragments and TCRs are thus considered as members of the immunoglobulin superfamily[104,210,211]. αβ T cell receptors are formed by α and β subunits each composed of variable and constant Ig-like domains that are linked by disulphide bonds, followed by a transmembrane domain and a short cytoplasmic tail which is responsible for interactions with the TCR complex and ultimately T cell signaling. The TCR variable regions have three hypervariable regions similar to the complementarity determining regions (CDR) of the immunoglobulin light and heavy chains. Each TCR subunit is composed of two invariant germline-encoded CDR1 and CDR2 regions, and a highly variable region formed by CDR3. These CDRs form the pMHC binding site **(Figure 4)**.

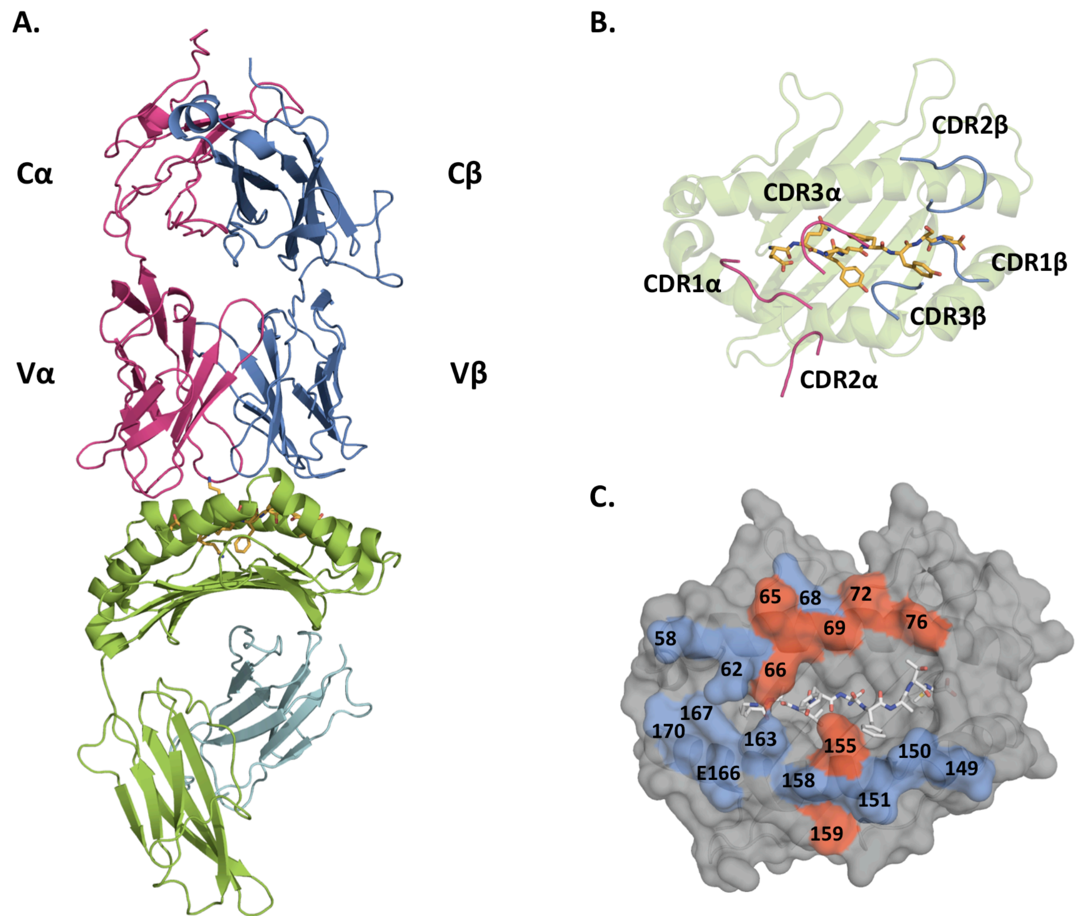

Figure 4. Structural overview of TCR-pMHC interactions. A. Overall side view of TCR-pMHC complex (PDB ID: 2CKB). TCRα (magenta), TCRβ (blue), MHC heavy chain (green) and β_2m (cyan) **B.** Top view of pMHC and TCR CDR loops, coloring is same as in A. **C.** Top view of pMHC representing "TCR footprints" on MHC. MHC surface is grey, TCR contact residues are colored either red or blue. TCR contact residues negatively effecting TCR recognition upon mutagenesis are colored as red[212-214]. Adapted from[213,215,216]. Note: TCR footprints and TCR contact residues can vary among different TCR-pMHC complexes.

The TCRα chain is located on chromosome 14 both in humans and mice. The TCRα gene consists of several functional segments formed by the variable (V), joining (J) and constant (C) regions. Successful rearrangement of these segments forms a functional TCRα chain which then couples with the TCRβ chain. The TCRβ chain is located on chromosome 6 of the mouse and chromosome 7 of the human genome. The TCRβ gene is formed by variable, joining and constant regions with an additional diversity (D) segment located between the variable and joining segments specifically forming the CDR3 of the TCRβ gene. Combinatorial joining of the VJ and VDJ segments generates a large number of random gene combinations for the TCR chains resembling over a million of αβTCR combinations. Additional diversity is achieved through nucleotide additions while combining VJ and VDJ segments. As CDR1 and CDR2 are only encoded in the V segment of the TCR loci, the variability of CDR1 and CDR2 is germline-encoded. On the other hand, the CDR3 regions of the TCR genes are generated through VJ and VDJ recombination with a potential for nucleotide addition, finally resulting in formation of the most variable regions of the TCR.

Recognition of pMHC by TCRs is mediated through interactions between the variable TCR regions and the composite surface established by the MHC α-helices and the antigenic peptide **(Figure 4)**. Most of the MHC-I specific TCRs concentrate their pMHC interactions towards the central regions of the α1 and α2 helices, binding to the pMHC in a diagonal and polarized manner, across the MHC α1 and α2 helices[104]. In general, the TCR α-chain contacts the N-terminal part of the peptide and docks on the MHC α2 helix. Conversely, the TCRβ chain interacts with the C-terminal part of the peptide and the MHC α1 helix. The focus of the most variable CDR3 loops of αβTCRs is concentrated on the peptide amino acids where the pivot point of the TCR on MHC is centered[80]. However, most of the TCR-MHC interactions are mediated through germline-encoded CDR1 and CDR2 loops establishing a gasket-like shape that surrounds the central CDR3-peptide interface[79]. TCRs usually directly contact 2-5 peptide-side chains, which bulge out of the peptide-binding groove, establishing functional hotspots where single subtle amino acid modifications can dramatically alter TCR specificity[217-219].

T cells scan cell surface MHC molecules in order to discover the best possible pMHC candidate that leads to appropriate TCR-pMHC interactions and successful T cell signaling[79]. It has been proposed that germline-encoded TCR CDR1 and CDR2 regions focus on the MHC, and this plays an essential role in TCR-mediated scanning of MHC molecules[220,221]. Structural analysis of TCR-pMHC complexes also revealed that it is quite possible that TCRs are evolutionarily biased for recognition of MHC through the germline-encoded CDR1 and CDR2 regions[79,80]. Even though TCR and MHC genes are located on different chromosomes, it has been demonstrated that specific CDR1 and CDR2 amino acid residues, such as Y29 of Vα CDR1 and Y48 Vβ CDR2, have a crucial importance in TCR-MHC interactions[79,80]. These residues generally contact/pair in the direct vicinity of other specific residues located on MHC α-helices and which are thus proposed to act as "TCR footprints on MHC"[213]. In general, tyrosine residues form van der Waals interactions with residues on MHC α-helices and flanking evolutionarily conserved CDR1 and CDR2 residues[80,222]. The formed interactions do not require a precise geometry, enabling the TCRs to be more flexible while docking on the pMHC and slide over the MHC α-helices allowing for different binding combinations during their quest for the optimal cognate peptide. This built-in flexibility of evolutionarily selected interactions results in a variation of TCR docking angles and pitches onto pMHC complexes that may compensate for differences in peptide and CDR3 sequences[80]. These features of evolutionarily conserved CDR residues ensure that every TCR has a residual affinity for most MHC molecules, which is important for positive selection and peripheral tolerance[79]. On the other hand, the peptide is important for selecting the most energetically and/or functionally optimal TCR-pMHC interactions. It also contributes to the final docking geometry of the TCR[79]. Initial contacts with MHC α-helices followed by a more meticulous scanning of the peptide-

binding groove by the CDR3 region suggests a two-step mechanism for TCR-pMHC interactions[104,221].

It is also important to note that most of the TCR-pMHC structures that have been determined until now belong to either Vβ8 in the mouse or Vβ13 in human and some more related variable regions[109,110,216,222-268]. Since these two Vβ families provide higher chances for success in crystallographic studies, most of the general conclusions about TCR/MHC interactions have been derived from these structural analyses. However it should be noted that evolutionarily selected interactions might be different for other TCR Vβ families. Furthermore, several other examples have been published in which the CDR1 and/or CDR2 loops directly contact the peptide[227,243] or in which the CDR3 loop plays a significant role in contacting MHC molecules[269-271].

1.7.1 Kinetics and thermodynamics of TCR and MHC/peptide interactions

When compared to antibody-antigen interactions, the affinity of TCR-pMHC interactions can be considered low, ranging from 1 to 200 µM[272-274]. The extreme specificity of T cells equips them with the ability to detect very low numbers of antigenic pMHC complexes, so that even one pMHC complex can be efficiently recognized and trigger a T cell response[275]. Our understanding of the kinetic and thermodynamic properties of TCR-pMHC interactions has improved dramatically following technical advances in recombinant expression of soluble TCR and pMHC molecules. In the next two sections, I will summarize the general principles regarding the biochemical basis of T cell biology, as well as the kinetics and thermodynamics underlying TCR-pMHC interactions.

1.7.1.1 Kinetics of T cell interactions

Surface Plasmon Resonance (SPR) enables kinetic measurements of TCR-pMHC interactions using soluble TCR-pMHC complexes. Initial observations led to the general conclusion that TCRs bind to pMHCs within a low affinity range and with very fast association rates that are difficult to estimate directly compared to the much slower disassociation rates. Concurrently, several hypothetical models have been proposed in attempts to explain the biology of pMHC-specific T cells, highlighting either the importance of affinity[276] or of the disassociation rates[19,277].

The "kinetic proofreading model" proposed by McKeithan, suggested that T cell activation was only possible if the amount of TCR-pMHC interactions reached a certain threshold[19]. More importantly, slower disassociation rates correlated with improved T cell activation. Later, Kalergis and Nathenson proposed that the correlation of T cell activation with the half-life of TCR-pMHC interactions may not always be linear[278], and that in some cases, longer TCR/pMHC half-lives could impair T cell responses. An optimal half-life is required for efficient T cell activation, whereas both too short or too long TCR-pMHC engagements would lead to T cell unresponsiveness[279]. This was also well in line with the serial engagement concept proposed by Lanzavecchia, where

serial engagement of TCRs to cognate pMHC can boost the internal signaling in T cells[20]. However, such boosting would be sustainable only if the half-life of a single interaction is not too prolonged. Thus, it seems important that while TCR-pMHC interactions must be long enough to fulfill the requirements of activation, they should also be short enough to avoid inhibition of serial engagement. Likewise, the affinity model suggested that T cell activation correlates with the number of receptors engaged. This was later confirmed by the observation that high affinity pMHC molecules can occupy a large number of TCRs and therefore trigger stronger responses[276,280-283]. It should be noted that, the hypothetical models introduced in this section are derived from various studies analyzing different TCR-pMHC pairs as well as different experimental setups and techniques. Therefore, a variety of experimental data support the different proposed models[19,276-279].

Furthermore, the discrepancy regarding whether the off-rate or the affinity correlates with the potency of T cell activation was recently addressed in a study[284], where a large group of pMHC ligands for a tumor-reactive $CD8^+$ TCR were categorically analyzed. The authors conclude that while affinity is the better predictor of T cell activation potency for pMHC ligands with faster association rates, those with slower association rates rely more on the off-rate of the interaction. This novel hypothetical model called "confinement time" also holds true in various cases of $CD4^+$ and $CD8^+$ TCR-pMHC interactions, including those compromised in previously incongruent studies [284]. The bottom line seems to be that among the multitude of various TCR-pMHC pairs that occur in nature, the variance in the biochemistry of interactions might reflect differences in biochemical and thermodynamic parameters[285].

Also, one should keep in mind that the juxtaposition of T cell and APC membranes most probably influences TCR-pMHC binding[286-288]. Thus, the evaluation of these interactions according to any single parameter, such as affinity or dissociation rate, is likely to be limited, although not wrong. The importance of the influence exerted by membrane interactions is further underlined by fluorescence resonance energy transfer (FRET) measurements of membrane-bound TCR-pMHC interactions, where dissociation rates in the context of cellular membranes were much faster compared to SPR measurements and the affinity was much higher when compared to measurements with molecules in solution[289]. Further support for this phenomenon is provided by mechanical assays showing a correlation between the kinetic parameters of membrane-bound TCR with T cell activation potency, while such correlation was lacking when measurements from assays with soluble molecules were used[290].

1.7.1.2 Thermodynamics of T cell interactions

Additional insights into TCR-pMHC interactions and their impact on T cell activation were gained by analyzing the thermodynamics of TCR engagement to cognate pMHC molecules. Initial measurements of TCR binding thermodynamics using JM22 (human TCR) and F5 (murine TCR) revealed that the binding of these two TCRs to cognate

pMHC molecules resulted in a combination of favorable enthalpy and unfavorable entropy changes[291]. Evaluation of kinetic and structural data also suggested, in these two examples, that rather than simple conformational adjustments upon binding, the CDR loops of the TCR were relatively flexible in the unbound state. This flexibility stands out as a key feature in TCR biology as it probably allows the TCR binding site to adapt slightly to different MHC ligands. Similar favorable enthalpy and unfavorable entropy changes were later demonstrated for the binding of the 2B4 TCR to its cognate pMHC ligand[292]. Although early thermodynamics data indicated a need for folding of TCR CDR loops upon recognition of pMHCs, the accumulation of more thermodynamic and structural data provided a more refined and revised view which still encompassed CDR loop adjustments but of different magnitude and seemed to consist of well defined structural shifts rather than the ordering of highly flexible backbones[223]. Overall, it was believed that CDR loop adjustments required to bind to pMHC complex is one of the major contributors to the observed unfavorable binding entropy.

The heat capacity change (ΔCp) measured during TCR-pMHC interactions probes the conformational changes. Davis and colleagues were first to assess ΔCp using the 2B4 TCR/MCC/HLA-E^k complex[292]. Since the heat capacity change is strongly influenced by changes in solvation[293] and since burial of hydrophobic surfaces contributes positively to ΔCp[294,295], such measurements provide important insights into differences that may alter solvent exposed surface areas[296] and thus conformational changes. A negative heat capacity is consistent with burial of hydrophobic surfaces[297], as well as entrapment of waters[298] and has also been connected to T-cell activation[285]. Although certain methodological caveats do exist, principally concerning the accuracy of Van't Hoff determined heat capacity changes, the occurrence of conformational changes between free and pMHC-bound TCRs seems to be a common event. Several structural studies have demonstrated conformational differences between free and pMHC-bound TCRs, as well as differences between the same TCR bound to different pMHC ligands, which established the grounds for the unfavorable binding entropy that reflects the loss of TCR conformational flexibility[237,260,299-301].

While this suggested thermodynamic signature presented very interesting implications for T cell biology, it was recently questioned when favorable entropy changes were demonstrated in the binding of A6 TCR Tax to HLA-A2[302]. Favorable entropy changes in protein-protein interactions commonly arise from desolvation, whereby ordered waters are expulsed from apolar surfaces upon binding, leading to an increase in total entropy of the system[303]. The release of water molecules upon TCR-pMHC binding can thus promote an entropic pathway of recognition, as clearly demonstrated in a previous study[270]. Following repeated confirmation of various similar favorable entropy cases[216,231,257,304], the notion that the biochemistry of each TCR-pMHC interaction is different has gained more momentum, concluding that unfavorable binding entropies are not necessarily a signature of these interactions.

On the other hand, poor shape complementarity between TCRs and pMHC interfaces may result in the formation of cavities that can trap waters, as observed in several TCR/pMHC crystal structures[238,270,305]. Trapped water molecules can form hydrogen bonds, thus contributing positively to the binding enthalpy of TCR interactions. In general, enthalpy changes are all favorable in correlation with the majority of protein-protein interactions[306], but there are several TCR-pMHC interactions with enthalpy changes that are very close to zero. In conclusion, it is becoming more and more clear that TCR-pMHC interactions are far from having a unique enthalpic/entropic signature. As Armstrong and Baker elegantly put it in a recent review: "It matters not how you form the TCR-pMHC complex, just that you do"[307].

While there is still a need for direct assays for the determination of interaction dynamics, especially regarding the flexibility of CDR loops, comparative thermodynamic analysis of free and pMHC-bound TCRs may still provide important insights, especially if the conformational changes occur due to a pre-existing conformational equilibrium in the unbound receptor, as hypothesized by Holler and Kranz[308] and recently observed by James and Tawfik using the SPE7 antibody[309].

Interestingly, the conformational changes that take place during TCR-pMHC binding are not limited to those of the TCR. Several studies have shown that conformational changes occur in the peptide between the free and bound forms of the pMHC complex. While two studies demonstrate rather small conformational changes[240,310], a third study reported the flattening of the extremely bulged HLA-A35-restricted EBV-derived peptide upon TCR binding[110]. Similarly, other studies have shown that peptides may adopt multiple conformations in the peptide binding groove of MHC or that disordered side chains may become ordered upon TCR engagement[111,311-318].

1.7.2 TCR cross-reactivity

Even though TCR must be highly pMHC-specific, T cells display dual specificity to self and non-self. Thus, T cells are also highly promiscuous with the ability to recognize MHC-restricted self-peptides and actually benefit from this feature to distinguish self and non-self. More specifically, T cells seem to be poly-specific[10]. Indeed, it has been suggested that a single TCR may react productively with approximately 10^6 different MHC-associated minimal peptide epitopes[319] and that different peptides can act as agonists for a given T cell, while a considerably larger number of peptide ligands induce weaker signals[10]. Several studies have demonstrated such TCR cross-reactivity to a variety of human and murine T cells[320-328]. Cross-reactivity roots from the developmental phases of T cells, where during thymic selection, only those expressing TCRs that optimally recognize self-pMHC complexes are allowed to maturate. Compared to the activation of mature T cells, weaker TCR signals are required for positive selection in the thymus, which is also peptide-dependent and can be influenced by both the density of the pMHC complex, as well as the affinity of the TCR

to that specific pMHC. Similarly, it has been observed in thymic organ cultures that low densities of agonist peptides, as well as normal densities of peptides deemed as weak agonists or antagonists for mature T cells, could drive positive selection[329-331]. Therefore, circulating mature T cells that have a vital role in detecting non-self pMHC complexes are already specific for one or several self-pMHC complexes.

TCR cross-reactivity can have a profound effect on the outcome of responses against invading pathogens, as demonstrated in murine models where $CD8^+$ T cells cross-reacted with peptides from two different viruses, LCMV and Pichinde virus (PV)[329-331]. Such examples put forward TCR cross-reactivity as a common phenomenon that maintains a functional significance by adding extra diversity to T cell responses. The maintenance of a fine balance between specificity and cross-reactivity is likely to contribute to a system where a sufficient number of T cells in a given individual is able to respond to a completely novel pathogen[332]. Studies have also shown that a relatively diverse TCR repertoire, even in the presence of only one MHC-II-restricted peptide can be generated and that these TCRs may have the capacity to react to peptides with no sequence identity to the selecting peptide[333]. This constitutes a proof-of-principle for T cell activation by peptides that are unrelated in sequence to the original selecting peptide, which also provides an additional insight into positive selection and TCR cross-reactivity.

A major mechanism underlying the phenomenon of cross-reactivity is the plasticity of the interactions between TCRs and pMHCs. Although this encounter might be described as a lock-and-key interaction, it is presently thought to be a more dynamic event where both the lock and the key may undergo minor rearrangements in order to establish an optimum fit. Several examples of TCR-pMHC complexes have provided clues regarding the importance of the plasticity of CDR loops on TCR recognition[249,300,301]. Structural rearrangements taking place during TCR engagement to pMHC seem to be of key importance for the stabilization of the interaction[271]. Moreover, this plasticity[110] allows the TCR to scan the surface of the pMHC in order to establish a favorable binding position, thereby increasing the chances for cross-reactivity towards another novel epitope or pMHC.

A prominent example of TCR plasticity was observed for the mouse TCR BM3.3 bound to three different pMHC ligands $H\text{-}2K^b$/VSV8, $H\text{-}2K^b$/pBM1 and $H\text{-}2K^{bm8}$/pBM8[260,301]. Comparative structural analysis of BM.3.3 in complex with all three pMHCs suggested the conformational fine-tuning of TCR CDR loops[260,301]. More specifically, during the docking of the BM3.3 TCR to the three different pMHCs, the CDR3α loop was significantly remodeled although the BM3.3 TCR bound to all pMHCs with very similar overall docking angles.

The first determined crystal structure of a ternary TCR-pMHC complex was the human A6 TCR and its human T-cell lymphotropic virus (HTLV-1)-derived cognate peptide Tax in complex with HLA-A2[240]. The same TCR cross-reacts with HLA-A2 in complex with

the *Saccharomyces cerevisiae*-derived Tel1p peptide that shares only five residues with the Tax peptide. Comparative structural analysis of HLA-A2/Tel1p and HLA-A2/Tax with or without the A6 TCR revealed that the plasticity of the Tax peptide played an important role in the observed cross-reactivity between the two pMHCs[225]. Interestingly, the conformation of the peptide in the A6 TCR/HLA-A2/Tax complex perfectly mimicked Tel1p in complex with HLA-A2 before TCR engagement. These results highlight the significant role of peptide plasticity in TCR cross-reactivity, where alternating peptide conformations can provide a similar antigenic landscape for a given TCR. Additionally, a similar form of plasticity was also seen at the MHC level, in the frame of TCR allorecognition, where cross-recognition of structurally similar polymorphic MHCs could be derived through both structural alterations and molecular mimicry[271].

Molecular mimicry has been defined as similar structures shared by molecules encoded by dissimilar genes[334], where shared key chemical and structural features of different pMHCs result in TCR cross-reactivity[335]. In principle, molecular mimicry allows a single TCR to engage cross-reactive ligands similarly. The role of molecular mimicry-based TCR cross-reactivity in transplantation and autoimmune diseases has been discussed elsewhere[336-339]. For example, T-cells specific for foreign peptides can also recognize structurally similar self pMHCs and initial triggering of T cells specific for foreign peptides can result in recognition of self peptides in complex with MHC in the periphery, potentially triggering autoimmune responses[323,337,340-346].

The murine P14 TCR recognizes the LCMV-derived H-2D^b-restricted immunodominant epitope gp33. The same TCR can also recognize a self-epitope derived from an enzyme expressed in the adrenal medulla called dopamine Β-mono-oxygenase (DBM)[344,347]. The sequence identity between the gp33 epitope (KAVYNFATC) and the mDBM epitope (KALYDYAPI) is restricted to only four residues. The P14 TCR can also recognize the rat homologue of DBM (rDBM), which has an additional identical residue at H-2D^b anchoring residue p5 (KALYNYAPI). However, although almost half of the residues of the gp33 and rDBM epitopes are not identical, both peptides in complex with H-2D^b displayed a perfect molecular mimicry explaining the basis of cross-recognition of gp33 and rDBM by P14 TCR[348].

1.8 ALTERED PEPTIDE LIGANDS (APL) AND PEPTIDE DESIGN

A promising strategy to improve T cell responses against MHC-restricted viral escape mutations and TAAs with low immunogenicity is the use of optimized peptides or altered peptide ligands (APL), whose amino acid sequences have been slightly modified in order to improve binding to HLA molecules or to increase efficiency of interaction with TCRs. The term APL was initially proposed by Brian Evavold “to describe analogues of immunogenic peptides in which the TCR contact residues have been manipulated”[349]. Since then, APLs have been used in several studies to assess and

possibly to improve TCR interactions with cognate pMHCs[43,217,297,350,351]. It should also be noted that the term APL was later broadened to include peptide analogues with modifications at MHC interacting positions.

The molecular characterization of TAAs that are recognized by T cells opens up for new possibilities for the design of well-defined and targeted therapeutic vaccines against cancer. Immunological eradication of tumors is often associated with a robust cytotoxic T cell response against TAAs. Since many TAAs are self-proteins or are closely related to self-proteins, they tend to be poorly immunogenic. Most T cells with high affinity and specificity to self-peptides are eliminated during T cell development. As a consequence of thymic selection, in which high affinity auto-reactive T cell clones are negatively selected[84,352], T cells specific for TAAs are generally low affinity cells, tightly controlled by peripheral tolerance[353] and are exposed to immunosuppressive pressure by the tumor milieu[354].

The conventional approach to design tumor-derived APLs is to optimize MHC anchor residues. The primary goal of this approach is to achieve high affinity peptides that would stabilize MHC complexes over a longer period of time to enable more efficient interactions between TCR and MHC/APL complexes, possibly resulting in the activation of T cells that would cross-react with wild-type pMHC complexes and hopefully eliminate tumor cells. Another way of designing APLs is to modify the peptide residues that contact TCRs. Two well-described examples are the p1L-variant analogue of the Mart127-35 epitope[355] and the p6D analogue of the carcinoembryonic antigen (CEA) CAP peptide[356,357].

The extensive use of APLs in several studies and TCR models has provided a significant improvement in the understanding of the potential immunomodulatory role of peptide-MHC affinity, TCR-pMHC affinity and the efficacy of immune responses triggered by TCR-pMHC interactions[43,191,272,325,326]. However, despite the promising results achieved in pre-clinical studies, peptide-based tumor vaccines have not yet fulfilled the expected clinical efficacy[358,359]. Therefore, it is essential to define the reasons for the partial inefficiency of previous peptide vaccination trials, such as the suppressive tumor milieu and immune escape strategies at the tumor cell level[360,361]. Additionally, one of the potential reasons that could lie behind the failure of APLs as effective cancer vaccines so far is that the T cells primed by APLs can not efficiently and productively cross-react with wild-type epitopes. Introduction of amino acid substitutions in order to enhance immunogenicity of peptides has the inherent risk of producing diverse and unpredictable changes to the TCR-pMHC interactions that could also lead to the expansion of a TCR repertoire, which may not cross-react with the native epitope[351].

The structural properties of TAAs are also still poorly understood and only few crystal structures of human MHC/TAA complexes have been reported. Several research groups have designed peptides with enhanced MHC binding by substituting anchor

residues to the most preferred residues of the MHC allele. Unfortunately, this approach does not fit well to most of the peptides since as mentioned before, many TAAs that are eluted or predicted to bind to the MHC allele using a specific algorithm already contain the preferred MHC anchoring residues. Additionally, a recent study from the group of Brian M. Baker elegantly demonstrated that an anchor residue modified variant of the Mart-1 epitope failed to improve the antigenicity of the peptide despite significantly improving the peptide-MHC affinity. The introduced modification enhanced the flexibility of the pMHC in which the modified peptide adopts two different conformations, thus negatively effecting TCR binding[362].

In contrast, the research presented in this thesis uses alternative ideas to design a new family of APLs, based on the comparative analysis of a large number of crystal structures of infection-associated and TAA peptides in complex with different MHC-I molecules[118] (Papers II and III). Here we aimed to achieve improved binding affinity and stabilization capacity of such modified peptides to MHC molecules through increased van der Waals and CH-π interactions with specific MHC heavy chain residues that are conserved among most known MHC-I (e.g. tyrosine residue Y159 and tryptophan residue 167), while keeping the conformation of the MHC/APL as similar as possible to the parent pMHC **(Figure 5)**.

Taken together, the current knowledge about engineering TCR-pMHC interactions highlights four fundamental rules of APL design[351]. First, it is important to improve peptide-MHC affinity and pMHC stability. Second, APL should trigger potent and rapid T cell responses. Third, expanded APL specific T cells should efficiently cross-react with the original peptide. Finally, APL-specific T cells should efficiently recognize and eliminate infected cells or tumor cells, which are naturally processing and presenting the WT epitope.

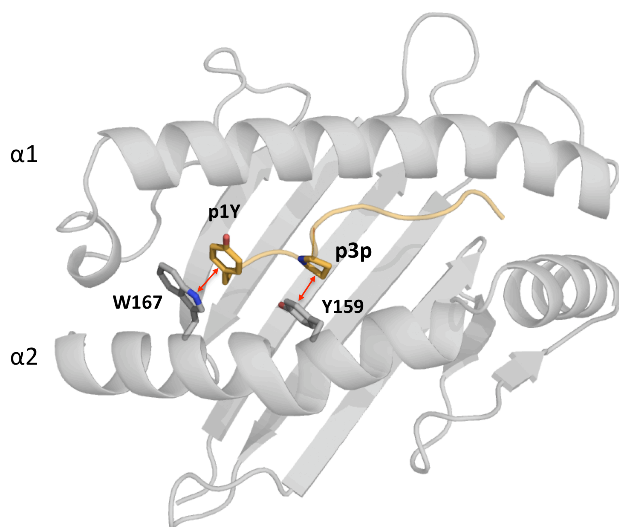

Figure 5. Targeting evolutionarily conserved residues in order to improve pMHC stability and immunogenicity. Introducing Y at p1 and P at p3 of the peptide (yellow) can increase van der Waals and CH-π interactions with MHC (grey) residues W167 and Y159, respectively.

2 GENERAL AIMS OF THIS THESIS

The overall aim of my PhD studies was to enhance our understanding of the molecular and functional basis underlying the potential modulation of $CD8^+$ T cell and NK cell responses by MHC-I-restricted altered peptide ligands (APLs). Using a combination of functional immunological assays, biochemistry and structural biology, I aimed to understand how specific peptide modifications could affect the recognition of MHC/peptide complexes and alter the functional outcomes. The results of these studies are important for the future design of novel peptide-based vaccines.

My specific aims were to develop a rational approach in order to design novel altered peptide ligands by:

1) Improving the peptide binding affinity and peptide-MHC stability of viral-derived and tumor-associated antigens to their cognate MHC-I molecules through localized molecular modifications.

2) Visualizing and understanding, by X-ray crystallography, how the introduced substitutions improved binding affinity and pMHC stability of the modified peptides without affecting their conformation (molecular mimicry).

3) Enhancing cytotoxic T cell responses to the modified epitopes in complex with their cognate MHC-I molecules and triggering a T cell population that efficiently cross-reacts with the original wild-type epitopes.

3 RESULTS AND DISCUSSION

Inflammation-associated nitrotyrosination affects TCR recognition through reduced stability and alteration of the molecular surface of the MHC complex (PAPER I)

The results of this study indicate that conversion of a specific peptide residue from tyrosine to nitrotyrosine may have very important implications for T cell recognition either through direct TCR contacts, or indirectly by MHC contact residues.

PTMs provide an elegant potential mechanism for the triggering of novel/different T cell responses and for avoiding the constraints of immunological self-tolerance, as well as for evading immunosurveillance through the presentation of MHC-restricted neo-antigens. PTMs are common during inflammation including viral infections and potentially compromise host defense mechanisms. Several others have highlighted the importance of PTMs as novel antigenic agents that can be used in the development of novel immunotherapy and vaccine design approaches[152,167,168]. For example, MUC1, a mucin type glycoprotein, is an over-expressed and abnormally glycosylated protein in cancer cells. MUC1-derived glycosylated epitopes have been used to break tolerance in order to target cancer cells more efficiently[363-365]. Recent crystal structures of pMHC complexes with phosphorylated epitopes demonstrated that PTM residues could interact directly with the TCR and affect pMHC stability[366,367]. Moreover, several studies have demonstrated that PTMs can break tolerance and influence the progress of autoimmune diseases including arthritis, lupus erythematosus, multiple sclerosis and type 1 diabetes[363,368-378]. For example, deamidated epitopes derived through the digestion of wheat gluten bind with high affinity to the predisposed HLA-DQ2 and/or HLA-DQ8 MHC alleles, eliciting strong T cell responses against the gastrointestinal lining in celiac disease[373-375].

Several studies have demonstrated that LCMV infection of C57BL/6 mice generates strong CTL responses towards H-2K^b and H-2D^b in complex with the immunodominant epitopes gp34-41 (AVYNFATM) and gp33-41 (KAVYNFATM), respectively[379,380]. Introduction of PTMs in these epitopes generates neoepitopes, which when presented by H-2D^b and H-2K^b may lead to altered CD8$^+$ T cell responses during LCMV infection[166]. More specifically, conversion of the tyrosine residue at positions 3 and 4 of the immunodominant peptide gp34 and gp33, respectively, to a nitrotyrosine (NY) greatly affected recognition of the immunodominant gp34 and gp33 epitopes by CD8$^+$ T cells, providing potential additional means for immune escape[166].

In this study, we investigated the structural and biochemical effects of such a modification on altered T cell recognition following nitrotyrosination of gp34. Comparison of the crystal structures of H-2K^b/gp34 and nitrotyrosinated H-2K^b/NY-gp34 provided a structural explanation for the observed differential TCR recognition. Hydrogen bond interactions between p3Y and the H-2K^b residue E152 were abolished

following the post-translational modification of p3Y to p3NY, resulting in a different conformation for E152 that indirectly altered TCR recognition **(Paper I Figure 3)**. Furthermore, significant negative effects of nitrotyrosination on MHC/peptide binding efficiency and stabilization capacity were observed **(Paper I, Figure 4)**. Importantly, although additional interactions were formed between the PTM peptide and H-2K^b, the conformation of a large amount of surrounding residues was strained, significantly reducing the overall stability of the MHC complex. Even though such strained interactions have not yet been reported for MHC-I complexes (besides our study), strained conformations are commonly observed in crystal structures of other proteins including spectrin SH3 core-domains, native serine protease inhibitors and thymidylate synthase[381-383]. Our research group has previously demonstrated that subtle modifications in MHC-restricted peptides can result in significant alterations of MHC stabilization[176], a phenomenon that should be taken into account and measured upon trying to design APLs for modulation of T cell responses[118,362]. Likewise, several other amino acid modifications caused by oxidative stress could result in similar effects on TCR recognition[384,385]. Taken together, the results of this study could provide additional structural and biochemical explanations to the underlying mechanisms through which PTMs may be used by viruses or other pathogens to evade immune surveillance. Most importantly, this study demonstrates that nitrotyrosination can directly affect TCR recognition by modifying the properties of key TCR-interacting residues on the presented peptide **(Paper I, Figure 5)** or by altering the conformations of other MHC residues that are of importance for TCR recognition. Furthermore, nitrotyrosination can also indirectly affect TCR recognition by severely destabilizing the MHC complex.

The fundamental basis that the above-mentioned mechanisms provide for MHC-dependent recognition, highlights the imminent dual impact of post-translational modifications, possibly allowing viral immune escape from TCR recognition but also potentially inducing the expansion of a subset of T cells that could induce autoreactivity[152,167,168]. Similar mechanisms are applied on self-peptides in the case of inflammation or oxidative stress, which could also lead to the formation of neo-epitopes leading to escape from central tolerance. Little is known about the impact of these modified *de novo* MHC complexes on the initiation of unwanted T cell responses that may result in autoreactivity. Future identification of disease-associated PTMs that break tolerance may improve both diagnosis and treatment of autoimmune diseases.

In conclusion, our study provides a novel structural platform explaining the functional effects of peptide nitrotyrosination on initiation of TCR responses and provides useful insight into the complexity of factors governing MHC-dependent immune surveillance. Besides answering basic questions regarding the effects of subtle changes in the peptide presented by the MHC molecule, the insight gained by this study might help to improve the arsenal of tools used to fine-tune T cell responses *in vivo* or *ex vivo*.

The unexpected T-cell recognition of an altered peptide ligand is driven by reversed thermodynamics (PAPER II)

This study investigates the molecular basis underlying the variable recognition by the same TCR of the MHC-I molecule H-2D^b in complex with four different APLs. In my opinion, the results contribute to understanding the molecular basis for TCR discrimination of MHC-restricted APLs and provide additional insights into the design of novel APLs for future peptide vaccine approaches. The TCR P14, specific for H-2D^b in complex with the immunodominant LCMV-derived peptide gp33 (KAVYNFATM), is highly sensitive to subtle modifications at the protruding peptide position 4. The naturally occurring escape mutation of the tyrosine residue at position 4 of gp33 to a phenylalanine (Y4F), completely abolishes recognition of the MHC complex by P14[107,176,181,182,386,387]. Thus, the removal of only a hydroxyl group is enough to abolish TCR down-regulation, P14 CD8$^+$ T cell proliferation, activation, maturation and effector functions. Furthermore, substitution of the tyrosine residue at position 4 to a serine (Y4S) abrogated P14 T cell responses similar to Y4F. Additionally, this APL antagonized the recognition of H-2D^b/gp33 by P14[387,388]. In contrast, P14 still recognizes H-2D^b in complex with Y4A, where an entire aromatic ring was substituted to an alanine. Thus the main question addressed by this study was why the same TCR, with such a high sensitivity to subtle changes as in Y4F, can retain the capacity to recognize other APLs with more dramatic modifications such as Y4A.

In order to address this phenomenon we utilized functional immunological assays, as well as several structural and biochemical techniques. The thermal stability of the four pMHCs included in the study, were analyzed by circular dichroism (CD) in order to assess if the immunogenic hierarchy was due to pMHC interactions. Additionally, the crystal structures of H-2D^b/Y4A and H-2D^b/Y4S were determined and compared to the previously published structures of H-2D^b/gp33 and H-2D^b/Y4F. Finally, the affinity of P14 TCR to each pMHC was measured using SPR and the thermodynamic characteristics of P14 TCR ligation to both gp33 and Y4A were also determined.

All functional assays, including Cr^{51} release cytotoxicity, TCR down-regulation, IFNγ production, T cell proliferation and CD107a degranulation, demonstrated that the four peptides followed the immunogenic hierarchy that can be described as gp33>Y4A>Y4S=Y4F **(Paper II, Figure 1)**. Furthermore, MHC-peptide affinities were measured for all APLs using the TAP-deficient H-2D^b positive cell line T2-D^b. All APLs displayed similar strong peptide binding affinities and capacity to stabilize cell surface MHC levels. In order to exclude the possibility that differences in pMHC stability were the main contributing factor for the immunogenic hierarchy, we also measured the melting temperatures of the different pMHC complexes using CD. Our results demonstrated that the observed immunogenic hierarchy could not be explained by differences in peptide MHC affinity and MHC stabilization capacity **(Paper II, Figure 2 and Supplementary Figure 1)**.

As a positive correlation between affinities of TCR/pMHC interactions and T cell responses has been previously demonstrated, we also assessed the affinity of soluble P14 to H-2D^b in complex with each peptide using SPR **(Paper II, Table I and supporting information Figure 2)**. The K_D values for the interaction of P14 with gp33 and Y4A were measured at 8.6μM and 58.6μM, respectively, displaying good correlation with the immunogenic hierarchy. The affinities of P14 to Y4F and Y4S were too low, impairing the possibility to derive an exact K_D value for these interactions with P14 TCR. Even though accurate values could not be achieved, it was clear that the binding affinities of these modified peptides were dramatically lower compared to both gp33 and Y4A, which is also in line with the observed immunological hierarchy.

Detailed comparison of the crystal structures of H-2D^b in complex with Y4A and Y4S with the previously published crystal structures of H-2D^b/gp33 and H-2D^b/Y4F[176] revealed that all peptides displayed similar conformations. However, subtle structural alterations were observed in H-2D^b and peptides **(Paper II, Figure 4)**. More specifically the side chains of peptide position 1 (p1K) and MHC residues E58 and R62 in H-2D^b/Y4A were different compared to other peptides in complex with H-2D^b. This conformational change at p1K and its surrounding has previously been described in the crystal structure of another semi agonist APL of gp33 (F6L) in complex with H-2D^b [176]. Additionally, this region of the pMHC has also been previously suggested as a secondary hotspot for P14 TCR recognition[389]. As position 1 of the peptide plays an important role in TCR recognition for several MHC alleles including H-2D^b [389], HLA-A2[390], H-2K^b [391] and HLA-B27[389,390,392], p1K was replaced in both gp33 and Y4A to a serine or a leucine in order to investigate the functional impact of this subtle conformational difference. p1S and p1L-substituted gp33 and Y4A could not trigger TCR down-regulation and almost abrogated T cell recognition **(Paper II Figure 5)**. Thus, in the frame of P14 TCR recognition peptide position 1 and MHC residues in its vicinity can act as a secondary hotspot that can compensate for the loss of main TCR protruding residues, providing a potential answer as to the observed recognition of Y4A by P14 but not Y4F and Y4S.

While the results above mainly support the immunogenic hierarchy rather than provide detailed information regarding the discrepant P14 TCR behavior against the peptides used in this study, further analysis was performed to understand how the interactions of P14 with H-2D^b/gp33 and H-2D^b/Y4A were thermodynamically different. As previously elegantly reviewed by Armstrong and Baker, the same TCR can recognize different pMHC complexes with altered thermodynamic signatures, indicating the potential importance of conformational changes and water release upon TCR binding[307].

Different thermodynamic signatures were clearly established for the recognition of the agonist H-2D^b/gp33 and the weak agonist H-2D^b/Y4A by P14 TCR. In contrast to the recognition of H-2D^b/gp33 that was strictly enthalpy-driven, recognition of H-2D^b/Y4A

by P14 was characterized by favorable entropy combined with a large reduction in the favorable enthalpy term **(Paper II, Figure 3)**. In protein-protein interactions, the resulting change in enthalpy can be described as the sum of favorable enthalpy that is derived from all inter- and intra-molecular interactions formed, and unfavorable enthalpy that results from e.g. the displacement of water molecules from the binding interface[393]. It is highly possible that the favorable ΔH measured for the P14/H-2D^b/gp33 interaction reflects the formation of several intermolecular contacts. Conversely, fewer intermolecular contacts following the mutation of tyrosine to alanine at p4 of gp33 may underline the observed drastic reduction in favorable ΔH for the P14/H-2D^b/Y4A interaction[257]. It is likely that the interactions formed between the CDR(s) of P14 and MHC regions such as around p1K in H-2D^b/Y4A may partially compensate for the loss of interactions between P14 and the region surrounding p4A. Such a phenomenon would also be well in line with previous data that demonstrated the adaptive capacity of TCRs to certain pMHC ligands through distinct conformations of the inherently flexible CDR3 loops, resulting in TCR cross-recognition[262,394-396].

Water molecules play an important role in both protein folding and protein-protein interactions. Prominently, the phenomenon of desolvation, where upon binding, the ordered water molecules are expulsed from apolar surfaces, leads to an increase of entropy and commonly gives rise to favorable entropy changes[303]. The expulsion of water molecules upon TCR binding to pMHC can result in favorable entropy for the recognition event[270]. On the other hand, poor shape complementarity between TCR and pMHC, may result in the formation of cavities that trap water molecules as frequently observed in several crystal structures[238,270]. These trapped water molecules might form hydrogen bonds and contribute positively to the binding enthalpy of pMHC recognition by TCR. In the case of H-2D^b/gp33 and H-2D^b/Y4A, we have observed an equivalent amount of water molecules with a similar degree of coordination **(Paper II, Supporting information Figure 4)**. Thus, the unfavorable entropy measured for P14/H-2D^b/gp33 likely reflects an inherent loss of flexibility consistent with the ordering of CDR loops and pMHC residues upon binding[397]. Conversely, the favorable entropy for recognition of H-2D^b/Y4A by P14 could be a result of the possible increase in desolvation entropy in combination with a reduction of entropic cost upon ordering the CDR loops which is also well in line with the reduced ΔH for this interaction.

Burial of hydrophobic surfaces[297] as well as entrapment of waters[298] might also reflect as negative heat capacity change and display a connection to T cell activation. Negative ΔCp-values were measured for both H-2D^b/gp33 and H-2D^b/Y4A interactions within the range of previously reported TCR-pMHC interactions[297]. However, the ΔCp-value for the ternary P14/H-2D^b/gp33 interaction was four times more negative compared to the P14/H-2D^b/Y4A interaction **(Paper II, Table I)**. This can possibly be explained by the increased water molecule displacement during the interaction of P14 with H-2D^b/Y4A

that also corresponded well with the favorable entropy change observed for this interaction.

Even though it is still not completely clear why H-2D^b/Y4S was not recognized by P14, our study might provide some potential explanations. First, in contrast to H-2D^b/Y4A, we have not observed any conformational change at p1K and the surrounding MHC residues, which may introduce a penalty for TCR docking and adaptation upon binding. Secondly, water molecules are more coordinated in the crystal structure of H-2D^b/Y4S, specifically around the hydroxyl tip of p4S **(Supporting information Figure 4)**. Thus, it is possible that amino acid substitutions in the peptide, which interfere with the release of coordinated water molecules upon TCR binding, leading to an increase in the enthalpy price for this interaction[274]. Therefore one may speculate that entrapped water molecules within the H-2D^b/Y4S complex represent one of the negative factors that affect P14 TCR recognition.

In conclusion, our study clearly demonstrates that the same TCR has the ability to respond to both the agonist H-2D^b/gp33 and the semi-agonist H-2D^b/Y4A through the use of different thermodynamic strategies. In the absence of main TCR hotspot residues, interaction with the first peptide position may take over serving as a secondary/alternative hotspot. Additionally, tightly coordinated water molecules may interfere with TCR recognition. Finally, in the frame of designing optimal peptide vaccines including amino acid modifications, we believe that one should take into consideration the conclusions derived from this study and keep in mind that several biochemical aspects can affect the efficiency of designed peptides, besides improving peptide binding affinity.

Induction of efficient CTL responses against a viral escape mutant through an unconventional peptide optimization (PAPER III)

The Achour research group has previously demonstrated that substitution at peptide positions 2 and 3 of the H-2D^b-restricted melanoma-associated epitope gp100 (EGSRNQDWL) to a glycine and proline, respectively, improved both the stability and the immunogenicity of H-2D^b in complex with the EGP peptide (EGPSRNQDWL)[398]. Most importantly, although activation with the wild-type EGS peptide did not result in any response against the EGS epitope, CD8$^+$ T cells activated using EGP cross-reacted to EGS and displayed a remarkable improvement of response against the H-2D^b/EGS complex. Comparative structural analysis of H-2D^b in complex with the two peptides indicated that the binding affinity of EGP to H-2D^b was increased through the formation of a combination of van der Waals and CH-π interactions with the side chain of the tyrosine residue 159 in H-2D^b. Importantly, analysis of the crystal structures also demonstrated that the introduction of glycine and proline in the non-anchoring peptide positions 2 and 3, respectively, did not affect the conformation of the modified

peptide EGP when compared to the initial wild-type peptide EGS, keeping a strict molecular mimicry between the two MHC complexes. This identical conformation probably lies at the heart of the efficient cross-reactivity of T cells activated by EGP towards EGS.

In the follow-up study presented in this paper, we applied the same p3P modification to the H-2D^b-restricted viral epitopes presented in paper II (gp33, Y4A and Y4F). An interesting aspect of the study was to analyze whether the introduced substitutions could revert the effects presented by the viral escape mutation Y4F. It is important to note that, in contrast to the EGS/EGP study, the gp33 peptide and its APLs bind efficiently to H-2D^b. Proline-substituted versions of gp33, Y4A and Y4F are annotated as V3P, PA and PF, respectively.

We used CD to determine the effect of p3P on the thermal stability of the pMHC complexes and SPR to assess the affinity of P14 to all the p3P modified pMHCs. Comparative analysis of the stabilization capacity of all the studied peptides using CD demonstrated that all the p3P-modified peptides displayed significantly increased thermal pMHC stability **(Paper III, Figure 1)**. Moreover, the affinity (K_D) of P14 to H-2D^b in complex with WT epitopes and p3P-modified variants was assessed demonstrating that pMHC complexes with the p3P-modified peptides exhibited consistently improved affinity to the P14 TCR **(Paper III, Figure 1)**. Similar results were obtained by measuring TCR downregulation, which is one of the first markers of T cell activation. Target cells presenting p3P-modified peptides significantly improved the P14 TCR down-regulation **(Paper III, Figure 1)**. Furthermore, all performed functional assays including Cr^{51} release cytotoxicity, TCR downregulation, intracellular IFNγ and TNF production, T cell proliferation and CD107a degranulation demonstrated a clear improvement of immune responses to all proline-substituted APLs resulting in the following novel hierarchical order V3P>gp33> PA>Y4A>PF>Y4F **(Paper III, Figure 3)**.

The crystal structures of all proline-modified peptides were determined **(Paper III, Figure 2 and supplementary Figure 2)**. Comparative structural analyses demonstrated a conserved peptide conformation and overall molecular mimicry between p3P-modified peptides and their wild-type counterparts.

Finally, *in vivo* investigation of the effects of the p3P modifications was performed using C57BL/6 mice, analyzing T cell responses against the viral escape epitope Y4F following immunization of the animals with gp33, the viral escape variant Y4F or PF. More importantly, vaccination with PF resulted in a significantly more focused CTL response towards Y4F, as demonstrated by the significantly enhanced ratio of Y4F/gp33-specific T cell responses **(Paper III, Figure 4)**. Interestingly, it has been previously demonstrated that a CTL clone specific to H-2D^b/Y4F could only partially cross-react with H-2D^b/gp33, by killing target cells loaded with gp33 but failing to proliferate and secrete IFNγ[399]. Additionally, several studies demonstrated that such viral escape variants induced anergy in cross-reactive T cells[400], apoptosis[401,402] or

diminished T cell activation[403]. Thus it is essential to develop novel approaches in designing peptide vaccines that specifically target viral escape mutants. Such vaccines should efficiently elicit distinct and more specific T cell responses against viral escape mutants **(Paper III, Figure 4 and 5)** in order to avoid negative impacts of immune interference, T cell antagonism, and T cell anergy. Therefore, the use of PF, which enabled the generation of a robust T cell response, highlights the potential use of such modifications in future vaccination attempts. In my opinion, the same kind of unconventional approaches targeting evolutionarily conserved MHC residues, can be adapted to other alleles. Indeed, these promising results could be adapted to immune evading tumor-associated antigens and/or to other viral escape mutants in several viral infection models such as HIV. Design of such modifications that increase pMHC stability and affinity, while preserving molecular mimicry in order to ensure cross-reactivity between the modified APL and the wild-type peptide stands out as a key point if such approaches are to be evaluated.

Natural killer cell tolerance persists despite significant reduction of self MHC class I on normal target cells in mice (PAPER IV)

As previously mentioned, MHC-I serves as a window into the cell, providing a basis for T cells to distinguish self from non-self by displaying internally processed peptides. This communication is mediated through specific interactions between MHC-I and TCR. This window of communication is reflective of the general status inside the cell and provides T cells with qualitative information that is used to determine their actions. Instead, NK cells are tuned to recognize and react to the presence or absence of this communicative window[39]. In this sense, NK and T cells play complementary roles regarding MHC-I recognition and thus immunosurveillance. Cell surface MHC-I expression can be downregulated during viral infections or malignant transformation in order to avoid T cell recognition[140,404-406]. While this ensures escape from T cell-mediated immunosurveillance, reduced amount of surface MHC-I can result in NK cell triggering. This role of NK cells is maintained in mice through interactions between MHC-I and the inhibitory Ly49 molecules that belong to the C-type lectin like receptor family[38,39,41,407].

In this study, MHC-deficient, MHC-homozygote, MHC-hemizygote and multiple MHC-expressing mouse models were used to investigate the extent of MHC-I downregulation required for normal cells to trigger NK cell effector functions. C57/BL6 mice normally express the H-2K^b and H-2D^b MHC-I alleles ($K^bD^{b+/+}$) and are considered homozygote since both parental alleles are transcribed. Additionally, single MHC-I homozygote mice ($K^{b+/+}$, $D^{b+/+}$ and $D^{d+/+}$), as well as hemizygote mice that have only one copy of the MHC-I gene were used. While MHC-deficient mice lack MHC-I expression, TAP-deficient mice display reduced levels of MHC-I expression. Finally, multiple MHC-I-expressing mice were also used in functional assays. The specific genotypes of these

mice are reported in **Table 1 of paper IV**. These systems with well defined MHC-I expression provide a useful platform to study NK effector functions *in vivo* and *in vitro*, where the principles for NK cell sensitivity and triggering were assessed by *in vivo* cytotoxicity assays as well as by examining the modulatory role of peptide-regulated MHC-I levels. Additionally, Ly49 receptor expression levels in each mouse model were determined in order to understand the effects of MHC-I expression levels on receptor down-modulation on cells expressing multiple Ly49 receptors.

The results of this study indicate that, when interacting with normal cells, NK cells are presented with an abundance of MHC-I molecules, which are much more than what is needed for establishing self-tolerance and to induce maximal Ly49 down-modulation. When homozygous mice expressing normal levels of MHC-I were injected with hemizygous splenocytes expressing approximately 50% less MHC-I of the same allele/s, naïve NK cells did not reject the cells over a period of four days **(Paper IV, Figure 2A-C)**. Moreover, even though the transferred cells expressed 50% lower levels of MHC-I, they were not rejected by pre-activated NK cells which were still tolerant **(Paper IV, Figure 2D)**. This clearly indicates that NK cell tolerance is robust since *in vivo* missing-self rejection of normal healthy cells requires more than a 50% reduction in MHC-I expression levels to occur. Interestingly, this effect was still maintained despite pre-activation of NK cells *in vivo*.

In order to determine the threshold for MHC-I-dependent NK cell activation, splenocytes from TAP-deficient mice, expressing low levels of MHC-I were used. MHC-I levels can be artificially increased through addition of exogenous peptides *in vitro*. We incubated these cells with the H-2K^b-restricted Moloney murine leukemia virus (MuLV) peptide (SSWDFITV)[408], which stabilized 10-80% of H-2K^b surface expression. Surprisingly, when adoptively transferred into $K^{b+/+}$ mice, only cells with less than 20% H-2K^b expression were rejected while all other cells were tolerated **(Figure 6 and Paper IV, Figure 3)**.

The modulatory effect of cell surface MHC-I expression levels on NK cell effector functions helps to fine-tune the activation status of the NK cell repertoire to avoid autoimmunity while keeping their ability to respond to viral infection or malignant transformation. Interestingly, NK cells seem to lack mechanisms for sensing an MHC-I downregulation of more than 50% when engaged upon normal cells or resting cells, at least in the case of Ly49 down-modulation. Therefore, it is possible that NK cells are relatively unresponsive to fluctuations in MHC-I expression of normal target cells.

This phenomenon can be explained by a model in which a certain threshold for NK cell activation can effectively be calibrated in order to maintain tolerance to varying levels of MHC-I expression on target cells. It is possible that tumor cells or virally infected

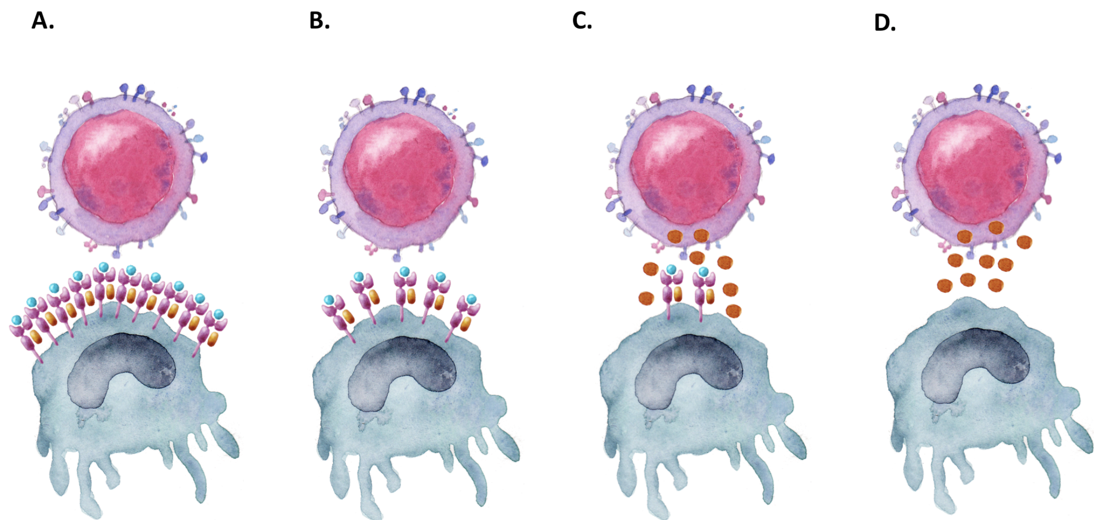

Figure 6. NK cell activation threshold requires at least 80% MHC-I downregulation A. Normal MHC-I expression inhibits NK cell activation B. 50% reduction of MHC-I does not activate NK cells **C.** NK cells get activated upon loss of more than 80% MHC-I **D.** Complete loss of MHC-I expression activates NK cells, "Missing-Self"

cells that display stress signals in the form of NK cell activating ligands would trigger NK cell activation more efficiently than normal splenocytes. Such mechanisms would act as a preventive system against autoimmune reactions and imply the necessity of other signals including the upregulation of activating ligands and/or the downregulation of other inhibitory ligands in order to ensure efficient NK cell responses. A more detailed[409] analysis of the role of MHC in NK cell education and tolerance is provided in the PhD thesis of Petter Brodin[410] and in other publications[44,411,412].

Study IV shows that the NK cell activation status can be modulated through the use of MHC-I-restricted peptides. Moreover, It has been demonstrated in other studies that MHC-I-bound peptides can modulate NK cell responses during interaction with KIRs[413-417]. Recently, Lena Fadda and Salim I. Khakoo demonstrated that MHC-I-restricted antagonist peptides that inhibited KIR signaling enhanced recognition of target cells by NK cells [418]. MHC-I-restricted peptide-mediated modulation of NK cells can have future implications in attempts to sensitize the immune system against previously inert targets. Moreover, quantitative use of MHC-I-restricted peptides can provide an additional tool to further investigate NK cell biology and regulate allele-specific NK cell responses. For example such an approach could be used to optimize *ex vivo* expansion and activation of NK cells with an increased capacity to target tumor cells. More specifically, this could be performed through the use of HLA-E-restricted peptides, in order to recruit activating receptor NKG2C^{+} NK cell populations, which are primed to target tumor cells that have increased levels of HLA-E expression. Since evolutionarily conserved MHC-I residues including W167 and Y159 also exists in HLA-E[409] and the peptide binding motif of HLA-E-restricted epitopes are similar to HLA-A2[419], combining the design of novel MHC-restricted APLs that are optimized with a similar approach used in study III with peptides that are more prone to attract the attention of NKG2C

than the inhibitory molecule NKG2A, could potentially maximize the efficiency for the generation of an optimal NK cell repertoire against a specific tumor target.

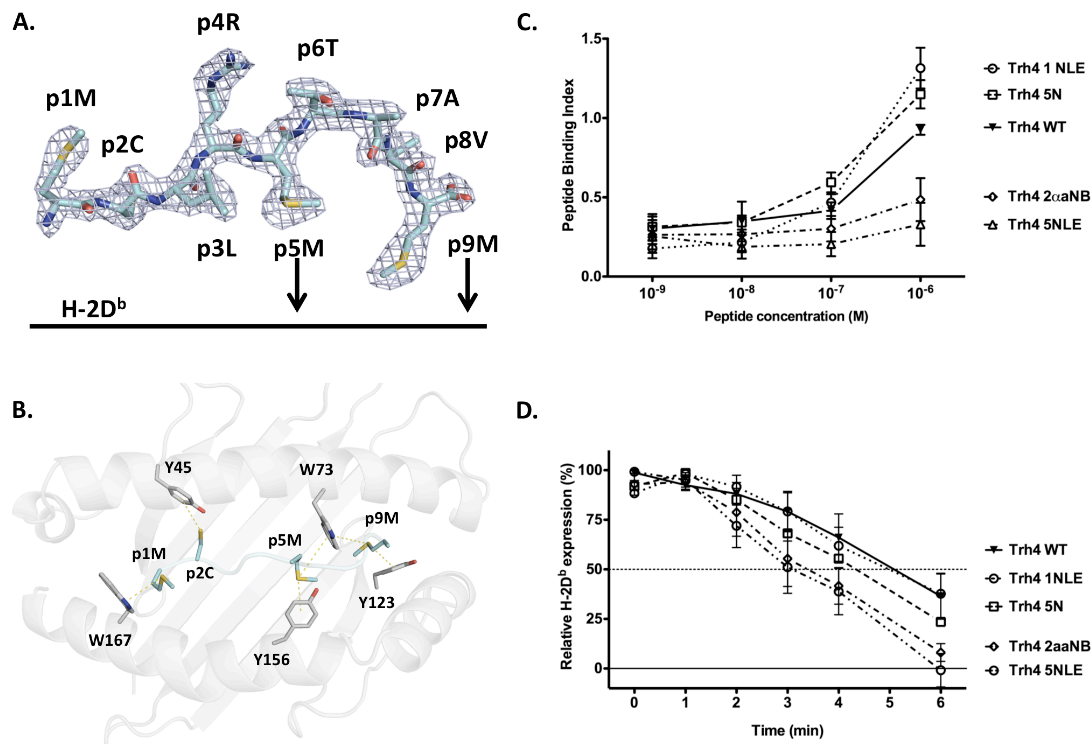

Figure 7. Sulphur-aromatic interactions mediate binding of TrH4 peptide to H-2D^b A. The electron density map shown around the Trh4 epitope (MCLRMTAVM) (generated with |2Fo|-|Fc| Fourier coefficients and contoured at 1 σ) as seen from the side of the α2 helix. **B.** Overview of sulphur-aromatic interactions. H-2D^b/Trh4 complex is seen from top. H-2D^b in light grey, peptide in cyan. **C.** Removal of sulphur at position 2 and 5 of Trh4 diminishes MHC/peptide affinity. NLE represents norleucine and 2αaNB represents α-Aminobutric acid **D.** Removal of sulphur at position 2 and 5 of Trh4 diminishes MHC/peptide stability.

3.1 ADDITIONAL RESULTS AND FUTURE IMPLICATIONS

The crystal structure of H-2D^b in complex with the neo-epitope Trh4 associated with impaired peptide processing reveals a non-canonical binding

A unique category of CTLs that can prevent the escape of tumors from recognition has been recently identified[151]. These CTLs target a specific alternative peptide repertoire that is not normally presented on the surface of healthy cells, and that is only presented on MHC-I at the surface of cells with impaired antigen processing such as impaired TAP, tapasin or proteasomal functions, often associated with tumor evasion. In this study, the crystal structure of the first example of such an immunogenic TEIPP neoantigen, was determined **(Figure 7A and B)**. In contrast to most H-2D^b-restricted peptides that make use of a binding motif composed of an asparagine and a hydrophobic residue at positions 5 and 9, respectively (p5Np9L), the TEIPP epitope Trh4 (MCLRMTAVM), binds to H-2D^b using an unusual binding motif, with a preponderance of sulphur-containing residues and SH-π interactions. Besides the cysteine residue at position 2 that protrudes towards the B-pocket of H-2D^b, two

methionine residues occupy the main anchor positions 5 and 9. Furthermore, the side chain of a third methionine residue, localized at position 1 of the peptide, interacts mainly with the side chains of the H-2D^b residue W167. Peptide binding and stabilization assays were performed to analyze the respective importance of each peptide position. We hypothesized that, similarly to the CH-π interactions that are formed between p3P and Y159 **(Paper III)**, the side chains of p1M, p2C, p5M and p9M could be important for the interaction of Trh4 with H-2D^b. Substitutions of p5M to norleucine or of p2C to α-amino-N-butyric acid clearly impaired the peptide binding capacity and cell surface MHC stability, suggesting the importance of SH-π interactions for the binding of Trh4 to H-2D^b **(Figure 7C and D)**. Thus, besides providing the first crystal structure of a TEIPP epitope, H-2D^b/Trh4, this study provides novel insights into the possible use of naturally occurring and/or modified sulphur-containing residues in order to increase binding affinity and possibly immunogenicity of targeted peptides.

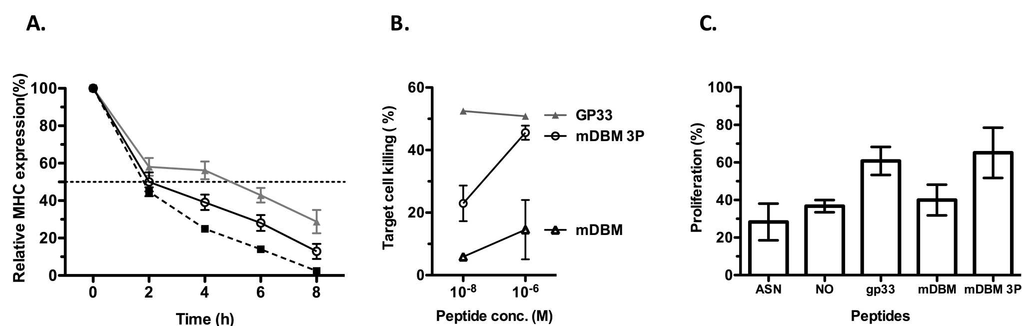

Figure 8. p3P modification increases pMHC stability and T cell cross-reactivity. A. p3P modification increases cell surface stability of MHC molecules. Black dashed line is mDBM. Black line is mDBM 3P and grey line is control peptide gp33. **B.** Target cells loaded with control peptide gp33 and mDBM 3P are efficiently killed by P14 T cells. **C.** P14 T cell proliferation increases upon stimulation with mDBM 3P. ASN (ASNENMETM) is influenza derived H-2D^b-restiricted epitope. Target cells without any peptide is NO.

Structural and biochemical studies of P14 cross-reactivity between gp33/H-2D^b and H-2D^b in complex with the mimotope of a self-peptide

It has been previously demonstrated that P14 slightly cross-reacts with H-2D^b in complex with the self-peptide mDBM (KALYDYAPI) derived from dopamine mono-oxygenase, as well as with its rat homolog rDBM (KALYNYAPI)[420]. Although both mDBM and rDBM share less than 50% sequence identity with gp33, the crystal structure of H-2D^b/rDBM revealed a striking conserved molecular mimicry between the surfaces of H-2D^b/gp33 and H-2D^b/rDBM[421]. The aim of the present study was to determine if we could increase the previously observed cross-reactivity of P14 to H-2D^b/mDBM by introducing a proline at peptide position 3 of the mDBM peptide. Our analysis reveals that, in contrast to mDBM, the proline substituted altered mDBM(3P) displayed significant capacity to bind and stabilize H-2D^b **(Figure 8A)**. Using P14 transgenic mice, TCR down-regulation, P14 T cell proliferation, TNF production and Cr^{51} release cytotoxicity assays demonstrated that recognition of mDBM(3P)-targets was significantly increased **(Figure 8B and C)**.

The obtained results should provide us with a better understanding of the rules steering enhanced recognition of MHC-I complexes by TCRs. As molecular mimicry is a (non-excluding) strong candidate for initiation of infection-mediated autoimmunity, this study could also provide some further insights into the levels of cross-reactivity required for potential initiation of disease. Currently, further immunological characterization of the p3P modification is ongoing in parallel with structural studies.

Enhancement of immunogenicity through structural modifications of the melanoma-associated HLA-A2-restricted peptide MC1R

Melanocortin receptor 1 (MC1R) is one of the receptor proteins for melanocyte-stimulating hormone (MSH) and is a genetic risk factor for melanoma and non-melanoma skin cancer. It has been previously demonstrated that an epitope derived from the MC1R protein, $MC1R_{291}$ (AIIDPLIYA), is recognized by human melanoma-specific HLA-A2 restricted CTLs[422]. $MC1R_{291}$ is classified as a tumor associated antigen and is a potential target for melanoma immunotherapy. In this study we aimed to increase MHC/peptide interactions and thus, T cell responses towards the $MC1R_{291}$ epitope through targeted peptide modifications. As the $MC1R_{291}$ epitope lacks the optimum HLA-A2 anchor residue at position 9 of the peptide, alanine was substituted with a preferred amino acid, valine (9V). Additionally, in order to improve MHC/peptide interactions through evolutionarily conserved MHC-I residue, W167, we have introduced a tyrosine at position 1 of $MC1R_{291}$ (1Y) as well as combined this approach with conventional anchor fixing modification (1Y9V). All designed $MCR1_{291}$ APLs had increased MHC binding affinity and pMHC cell-surface stability **(Figure 9A)**. Furthermore, $MC1R_{291}$ APLs were used to stimulate $MC1R_{291}$-specific CTLs from peripheral blood mononuclear cells (PBMC) of HLA-A2$^+$ healthy donors. T cells stimulated with APLs could expand more efficiently than those stimulated with the wild-type peptide; however, activation and expansion of $MC1R_{291}$ CTLs were surprisingly donor-specific as different donors had distinct preferences for one or more of the modified peptide(s). Importantly, CTLs stimulated with preferred modified peptide efficiently cross-reacted with the weakly immunogenic wild-type $MC1R_{291}$ epitope **(Figure 9B-D)**.

In conclusion, we have designed altered peptide ligands of a TAA, which could improve CTL responses towards cells presenting the tumor-associated wild-type peptide. Additionally, observed differential peptide preferences of donors could provide a potential explanation for the failure of previous vaccination trials with modified peptides. Since, MHC allelic diversity of donors/patients as well as history of infectious diseases could affect the T cell repertoire and thus T cell responses, I think it is essential to test a diverse array of APLs in order to optimize personalized treatment of cancer or viral infections.

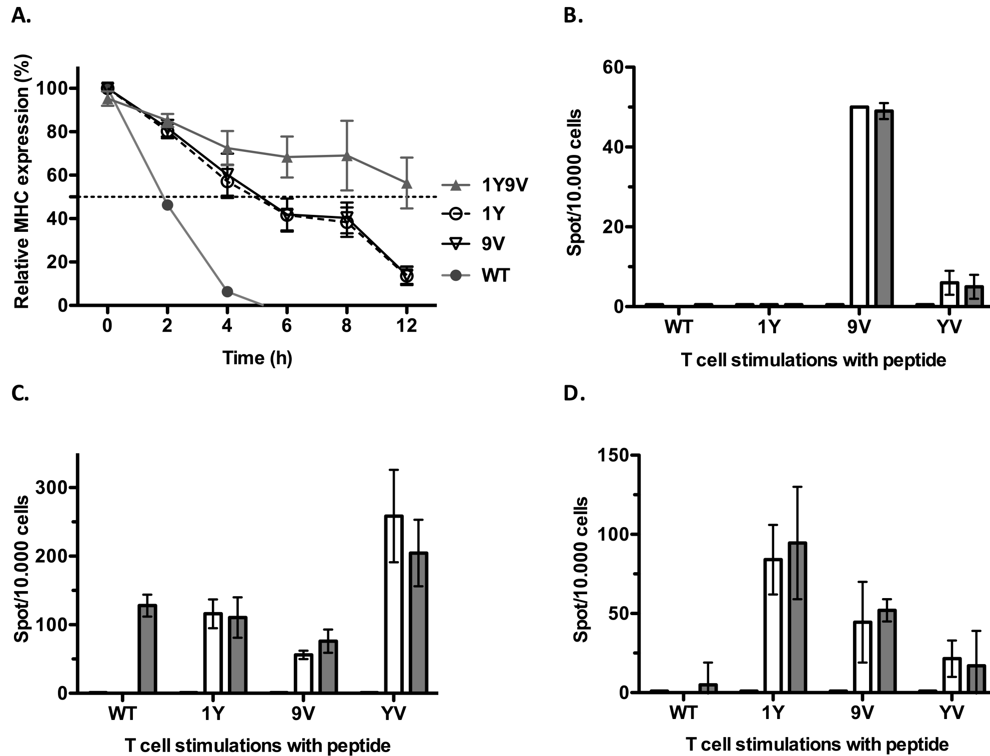

Figure 9. Anchor fixing and/or targeting evolutionarily conserved MHC residue W167, results in improved pMHC stability and donor-specific immunogenicity. A. Cell surface HLA-A2 levels are stabilized with modified peptides. WT: MC1R291 (AIIDPLIYA), 9V: A9V anchor fixing modification. 1Y: A1Y modification. 1Y9V: combination of 1Y and 9V modifications. **B, C, D.** T cell expansions from three different HLA-A2 positive donors with modified peptides resulted in donor-specific MC1R WT epitope cross-reactive IFNγ secreting T cell populations (IFNγ ELISpot). Against WT (grey), modified (white).

Design and analysis of structural modifications in HLA-A2-restricted multiple-myeloma-associated peptides; Implications for increased binding stability and immunogenicity

A set of HLA-A2-restricted multiple myeloma-associated tumor antigens (MM-TAAs) were selected, including the well-known cancer-associated peptides such as NY-ESO1 and MUC_{79-87}, $MUC_{167-175}$, $MUC_{264-272}$ but also a large array of other peptides such as FR3, FR4, FR11, FR16, FR20, $PRAME_{100}$, $PRAME_{142}$, $PRAME_{300}$, $PRAME_{425}$, $WT1_{126}$, $WT1_{187}$, $WT1_{235}$, HER2/NEU E75, ETV6-AML1 $PR1_{169}$, and $DKK_{120-129}$ [423-426]. The first aim of this study was to determine the crystal structures of HLA-A2 in complex with MM-TAAs in order to establish a first structural library that can be used as a template for future design of modifications in the targeted MM-TAAs. The second aim was to introduce modifications that could increase their capacity to stabilize HLA-A2 complexes while conserving the molecular mimicry when compared to wild-type MM-TAAs. The ultimate target is to increase immunogenicity of specific CTLs towards these cancer-associated targets. In collaboration with the research group of Prof. Ton Schumacher (Netherlands Cancer Institute, Amsterdam), we made use of *in crystallo* peptide substitution[427] to achieve these structural objectives. Using this method, the

crystal structure of HLA-A2/FR20 was determined. The unusual binding mode of FR20 provided important indications regarding the positions to be modified in order to potentially enhance its immunogenicity. The capacity of APLs of several MM-TAAs to bind and stabilize HLA-A2 complexes were analyzed, providing important individual information and displaying some important differences between how MM-TAAs should be approached. T cell response characterization of the effects of these introduced modifications will be performed by the Achour group in the future.

4 CONCLUDING REMARKS

This thesis focuses on the importance of MHC-I molecules in the regulation of T cell and NK cell responses, aiming to identify molecular mechanisms behind this phenomenon and possible interventions for optimizing these interactions.

In Paper I provides a structural platform explaining the functional effects of post-translational peptide modifications on initiation of T cell responses. Analysis of these effects presents useful insights into the complexity of factors governing MHC-dependent immune surveillance and could contribute to the improvement of the arsenal of tools used to fine-tune T cell responses *in vivo* or *ex vivo*.

In order to better understand the mechanisms of peptide discrimination by TCRs, in Paper II, we demonstrated that the same TCR has the ability to respond to both agonist and semi-agonist peptides through the use of different thermodynamic strategies. In the absence of main TCR hotspot residues, secondary/alternative hotspots may take over the responsibility. Additionally, tightly coordinated water molecules can interfere with TCR recognition and higher enthalpic prices may be an inevitable compromise for allowing the interaction to occur.

Moreover, in Paper III, we used altered peptide ligands modified at secondary anchoring residues as tools to increase the immunogenicity of peptides with already optimal MHC/peptide affinity. Importantly, a naturally occurring antagonist escape peptide variant of an LCMV-derived immunodominant MHC-I-restricted epitope was converted to an agonist through peptide modification, resulting in increased pMHC stability and TCR affinity, as well as conserved molecular mimicry which allowed T-cell cross-recognition.

Finally, in Paper IV, we analyzed the importance of MHC-I engagement in the activation of NK cells, using both differentially MHC expressing mouse models and peptide-mediated fine tuning of MHC presence on the cell surface. Our results indicate a certain threshold for NK cell activation, which is effectively calibrated to maintain tolerance to varying levels of MHC-I expression on target cells and draws attention to the necessity of other signals, such as upregulation of activating ligands or downregulation of inhibitory receptors for NK cell activation.

Conclusively, the studies included in this thesis provide important novel insights that can be used in alternative approaches for vaccine design. Modulation of NK cell responses through peptide-mediated tuning of MHC on target cells, as well as activation of T cells with APLs that conserve molecular mimicry to the wild-type peptides and result in cross-reaction, stand out as important findings that both elucidate important aspects of MHC-I biology and provide new strategies for the optimization of the design of novel anti-viral or anti-tumor peptide-based vaccines.

5 ACKNOWLEDGEMENTS

August 27, 2012

Kungshamra 4:54 am

Dear reader,

I am extremely happy that my PhD studies are coming to an end. It was a long, difficult and interesting journey for me that I really do not remember the first day of it. This period of agony taught me so many things and I am sure it prepared me very well for the upcoming challenges of life. This end or in other words the next chapter of my life would not come if I did not feel the support, friendship, and love of so many people that I would like thank:

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6 REFERENCES

1. Beschin A, Bilej M, Torreele E, De Baetselier P. On the existence of cytokines in invertebrates. *Cellular and molecular life sciences : CMLS*. 2001;58(5-6):801-814.
2. Blumbach B, Pancer Z, Diehl-Seifert B, et al. The putative sponge aggregation receptor. Isolation and characterization of a molecule composed of scavenger receptor cysteine-rich domains and short consensus repeats. *Journal of cell science*. 1998;111 (Pt 17):2635-2644.
3. Waddell DR, Duffy KT. Breakdown of self/nonself recognition in cannibalistic strains of the predatory slime mold, Dictyostelium caveatum. *The Journal of cell biology*. 1986;102(1):298-305.
4. Ottaviani E, Franceschi C. The invertebrate phagocytic immunocyte: clues to a common evolution of immune and neuroendocrine systems. *Immunology today*. 1997;18(4):169-174.
5. Rast JP, Anderson MK, Ota T, et al. Immunoglobulin light chain class multiplicity and alternative organizational forms in early vertebrate phylogeny. *Immunogenetics*. 1994;40(2):83-99.
6. Rast JP, Litman GW. T-cell receptor gene homologs are present in the most primitive jawed vertebrates. *Proceedings of the National Academy of Sciences of the United States of America*. 1994;91(20):9248-9252.
7. Dzik JM, Zielinski Z, Ciesla J, Walajtys-Rode E. Trichinella spiralis infection enhances protein kinase C phosphorylation in guinea pig alveolar macrophages. *Parasite immunology*. 2010;32(3):209-220.
8. Middleton D, Menchaca L, Rood H, Komerofsky R. New allele frequency database: http://www.allelefrequencies.net. *Tissue antigens*. 2003;61(5):403-407.
9. Gonzalez-Galarza FF, Christmas S, Middleton D, Jones AR. Allele frequency net: a database and online repository for immune gene frequencies in worldwide populations. *Nucleic acids research*. 2011;39(Database issue):D913-919.
10. Wucherpfennig KW, Allen PM, Celada F, et al. Polyspecificity of T cell and B cell receptor recognition. *Seminars in immunology*. 2007;19(4):216-224.
11. Nolz JC, Harty JT. Strategies and implications for prime-boost vaccination to generate memory CD8 T cells. *Advances in experimental medicine and biology*. 2011;780:69-83.
12. Alarcon B, Mestre D, Martinez-Martin N. The immunological synapse: a cause or consequence of T-cell receptor triggering? *Immunology*. 2011;133(4):420-425.
13. Dustin ML, Depoil D. New insights into the T cell synapse from single molecule techniques. *Nature reviews Immunology*. 2011;11(10):672-684.
14. Thummler K, Leipe J, Ramming A, Schulze-Koops H, Skapenko A. Immune regulation by peripheral suppressor T cells induced upon homotypic T cell/T cell interactions. *Journal of leukocyte biology*. 2010;88(5):1041-1050.
15. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. *Nature immunology*. 2010;11(1):90-96.
16. Fahmy TM, Bieler JG, Edidin M, Schneck JP. Increased TCR avidity after T cell activation: a mechanism for sensing low-density antigen. *Immunity*. 2001;14(2):135-143.
17. Schamel WW, Arechaga I, Risueno RM, et al. Coexistence of multivalent and monovalent TCRs explains high sensitivity and wide range of response. *The Journal of experimental medicine*. 2005;202(4):493-503.
18. Anikeeva N, Lebedeva T, Clapp AR, et al. Quantum dot/peptide-MHC biosensors reveal strong CD8-dependent cooperation between self and viral antigens that augment the T cell response. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(45):16846-16851.
19. McKeithan TW. Kinetic proofreading in T-cell receptor signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(11):5042-5046.
20. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature*. 1995;375(6527):148-151.
21. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol*. 1975;5(2):117-121.
22. Kiessling R, Klein E, Wigzell H. "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Eur J Immunol*. 1975;5(2):112-117.
23. Ruggeri L, Capanni M, Mancusi A, Martelli MF, Velardi A. The impact of donor natural killer cell alloreactivity on allogeneic hematopoietic transplantation. *Transpl Immunol*. 2005;14(3-4):203-206.
24. Costello RT, Fauriat C, Sivori S, Marcenaro E, Olive D. NK cells: innate immunity against hematological malignancies? *Trends Immunol*. 2004;25(6):328-333.
25. Sutlu T, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. *J Intern Med*. 2009;266(2):154-181.
26. Stern P, Gidlund M, Orn A, Wigzell H. Natural killer cells mediate lysis of embryonal carcinoma cells lacking MHC. *Nature*. 1980;285(5763):341-342.
27. Lanier LL. Natural killer cell receptor signaling. *Curr Opin Immunol*. 2003;15(3):308-314.

28. Chiesa S, Tomasello E, Vivier E, Vely F. Coordination of activating and inhibitory signals in natural killer cells. *Mol Immunol*. 2005;42(4):477-484.

29. Long EO. Negative signaling by inhibitory receptors: the NK cell paradigm. *Immunological reviews*. 2008;224:70-84.

30. Lanier LL. Up on the tightrope: natural killer cell activation and inhibition. *Nature immunology*. 2008;9(5):495-502.

31. Yokoyama WM. What goes up must come down: the emerging spectrum of inhibitory receptors. *The Journal of experimental medicine*. 1997;186(11):1803-1808.

32. Peterson ME, Long EO. Inhibitory receptor signaling via tyrosine phosphorylation of the adaptor Crk. *Immunity*. 2008;29(4):578-588.

33. Moretta A, Biassoni R, Bottino C, Mingari MC, Moretta L. Natural cytotoxicity receptors that trigger human NK-cell-mediated cytolysis. *Immunology today*. 2000;21(5):228-234.

34. Pessino A, Sivori S, Bottino C, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *The Journal of experimental medicine*. 1998;188(5):953-960.

35. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *The Journal of experimental medicine*. 1998;187(12):2065-2072.

36. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *The Journal of experimental medicine*. 1999;190(10):1505-1516.

37. Houchins JP, Yabe T, McSherry C, Bach FH. DNA sequence analysis of NKG2, a family of related cDNA clones encoding type II integral membrane proteins on human natural killer cells. *The Journal of experimental medicine*. 1991;173(4):1017-1020.

38. Levitsky HI, Lazenby A, Hayashi RJ, Pardoll DM. In vivo priming of two distinct antitumor effector populations: the role of MHC class I expression. *J Exp Med*. 1994;179(4):1215-1224.

39. Karre K, Ljunggren HG, Piontek G, Kiessling R. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature*. 1986;319(6055):675-678.

40. Bosse D, Ades E. Studies of adenovirus subtypes and down-regulation of HLA class I expression: correlations to natural-killer-mediated cytolysis. *Pathobiology*. 1991;59(5):313-315.

41. Karre K. NK cells, MHC class I molecules and the missing self. *Scand J Immunol*. 2002;55(3):221-228.

42. McVicar DW, Burshtyn DN. Intracellular signaling by the killer immunoglobulin-like receptors and Ly49. *Sci STKE*. 2001;2001(75):RE1.

43. Edwards LJ, Evavold BD. T cell recognition of weak ligands: roles of signaling, receptor number, and affinity. *Immunologic research*. 2011;50(1):39-48.

44. Hoglund P, Brodin P. Current perspectives of natural killer cell education by MHC class I molecules. *Nature reviews Immunology*. 2010;10(10):724-734.

45. Andersson S, Fauriat C, Malmberg JA, Ljunggren HG, Malmberg KJ. KIR acquisition probabilities are independent of self-HLA class I ligands and increase with cellular KIR expression. *Blood*. 2009;114(1):95-104.

46. Johansson S, Salmon-Divon M, Johansson MH, et al. Probing natural killer cell education by Ly49 receptor expression analysis and computational modelling in single MHC class I mice. *PloS one*. 2009;4(6):e6046.

47. Schonberg K, Sribar M, Enczmann J, Fischer JC, Uhrberg M. Analyses of HLA-C-specific KIR repertoires in donors with group A and B haplotypes suggest a ligand-instructed model of NK cell receptor acquisition. *Blood*. 2011;117(1):98-107.

48. Moretta A, Bottino C, Vitale M, et al. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol*. 2001;19:197-223.

49. Weissman AM, Hou D, Orloff DG, et al. Molecular cloning and chromosomal localization of the human T-cell receptor zeta chain: distinction from the molecular CD3 complex. *Proc Natl Acad Sci U S A*. 1988;85(24):9709-9713.

50. Lanier LL, Corliss BC, Wu J, Leong C, Phillips JH. Immunoreceptor DAP12 bearing a tyrosine-based activation motif is involved in activating NK cells. *Nature*. 1998;391(6668):703-707.

51. Reth M. Antigen receptor tail clue. *Nature*. 1989;338(6214):383-384.

52. Teng MW, Kershaw MH, Hayakawa Y, et al. T cells gene-engineered with DAP12 mediate effector function in an NKG2D-dependent and major histocompatibility complex-independent manner. *J Biol Chem*. 2005;280(46):38235-38241.

53. Raulet DH, Vance RE. Self-tolerance of natural killer cells. *Nature reviews Immunology*. 2006;6(7):520-531.

54. Bauer S, Groh V, Wu J, et al. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science*. 1999;285(5428):727-729.

55. Watzl C. The NKG2D receptor and its ligands-recognition beyond the "missing self"? *Microbes and infection / Institut Pasteur*. 2003;5(1):31-37.

56. Herberman RB, Nunn ME, Holden HT, Lavrin DH. Natural cytotoxic reactivity of mouse lymphoid cells against syngeneic and allogeneic tumors. II. Characterization of effector cells. *International journal of cancer Journal international du cancer*. 1975;16(2):230-239.

57. Kiessling R, Klein E, Pross H, Wigzell H. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *European journal of immunology*. 1975;5(2):117-121.

58. Su HC, Ishikawa R, Biron CA. Transforming growth factor-beta expression and natural killer cell responses during virus infection of normal, nude, and SCID mice. *Journal of immunology*. 1993;151(9):4874-4890.

59. Vosshenrich CA, Garcia-Ojeda ME, Samson-Villeger SI, et al. A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nature immunology*. 2006;7(11):1217-1224.

60. Andrews DM, Smyth MJ. A potential role for RAG-1 in NK cell development revealed by analysis of NK cells during ontogeny. *Immunology and cell biology*. 2010;88(2):107-116.

61. Freud AG, Becknell B, Roychowdhury S, et al. A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells. *Immunity*. 2005;22(3):295-304.

62. Kim S, Iizuka K, Kang HS, et al. In vivo developmental stages in murine natural killer cell maturation. *Nature immunology*. 2002;3(6):523-528.

63. Borghesi L, Hsu LY, Miller JP, et al. B lineage-specific regulation of V(D)J recombinase activity is established in common lymphoid progenitors. *The Journal of experimental medicine*. 2004;199(4):491-502.

64. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. *Immunity*. 2002;17(2):117-130.

65. Kouro T, Kumar V, Kincade PW. Relationships between early B- and NK-lineage lymphocyte precursors in bone marrow. *Blood*. 2002;100(10):3672-3680.

66. Pilbeam K, Basse P, Brossay L, et al. The ontogeny and fate of NK cells marked by permanent DNA rearrangements. *Journal of immunology*. 2008;180(3):1432-1441.

67. Yokota T, Kouro T, Hirose J, et al. Unique properties of fetal lymphoid progenitors identified according to RAG1 gene expression. *Immunity*. 2003;19(3):365-375.

68. Shi FD, Ljunggren HG, La Cava A, Van Kaer L. Organ-specific features of natural killer cells. *Nature reviews Immunology*. 2011;11(10):658-671.

69. Chiossone L, Chaix J, Fuseri N, Roth C, Vivier E, Walzer T. Maturation of mouse NK cells is a 4-stage developmental program. *Blood*. 2009;113(22):5488-5496.

70. Huntington ND, Tabarias H, Fairfax K, et al. NK cell maturation and peripheral homeostasis is associated with KLRG1 up-regulation. *Journal of immunology*. 2007;178(8):4764-4770.

71. Sun JC, Lanier LL. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nature reviews Immunology*. 2011;11(10):645-657.

72. Nandi D, Gross JA, Allison JP. CD28-mediated costimulation is necessary for optimal proliferation of murine NK cells. *Journal of immunology*. 1994;152(7):3361-3369.

73. Andoniou CE, van Dommelen SL, Voigt V, et al. Interaction between conventional dendritic cells and natural killer cells is integral to the activation of effective antiviral immunity. *Nature immunology*. 2005;6(10):1011-1019.

74. Andrews DM, Scalzo AA, Yokoyama WM, Smyth MJ, Degli-Esposti MA. Functional interactions between dendritic cells and NK cells during viral infection. *Nature immunology*. 2003;4(2):175-181.

75. Krug A, French AR, Barchet W, et al. TLR9-dependent recognition of MCMV by IPC and DC generates coordinated cytokine responses that activate antiviral NK cell function. *Immunity*. 2004;21(1):107-119.

76. Williams MA, Bevan MJ. Effector and memory CTL differentiation. *Annual review of immunology*. 2007;25:171-192.

77. Berg RE, Crossley E, Murray S, Forman J. Memory CD8+ T cells provide innate immune protection against Listeria monocytogenes in the absence of cognate antigen. *The Journal of experimental medicine*. 2003;198(10):1583-1593.

78. Tough DF, Borrow P, Sprent J. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science*. 1996;272(5270):1947-1950.

79. Garcia KC, Adams JJ, Feng D, Ely LK. The molecular basis of TCR germline bias for MHC is surprisingly simple. *Nature immunology*. 2009;10(2):143-147.

80. Marrack P, Scott-Browne JP, Dai S, Gapin L, Kappler JW. Evolutionarily conserved amino acids that control TCR-MHC interaction. *Annual review of immunology*. 2008;26:171-203.

81. Ganju RK, Smiley ST, Bajorath J, Novotny J, Reinherz EL. Similarity between fluorescein-specific T-cell receptor and antibody in chemical details of antigen recognition. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;89(23):11552-11556.

82. Van Laethem F, Sarafova SD, Park JH, et al. Deletion of CD4 and CD8 coreceptors permits generation of alphabetaT cells that recognize antigens independently of the MHC. *Immunity*. 2007;27(5):735-750.

83. Illing PT, Vivian JP, Dudek NL, et al. Immune self-reactivity triggered by drug-modified HLA-peptide repertoire. *Nature*. 2012;486(7404):554-558.

84. Huseby ES, White J, Crawford F, et al. How the T cell repertoire becomes peptide and MHC specific. *Cell*. 2005;122(2):247-260.

85. Ljunggren HG, Karre K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunology today*. 1990;11(7):237-244.

86. Anfossi N, Andre P, Guia S, et al. Human NK cell education by inhibitory receptors for MHC class I. *Immunity*. 2006;25(2):331-342.

87. Chalifour A, Scarpellino L, Back J, et al. A Role for cis Interaction between the Inhibitory Ly49A receptor and MHC class I for natural killer cell education. *Immunity*. 2009;30(3):337-347.

88. Fernandez NC, Treiner E, Vance RE, Jamieson AM, Lemieux S, Raulet DH. A subset of natural killer cells achieves self-tolerance without expressing inhibitory receptors specific for self-MHC molecules. *Blood*. 2005;105(11):4416-4423.

89. Johansson S, Johansson M, Rosmaraki E, et al. Natural killer cell education in mice with single or multiple major histocompatibility complex class I molecules. *The Journal of experimental medicine*. 2005;201(7):1145-1155.

90. Kim S, Poursine-Laurent J, Truscott SM, et al. Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature*. 2005;436(7051):709-713.

91. Yokoyama WM, Kim S. How do natural killer cells find self to achieve tolerance? *Immunity*. 2006;24(3):249-257.

92. Elliott JM, Wahle JA, Yokoyama WM. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *The Journal of experimental medicine*. 2010;207(10):2073-2079.

93. Joncker NT, Shifrin N, Delebecque F, Raulet DH. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *The Journal of experimental medicine*. 2010;207(10):2065-2072.

94. Sun JC, Lanier LL. Cutting edge: viral infection breaks NK cell tolerance to "missing self". *Journal of immunology*. 2008;181(11):7453-7457.

95. Yokoyama WM, Kim S. Licensing of natural killer cells by self-major histocompatibility complex class I. *Immunological reviews*. 2006;214:143-154.

96. Hsu KC, Keever-Taylor CA, Wilton A, et al. Improved outcome in HLA-identical sibling hematopoietic stem-cell transplantation for acute myelogenous leukemia predicted by KIR and HLA genotypes. *Blood*. 2005;105(12):4878-4884.

97. Miller JS, Cooley S, Parham P, et al. Missing KIR ligands are associated with less relapse and increased graft-versus-host disease (GVHD) following unrelated donor allogeneic HCT. *Blood*. 2007;109(11):5058-5061.

98. Orr MT, Murphy WJ, Lanier LL. 'Unlicensed' natural killer cells dominate the response to cytomegalovirus infection. *Nature immunology*. 2010;11(4):321-327.

99. Ogasawara K, Benjamin J, Takaki R, Phillips JH, Lanier LL. Function of NKG2D in natural killer cell-mediated rejection of mouse bone marrow grafts. *Nature immunology*. 2005;6(9):938-945.

100. Oppenheim DE, Roberts SJ, Clarke SL, et al. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nature immunology*. 2005;6(9):928-937.

101. Sun JC, Lanier LL. Tolerance of NK cells encountering their viral ligand during development. *The Journal of experimental medicine*. 2008;205(8):1819-1828.

102. Tripathy SK, Keyel PA, Yang L, et al. Continuous engagement of a self-specific activation receptor induces NK cell tolerance. *The Journal of experimental medicine*. 2008;205(8):1829-1841.

103. Maenaka K, Jones EY. MHC superfamily structure and the immune system. *Current opinion in structural biology*. 1999;9(6):745-753.

104. Rudolph MG, Stanfield RL, Wilson IA. How TCRs bind MHCs, peptides, and coreceptors. *Annual review of immunology*. 2006;24:419-466.

105. Leddon SA, Sant AJ. Generation of MHC class II-peptide ligands for CD4 T-cell allorecognition of MHC class II molecules. *Current opinion in organ transplantation*. 2010;15(4):505-511.

106. Li XC, Raghavan M. Structure and function of major histocompatibility complex class I antigens. *Current opinion in organ transplantation*. 2010;15(4):499-504.

107. Achour A, Michaelsson J, Harris RA, et al. A structural basis for LCMV immune evasion: subversion of H-2D(b) and H-2K(b) presentation of gp33 revealed by comparative crystal structure.Analyses. *Immunity*. 2002;17(6):757-768.

108. Stern LJ, Wiley DC. Antigenic peptide binding by class I and class II histocompatibility proteins. *Structure*. 1994;2(4):245-251.

109. Tynan FE, Burrows SR, Buckle AM, et al. T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. *Nature immunology*. 2005;6(11):1114-1122.

110. Tynan FE, Reid HH, Kjer-Nielsen L, et al. A T cell receptor flattens a bulged antigenic peptide presented by a major histocompatibility complex class I molecule. *Nature immunology*. 2007;8(3):268-276.

111. Speir JA, Stevens J, Joly E, Butcher GW, Wilson IA. Two different, highly exposed, bulged structures for an unusually long peptide bound to rat MHC class I RT1-Aa. *Immunity*. 2001;14(1):81-92.

112. Ebert LM, Liu YC, Clements CS, et al. A long, naturally presented immunodominant epitope from NY-ESO-1 tumor antigen: implications for cancer vaccine design. *Cancer research*. 2009;69(3):1046-1054.

113. Miles JJ, Elhassen D, Borg NA, et al. CTL recognition of a bulged viral peptide involves biased TCR selection. *Journal of immunology*. 2005;175(6):3826-3834.

114. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature*. 1987;329(6139):506-512.

115. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature*. 1987;329(6139):512-518.

116. Vita R, Zarebski L, Greenbaum JA, et al. The immune epitope database 2.0. *Nucleic acids research*. 2010;38(Database issue):D854-862.

117. Butler NS, Theodossis A, Webb AI, et al. Prevention of cytotoxic T cell escape using a heteroclitic subdominant viral T cell determinant. *PLoS pathogens*. 2008;4(10):e1000186.

118. van Stipdonk MJ, Badia-Martinez D, Sluijter M, Offringa R, van Hall T, Achour A. Design of agonistic altered peptides for the robust induction of CTL directed towards H-2Db in complex with the melanoma-associated epitope gp100. *Cancer research*. 2009;69(19):7784-7792.

119. Nicholls S, Piper KP, Mohammed F, et al. Secondary anchor polymorphism in the HA-1 minor histocompatibility antigen critically affects MHC stability and TCR recognition. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(10):3889-3894.

120. Joseph MA, Mitchell ML, Evanseck JD, et al. Secondary anchor substitutions in an HLA-A*0201-restricted T-cell epitope derived from Her-2/neu. *Molecular immunology*. 2007;44(4):322-331.

121. Pinilla-Ibarz J, May RJ, Korontsvit T, et al. Improved human T-cell responses against synthetic HLA-0201 analog peptides derived from the WT1 oncoprotein. *Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, UK*. 2006;20(11):2025-2033.

122. Groll M, Ditzel L, Lowe J, et al. Structure of 20S proteasome from yeast at 2.4 A resolution. *Nature*. 1997;386(6624):463-471.

123. Wright CA, Kozik P, Zacharias M, Springer S. Tapasin and other chaperones: models of the MHC class I loading complex. *Biological chemistry*. 2004;385(9):763-778.

124. Neefjes J, Jongsma ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews Immunology*. 2011;11(12):823-836.

125. Praveen PV, Yaneva R, Kalbacher H, Springer S. Tapasin edits peptides on MHC class I molecules by accelerating peptide exchange. *European journal of immunology*. 2010;40(1):214-224.

126. Agarwal AK, Xing C, DeMartino GN, et al. PSMB8 encoding the beta5i proteasome subunit is mutated in joint contractures, muscle atrophy, microcytic anemia, and panniculitis-induced lipodystrophy syndrome. *American journal of human genetics*. 2010;87(6):866-872.

127. Guillaume B, Chapiro J, Stroobant V, et al. Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(43):18599-18604.

128. Strehl B, Seifert U, Kruger E, Heink S, Kuckelkorn U, Kloetzel PM. Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. *Immunological reviews*. 2005;207:19-30.

129. Sijts EJ, Kloetzel PM. The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cellular and molecular life sciences : CMLS*. 2011;68(9):1491-1502.

130. Yewdell JW. Immunoproteasomes: regulating the regulator. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(26):9089-9090.

131. Basler M, Youhnovski N, Van Den Broek M, Przybylski M, Groettrup M. Immunoproteasomes down-regulate presentation of a subdominant T cell epitope from lymphocytic choriomeningitis virus. *Journal of immunology*. 2004;173(6):3925-3934.

132. Schultz ES, Chapiro J, Lurquin C, et al. The production of a new MAGE-3 peptide presented to cytolytic T lymphocytes by HLA-B40 requires the immunoproteasome. *The Journal of experimental medicine*. 2002;195(4):391-399.

133. Morel S, Levy F, Burlet-Schiltz O, et al. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. *Immunity*. 2000;12(1):107-117.

134. Murata S, Sasaki K, Kishimoto T, et al. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science*. 2007;316(5829):1349-1353.

135. Nitta T, Murata S, Sasaki K, et al. Thymoproteasome shapes immunocompetent repertoire of CD8+ T cells. *Immunity*. 2010;32(1):29-40.

136. Takahama Y, Takada K, Murata S, Tanaka K. beta5t-containing thymoproteasome: specific expression in thymic cortical epithelial cells and role in positive selection of CD8+ T cells. *Current opinion in immunology*. 2012;24(1):92-98.

137. Yewdell JW. DRiPs solidify: progress in understanding endogenous MHC class I antigen processing. *Trends in immunology*. 2011;32(11):548-558.

138. Starck SR, Shastri N. Non-conventional sources of peptides presented by MHC class I. *Cellular and molecular life sciences : CMLS*. 2011;68(9):1471-1479.

139. Donaldson JG, Williams DB. Intracellular assembly and trafficking of MHC class I molecules. *Traffic*. 2009;10(12):1745-1752.

140. Hansen TH, Bouvier M. MHC class I antigen presentation: learning from viral evasion strategies. *Nature reviews Immunology*. 2009;9(7):503-513.

141. Loch S, Tampe R. Viral evasion of the MHC class I antigen-processing machinery. *Pflugers Archiv : European journal of physiology*. 2005;451(3):409-417.

142. Griffin BD, Verweij MC, Wiertz EJ. Herpesviruses and immunity: the art of evasion. *Veterinary microbiology*. 2010;143(1):89-100.

143. Shen L, Sigal LJ, Boes M, Rock KL. Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo. *Immunity*. 2004;21(2):155-165.

144. Di Pucchio T, Chatterjee B, Smed-Sorensen A, et al. Direct proteasome-independent cross-presentation of viral antigen by plasmacytoid dendritic cells on major histocompatibility complex class I. *Nature immunology*. 2008;9(5):551-557.

145. Hunt DF, Henderson RA, Shabanowitz J, et al. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science*. 1992;255(5049):1261-1263.

146. Weinzierl AO, Rudolf D, Hillen N, et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur J Immunol*. 2008;38(6):1503-1510.

147. El Hage F, Stroobant V, Vergnon I, et al. Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 2008;105(29):10119-10124.

148. Wolfel C, Drexler I, Van Pel A, et al. Transporter (TAP)- and proteasome-independent presentation of a melanoma-associated tyrosinase epitope. *International journal of cancer Journal international du cancer*. 2000;88(3):432-438.

149. Chambers B, Grufman P, Fredriksson V, et al. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer research*. 2007;67(18):8450-8455.

150. Oliveira CC, Querido B, Sluijter M, Derbinski J, van der Burg SH, van Hall T. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. *Eur J Immunol*. 2011;41(11):3114-3124.

151. van Hall T, Wolpert EZ, van Veelen P, et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat Med*. 2006;12(4):417-424.

152. Petersen J, Purcell AW, Rossjohn J. Post-translationally modified T cell epitopes: immune recognition and immunotherapy. *Journal of molecular medicine*. 2009;87(11):1045-1051.

153. Krueger KE, Srivastava S. Posttranslational protein modifications: current implications for cancer detection, prevention, and therapeutics. *Molecular & cellular proteomics : MCP*. 2006;5(10):1799-1810.

154. Atassi MZ, Casali P. Molecular mechanisms of autoimmunity. *Autoimmunity*. 2008;41(2):123-132.

155. Hetzer C, Dormeyer W, Schnolzer M, Ott M. Decoding Tat: the biology of HIV Tat posttranslational modifications. *Microbes and infection / Institut Pasteur*. 2005;7(13):1364-1369.

156. Anderton SM. Post-translational modifications of self antigens: implications for autoimmunity. *Current opinion in immunology*. 2004;16(6):753-758.

157. Doyle HA, Mamula MJ. Post-translational protein modifications in antigen recognition and autoimmunity. *Trends Immunol*. 2001;22(8):443-449.

158. Utz PJ, Anderson P. Posttranslational protein modifications, apoptosis, and the bypass of tolerance to autoantigens. *Arthritis Rheum*. 1998;41(7):1152-1160.

159. Skipper JC, Hendrickson RC, Gulden PH, et al. An HLA-A2-restricted tyrosinase antigen on melanoma cells results from posttranslational modification and suggests a novel pathway for processing of membrane proteins. *The Journal of experimental medicine*. 1996;183(2):527-534.

160. Meadows L, Wang W, den Haan JM, et al. The HLA-A*0201-restricted H-Y antigen contains a posttranslationally modified cysteine that significantly affects T cell recognition. *Immunity*. 1997;6(3):273-281.

161. Pierce RA, Field ED, den Haan JM, et al. Cutting edge: the HLA-A*0101-restricted HY minor histocompatibility antigen originates from DFFRY and contains a cysteinylated cysteine residue as identified by a novel mass spectrometric technique. *J Immunol*. 1999;163(12):6360-6364.

162. Backlund J, Carlsen S, Hoger T, et al. Predominant selection of T cells specific for the glycosylated collagen type II epitope (263-270) in humanized transgenic mice and in rheumatoid arthritis. *Proceedings of the National Academy of Sciences of the United States of America*. 2002;99(15):9960-9965.

163. Haurum JS, Hoier IB, Arsequell G, et al. Presentation of cytosolic glycosylated peptides by human class I major histocompatibility complex molecules in vivo. *The Journal of experimental medicine*. 1999;190(1):145-150.

164. Andersen MH, Bonfill JE, Neisig A, et al. Phosphorylated peptides can be transported by TAP molecules, presented by class I MHC molecules, and recognized by phosphopeptide-specific CTL. *Journal of immunology*. 1999;163(7):3812-3818.

165. Zarling AL, Ficarro SB, White FM, Shabanowitz J, Hunt DF, Engelhard VH. Phosphorylated peptides are naturally processed and presented by major histocompatibility complex class I molecules in vivo. *The Journal of experimental medicine*. 2000;192(12):1755-1762.

166. Hardy LL, Wick DA, Webb JR. Conversion of tyrosine to the inflammation-associated analog 3'-nitrotyrosine at either TCR- or MHC-contact positions can profoundly affect recognition of the MHC class I-restricted epitope of lymphocytic choriomeningitis virus glycoprotein 33 by CD8 T cells. *Journal of immunology*. 2008;180(9):5956-5962.

167. Dunne JL, Overbergh L, Purcell AW, Mathieu C. Posttranslational modifications of proteins in type 1 diabetes: the next step in finding the cure? *Diabetes*. 2012;61(8):1907-1914.

168. Kastrup IB, Andersen MH, Elliott T, Haurum JS. MHC-restricted T cell responses against posttranslationally modified peptide antigens. *Advances in immunology*. 2001;78:267-289.

169. Rammensee HG, Friede T, Stevanoviic S. MHC ligands and peptide motifs: first listing. *Immunogenetics*. 1995;41(4):178-228.

170. Rammensee H, Bachmann J, Emmerich NP, Bachor OA, Stevanovic S. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics*. 1999;50(3-4):213-219.

171. Reche PA, Reinherz EL. Prediction of peptide-MHC binding using profiles. *Methods in molecular biology*. 2007;409:185-200.

172. Hakenberg J, Nussbaum AK, Schild H, et al. MAPPP: MHC class I antigenic peptide processing prediction. *Applied bioinformatics*. 2003;2(3):155-158.

173. Sette A, Fikes J. Epitope-based vaccines: an update on epitope identification, vaccine design and delivery. *Current opinion in immunology*. 2003;15(4):461-470.

174. Sant AJ, Chaves FA, Krafcik FR, et al. Immunodominance in CD4 T-cell responses: implications for immune responses to influenza virus and for vaccine design. *Expert review of vaccines*. 2007;6(3):357-368.

175. Chen W, McCluskey J. Immunodominance and immunodomination: critical factors in developing effective CD8+ T-cell-based cancer vaccines. *Advances in cancer research*. 2006;95:203-247.

176. Velloso LM, Michaelsson J, Ljunggren HG, Schneider G, Achour A. Determination of structural principles underlying three different modes of lymphocytic choriomeningitis virus escape from CTL recognition. *Journal of immunology*. 2004;172(9):5504-5511.

177. Tissot AC, Ciatto C, Mittl PR, Grutter MG, Pluckthun A. Viral escape at the molecular level explained by quantitative T-cell receptor/peptide/MHC interactions and the crystal structure of a peptide/MHC complex. *Journal of molecular biology*. 2000;302(4):873-885.

178. Goulder PJ, Watkins DI. HIV and SIV CTL escape: implications for vaccine design. *Nature reviews Immunology*. 2004;4(8):630-640.

179. Bowen DG, Walker CM. Mutational escape from CD8+ T cell immunity: HCV evolution, from chimpanzees to man. *The Journal of experimental medicine*. 2005;201(11):1709-1714.

180. Valkenburg SA, Gras S, Guillonneau C, et al. Protective efficacy of cross-reactive CD8+ T cells recognising mutant viral epitopes depends on peptide-MHC-I structural interactions and T cell activation threshold. *PLoS pathogens*. 2010;6(8).

181. Pircher H, Moskophidis D, Rohrer U, Burki K, Hengartner H, Zinkernagel RM. Viral escape by selection of cytotoxic T cell-resistant virus variants in vivo. *Nature*. 1990;346(6285):629-633.

182. Martin S, Kohler H, Weltzien HU, Leipner C. Selective activation of CD8 T cell effector functions by epitope variants of lymphocytic choriomeningitis virus glycoprotein. *J Immunol*. 1996;157(6):2358-2365.

183. Schnell FJ, Alberts-Grill N, Evavold BD. CD8+ T cell responses to a viral escape mutant epitope: active suppression via altered SHP-1 activity. *Journal of immunology*. 2009;182(4):1829-1835.

184. Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annual review of immunology*. 2003;21:139-176.

185. Spiotto MT, Fu YX, Schreiber H. Tumor immunity meets autoimmunity: antigen levels and dendritic cell maturation. *Current opinion in immunology*. 2003;15(6):725-730.

186. Dutoit V, Rubio-Godoy V, Dietrich PY, et al. Heterogeneous T-cell response to MAGE-A10(254-262): high avidity-specific cytolytic T lymphocytes show superior antitumor activity. *Cancer research*. 2001;61(15):5850-5856.

187. Yee C, Savage PA, Lee PP, Davis MM, Greenberg PD. Isolation of high avidity melanoma-reactive CTL from heterogeneous populations using peptide-MHC tetramers. *Journal of immunology*. 1999;162(4):2227-2234.

188. Zeh HJ, 3rd, Perry-Lalley D, Dudley ME, Rosenberg SA, Yang JC. High avidity CTLs for two self-antigens demonstrate superior in vitro and in vivo antitumor efficacy. *Journal of immunology*. 1999;162(2):989-994.

189. Boel P, Wildmann C, Sensi ML, et al. BAGE: a new gene encoding an antigen recognized on human melanomas by cytolytic T lymphocytes. *Immunity*. 1995;2(2):167-175.

190. van der Bruggen P, Van den Eynde B. Molecular definition of tumor antigens recognized by T lymphocytes. *Current opinion in immunology*. 1992;4(5):608-612.

191. Brichard V, Van Pel A, Wolfel T, et al. The tyrosinase gene codes for an antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *The Journal of experimental medicine*. 1993;178(2):489-495.

192. Coulie PG, Brichard V, Van Pel A, et al. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *The Journal of experimental medicine*. 1994;180(1):35-42.

193. Kawakami Y, Eliyahu S, Sakaguchi K, et al. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2-restricted tumor infiltrating lymphocytes. *The Journal of experimental medicine*. 1994;180(1):347-352.

194. Wolfel T, Hauer M, Schneider J, et al. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science*. 1995;269(5228):1281-1284.

195. Guilloux Y, Lucas S, Brichard VG, et al. A peptide recognized by human cytolytic T lymphocytes on HLA-A2 melanomas is encoded by an intron sequence of the N-acetylglucosaminyltransferase V gene. *The Journal of experimental medicine*. 1996;183(3):1173-1183.

196. Moreau-Aubry A, Le Guiner S, Labarriere N, Gesnel MC, Jotereau F, Breathnach R. A processed pseudogene codes for a new antigen recognized by a CD8(+) T cell clone on melanoma. *The Journal of experimental medicine*. 2000;191(9):1617-1624.

197. Haas GG, Jr., D'Cruz OJ, De Bault LE. Distribution of human leukocyte antigen-ABC and -D/DR antigens in the unfixed human testis. *American journal of reproductive immunology and microbiology : AJRIM*. 1988;18(2):47-51.

198. Chen YT, Scanlan MJ, Sahin U, et al. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(5):1914-1918.

199. Lucas S, De Smet C, Arden KC, et al. Identification of a new MAGE gene with tumor-specific expression by representational difference analysis. *Cancer research*. 1998;58(4):743-752.

200. Lethe B, Lucas S, Michaux L, et al. LAGE-1, a new gene with tumor specificity. *International journal of cancer Journal international du cancer*. 1998;76(6):903-908.

201. Kawakami Y, Eliyahu S, Jennings C, et al. Recognition of multiple epitopes in the human melanoma antigen gp100 by tumor-infiltrating T lymphocytes associated with in vivo tumor regression. *Journal of immunology*. 1995;154(8):3961-3968.

202. Zarour H, De Smet C, Lehmann F, et al. The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100. *The Journal of investigative dermatology*. 1996;107(1):63-67.

203. Vigneron N, Ooms A, Morel S, Ma W, Degiovanni G, Van den Eynde BJ. A peptide derived from melanocytic protein gp100 and presented by HLA-B35 is recognized by autologous cytolytic T lymphocytes on melanoma cells. *Tissue antigens*. 2005;65(2):156-162.

204. Wang RF, Appella E, Kawakami Y, Kang X, Rosenberg SA. Identification of TRP-2 as a human tumor antigen recognized by cytotoxic T lymphocytes. *The Journal of experimental medicine*. 1996;184(6):2207-2216.

205. Robbins PF, El-Gamil M, Li YF, et al. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. *The Journal of experimental medicine*. 1996;183(3):1185-1192.

206. Rubinfeld B, Robbins P, El-Gamil M, Albert I, Porfiri E, Polakis P. Stabilization of beta-catenin by genetic defects in melanoma cell lines. *Science*. 1997;275(5307):1790-1792.

207. Fisk B, Blevins TL, Wharton JT, Ioannides CG. Identification of an immunodominant peptide of HER-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic T lymphocyte lines. *The Journal of experimental medicine*. 1995;181(6):2109-2117.

208. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related proto-oncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. *The EMBO journal*. 1987;6(3):605-610.

209. Slamon DJ, Godolphin W, Jones LA, et al. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science*. 1989;244(4905):707-712.

210. Claverie JM, Prochnicka-Chalufour A, Bougueleret L. Implications of a Fab-like structure for the T-cell receptor. *Immunology today*. 1989;10(1):10-14.

211. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. *Nature*. 1988;334(6181):395-402.

212. Hornell TM, Solheim JC, Myers NB, et al. Alloreactive and syngeneic CTL are comparably dependent on interaction with MHC class I alpha-helical residues. *Journal of immunology*. 1999;163(6):3217-3225.

213. Baker BM, Turner RV, Gagnon SJ, Wiley DC, Biddison WE. Identification of a crucial energetic footprint on the alpha1 helix of human histocompatibility leukocyte antigen (HLA)-A2 that provides functional interactions for recognition by tax peptide/HLA-A2-specific T cell receptors. *The Journal of experimental medicine*. 2001;193(5):551-562.

214. Baxter TK, Gagnon SJ, Davis-Harrison RL, et al. Strategic mutations in the class I major histocompatibility complex HLA-A2 independently affect both peptide binding and T cell receptor recognition. *The Journal of biological chemistry*. 2004;279(28):29175-29184.

215. Varani L, Bankovich AJ, Liu CW, et al. Solution mapping of T cell receptor docking footprints on peptide-MHC. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(32):13080-13085.

216. Mazza C, Auphan-Anezin N, Gregoire C, et al. How much can a T-cell antigen receptor adapt to structurally distinct antigenic peptides? *The EMBO journal*. 2007;26(7):1972-1983.

217. Rudolph MG, Wilson IA. The specificity of TCR/pMHC interaction. *Current opinion in immunology*. 2002;14(1):52-65.

218. Corse E, Gottschalk RA, Allison JP. Strength of TCR-peptide/MHC interactions and in vivo T cell responses. *Journal of immunology*. 2011;186(9):5039-5045.

219. Schwartz RH. T-lymphocyte recognition of antigen in association with gene products of the major histocompatibility complex. *Annual review of immunology*. 1985;3:237-261.

220. Matsui K, Boniface JJ, Reay PA, Schild H, Fazekas de St Groth B, Davis MM. Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science*. 1991;254(5039):1788-1791.

221. Wu LC, Tuot DS, Lyons DS, Garcia KC, Davis MM. Two-step binding mechanism for T-cell receptor recognition of peptide MHC. *Nature*. 2002;418(6897):552-556.

222. Feng D, Bond CJ, Ely LK, Maynard J, Garcia KC. Structural evidence for a germline-encoded T cell receptor-major histocompatibility complex interaction 'codon'. *Nature immunology*. 2007;8(9):975-983.

223. Adams JJ, Narayanan S, Liu B, et al. T cell receptor signaling is limited by docking geometry to peptide-major histocompatibility complex. *Immunity*. 2011;35(5):681-693.

224. Archbold JK, Macdonald WA, Gras S, et al. Natural micropolymorphism in human leukocyte antigens provides a basis for genetic control of antigen recognition. *The Journal of experimental medicine*. 2009;206(1):209-219.

225. Borbulevych OY, Piepenbrink KH, Gloor BE, et al. T cell receptor cross-reactivity directed by antigen-dependent tuning of peptide-MHC molecular flexibility. *Immunity*. 2009;31(6):885-896.

226. Borbulevych OY, Santhanagopolan SM, Hossain M, Baker BM. TCRs used in cancer gene therapy cross-react with MART-1/Melan-A tumor antigens via distinct mechanisms. *Journal of immunology*. 2011;187(5):2453-2463.

227. Burrows SR, Chen Z, Archbold JK, et al. Hard wiring of T cell receptor specificity for the major histocompatibility complex is underpinned by TCR adaptability. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(23):10608-10613.

228. Buslepp J, Wang H, Biddison WE, Appella E, Collins EJ. A correlation between TCR Valpha docking on MHC and CD8 dependence: implications for T cell selection. *Immunity*. 2003;19(4):595-606.

229. Chen JL, Stewart-Jones G, Bossi G, et al. Structural and kinetic basis for heightened immunogenicity of T cell vaccines. *The Journal of experimental medicine*. 2005;201(8):1243-1255.

230. Cole DK, Yuan F, Rizkallah PJ, et al. Germ line-governed recognition of a cancer epitope by an immunodominant human T-cell receptor. *The Journal of biological chemistry*. 2009;284(40):27281-27289.

231. Colf LA, Bankovich AJ, Hanick NA, et al. How a single T cell receptor recognizes both self and foreign MHC. *Cell*. 2007;129(1):135-146.

232. Dai S, Huseby ES, Rubtsova K, et al. Crossreactive T Cells spotlight the germline rules for alphabeta T cell-receptor interactions with MHC molecules. *Immunity*. 2008;28(3):324-334.

233. Day EB, Guillonneau C, Gras S, et al. Structural basis for enabling T-cell receptor diversity within biased virus-specific CD8+ T-cell responses. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(23):9536-9541.

234. Degano M, Garcia KC, Apostolopoulos V, Rudolph MG, Teyton L, Wilson IA. A functional hot spot for antigen recognition in a superagonist TCR/MHC complex. *Immunity*. 2000;12(3):251-261.

235. Deng L, Langley RJ, Brown PH, et al. Structural basis for the recognition of mutant self by a tumor-specific, MHC class II-restricted T cell receptor. *Nature immunology*. 2007;8(4):398-408.

236. Denton AE, Wesselingh R, Gras S, et al. Affinity thresholds for naive CD8+ CTL activation by peptides and engineered influenza A viruses. *Journal of immunology*. 2011;187(11):5733-5744.

237. Ding YH, Baker BM, Garboczi DN, Biddison WE, Wiley DC. Four A6-TCR/peptide/HLA-A2 structures that generate very different T cell signals are nearly identical. *Immunity*. 1999;11(1):45-56.

238. Ding YH, Smith KJ, Garboczi DN, Utz U, Biddison WE, Wiley DC. Two human T cell receptors bind in a similar diagonal mode to the HLA-A2/Tax peptide complex using different TCR amino acids. *Immunity*. 1998;8(4):403-411.

239. Dunn SM, Rizkallah PJ, Baston E, et al. Directed evolution of human T cell receptor CDR2 residues by phage display dramatically enhances affinity for cognate peptide-MHC without increasing apparent cross-reactivity. *Protein science : a publication of the Protein Society*. 2006;15(4):710-721.

240. Garboczi DN, Ghosh P, Utz U, Fan QR, Biddison WE, Wiley DC. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature*. 1996;384(6605):134-141.

241. Garboczi DN, Utz U, Ghosh P, et al. Assembly, specific binding, and crystallization of a human TCR-alphabeta with an antigenic Tax peptide from human T lymphotropic virus type 1 and the class I MHC molecule HLA-A2. *Journal of immunology*. 1996;157(12):5403-5410.

242. Gras S, Burrows SR, Kjer-Nielsen L, et al. The shaping of T cell receptor recognition by self-tolerance. *Immunity*. 2009;30(2):193-203.

243. Gras S, Chen Z, Miles JJ, et al. Allelic polymorphism in the T cell receptor and its impact on immune responses. *The Journal of experimental medicine*. 2010;207(7):1555-1567.

244. Gras S, Saulquin X, Reiser JB, et al. Structural bases for the affinity-driven selection of a public TCR against a dominant human cytomegalovirus epitope. *Journal of immunology*. 2009;183(1):430-437.

245. Hahn M, Nicholson MJ, Pyrdol J, Wucherpfennig KW. Unconventional topology of self peptide-major histocompatibility complex binding by a human autoimmune T cell receptor. *Nature immunology*. 2005;6(5):490-496.

246. Hoare HL, Sullivan LC, Pietra G, et al. Structural basis for a major histocompatibility complex class Ib-restricted T cell response. *Nature immunology*. 2006;7(3):256-264.

247. Ishizuka J, Stewart-Jones GB, van der Merwe A, Bell JI, McMichael AJ, Jones EY. The structural dynamics and energetics of an immunodominant T cell receptor are programmed by its Vbeta domain. *Immunity*. 2008;28(2):171-182.

248. Jones LL, Colf LA, Stone JD, Garcia KC, Kranz DM. Distinct CDR3 conformations in TCRs determine the level of cross-reactivity for diverse antigens, but not the docking orientation. *Journal of immunology*. 2008;181(9):6255-6264.

249. Kjer-Nielsen L, Clements CS, Purcell AW, et al. A structural basis for the selection of dominant alphabeta T cell receptors in antiviral immunity. *Immunity*. 2003;18(1):53-64.

250. Li Y, Huang Y, Lue J, Quandt JA, Martin R, Mariuzza RA. Structure of a human autoimmune TCR bound to a myelin basic protein self-peptide and a multiple sclerosis-associated MHC class II molecule. *The EMBO journal*. 2005;24(17):2968-2979.

251. Liu YC, Chen Z, Burrows SR, et al. The energetic basis underpinning T-cell receptor recognition of a super-bulged peptide bound to a major histocompatibility complex class I molecule. *The Journal of biological chemistry*. 2012;287(15):12267-12276.

252. Luz JG, Huang M, Garcia KC, et al. Structural comparison of allogeneic and syngeneic T cell receptor-peptide-major histocompatibility complex complexes: a buried alloreactive mutation subtly alters peptide presentation substantially increasing V(beta) Interactions. *The Journal of experimental medicine*. 2002;195(9):1175-1186.

253. Macdonald WA, Chen Z, Gras S, et al. T cell allorecognition via molecular mimicry. *Immunity*. 2009;31(6):897-908.

254. Manning TC, Schlueter CJ, Brodnicki TC, et al. Alanine scanning mutagenesis of an alphabeta T cell receptor: mapping the energy of antigen recognition. *Immunity*. 1998;8(4):413-425.

255. Maynard J, Petersson K, Wilson DH, et al. Structure of an autoimmune T cell receptor complexed with class II peptide-MHC: insights into MHC bias and antigen specificity. *Immunity*. 2005;22(1):81-92.

256. Miles JJ, Bulek AM, Cole DK, et al. Genetic and structural basis for selection of a ubiquitous T cell receptor deployed in Epstein-Barr virus infection. *PLoS pathogens*. 2010;6(11):e1001198.

257. Miller PJ, Pazy Y, Conti B, Riddle D, Appella E, Collins EJ. Single MHC mutation eliminates enthalpy associated with T cell receptor binding. *J Mol Biol*. 2007;373(2):315-327.

258. Newell EW, Ely LK, Kruse AC, et al. Structural basis of specificity and cross-reactivity in T cell receptors specific for cytochrome c-I-E(k). *Journal of immunology*. 2011;186(10):5823-5832.

259. Reiser JB, Darnault C, Guimezanes A, et al. Crystal structure of a T cell receptor bound to an allogeneic MHC molecule. *Nature immunology*. 2000;1(4):291-297.

260. Reiser JB, Gregoire C, Darnault C, et al. A T cell receptor CDR3beta loop undergoes conformational changes of unprecedented magnitude upon binding to a peptide/MHC class I complex. *Immunity*. 2002;16(3):345-354.

261. Sami M, Rizkallah PJ, Dunn S, et al. Crystal structures of high affinity human T-cell receptors bound to peptide major histocompatibility complex reveal native diagonal binding geometry. *Protein engineering, design & selection : PEDS*. 2007;20(8):397-403.

262. Scott DR, Borbulevych OY, Piepenbrink KH, Corcelli SA, Baker BM. Disparate degrees of hypervariable loop flexibility control T-cell receptor cross-reactivity, specificity, and binding mechanism. *J Mol Biol*. 2011;414(3):385-400.

263. Simpson AA, Mohammed F, Salim M, et al. Structural and energetic evidence for highly peptide-specific tumor antigen targeting via allo-MHC restriction. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108(52):21176-21181.

264. Stadinski BD, Trenh P, Smith RL, et al. A role for differential variable gene pairing in creating T cell receptors specific for unique major histocompatibility ligands. *Immunity*. 2011;35(5):694-704.

265. Stewart-Jones GB, McMichael AJ, Bell JI, Stuart DI, Jones EY. A structural basis for immunodominant human T cell receptor recognition. *Nature immunology*. 2003;4(7):657-663.

266. Yin L, Huseby E, Scott-Browne J, et al. A single T cell receptor bound to major histocompatibility complex class I and class II glycoproteins reveals switchable TCR conformers. *Immunity*. 2011;35(1):23-33.

267. Yin Y, Wang XX, Mariuzza RA. Crystal structure of a complete ternary complex of T-cell receptor, peptide-MHC, and CD4. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(14):5405-5410.

268. Yoshida K, Corper AL, Herro R, Jabri B, Wilson IA, Teyton L. The diabetogenic mouse MHC class II molecule I-Ag7 is endowed with a switch that modulates TCR affinity. *The Journal of clinical investigation*. 2010;120(5):1578-1590.

269. Borg NA, Ely LK, Beddoe T, et al. The CDR3 regions of an immunodominant T cell receptor dictate the 'energetic landscape' of peptide-MHC recognition. *Nature immunology*. 2005;6(2):171-180.

270. Ely LK, Beddoe T, Clements CS, et al. Disparate thermodynamics governing T cell receptor-MHC-I interactions implicate extrinsic factors in guiding MHC restriction. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(17):6641-6646.

271. Gras S, Kjer-Nielsen L, Chen Z, Rossjohn J, McCluskey J. The structural bases of direct T-cell allorecognition: implications for T-cell-mediated transplant rejection. *Immunology and cell biology*. 2011;89(3):388-395.

272. Davis MM, Boniface JJ, Reich Z, et al. Ligand recognition by alpha beta T cell receptors. *Annual review of immunology*. 1998;16:523-544.

273. Germain RN, Stefanova I. The dynamics of T cell receptor signaling: complex orchestration and the key roles of tempo and cooperation. *Annual review of immunology*. 1999;17:467-522.

274. Huseby ES, Crawford F, White J, Marrack P, Kappler JW. Interface-disrupting amino acids establish specificity between T cell receptors and complexes of major histocompatibility complex and peptide. *Nat Immunol*. 2006;7(11):1191-1199.

275. Sykulev Y, Joo M, Vturina I, Tsomides TJ, Eisen HN. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity*. 1996;4(6):565-571.

276. Tian S, Maile R, Collins EJ, Frelinger JA. CD8+ T cell activation is governed by TCR-peptide/MHC affinity, not dissociation rate. *Journal of immunology*. 2007;179(5):2952-2960.

277. Rabinowitz JD, Beeson C, Lyons DS, Davis MM, McConnell HM. Kinetic discrimination in T-cell activation. *Proceedings of the National Academy of Sciences of the United States of America*. 1996;93(4):1401-1405.

278. Kalergis AM, Boucheron N, Doucey MA, et al. Efficient T cell activation requires an optimal dwell-time of interaction between the TCR and the pMHC complex. *Nature immunology*. 2001;2(3):229-234.

279. Carreno LJ, Gonzalez PA, Kalergis AM. Modulation of T cell function by TCR/pMHC binding kinetics. *Immunobiology*. 2006;211(1-2):47-64.

280. Sykulev Y, Cohen RJ, Eisen HN. The law of mass action governs antigen-stimulated cytolytic activity of CD8+ cytotoxic T lymphocytes. *Proceedings of the National Academy of Sciences of the United States of America*. 1995;92(26):11990-11992.

281. Schodin BA, Tsomides TJ, Kranz DM. Correlation between the number of T cell receptors required for T cell activation and TCR-ligand affinity. *Immunity*. 1996;5(2):137-146.

282. Baker BM, Gagnon SJ, Biddison WE, Wiley DC. Conversion of a T cell antagonist into an agonist by repairing a defect in the TCR/peptide/MHC interface: implications for TCR signaling. *Immunity*. 2000;13(4):475-484.

283. Malherbe L, Hausl C, Teyton L, McHeyzer-Williams MG. Clonal selection of helper T cells is determined by an affinity threshold with no further skewing of TCR binding properties. *Immunity*. 2004;21(5):669-679.

284. Aleksic M, Dushek O, Zhang H, et al. Dependence of T cell antigen recognition on T cell receptor-peptide MHC confinement time. *Immunity*. 2010;32(2):163-174.

285. Krogsgaard M, Prado N, Adams EJ, et al. Evidence that structural rearrangements and/or flexibility during TCR binding can contribute to T cell activation. *Molecular cell*. 2003;12(6):1367-1378.

286. Dustin ML, Golan DE, Zhu DM, et al. Low affinity interaction of human or rat T cell adhesion molecule CD2 with its ligand aligns adhering membranes to achieve high physiological affinity. *The Journal of biological chemistry*. 1997;272(49):30889-30898.

287. Qi S, Krogsgaard M, Davis MM, Chakraborty AK. Molecular flexibility can influence the stimulatory ability of receptor-ligand interactions at cell-cell junctions. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(12):4416-4421.

288. Dustin ML, Zhu C. T cells like a firm molecular handshake. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(12):4335-4336.

289. Huppa JB, Axmann M, Mortelmaier MA, et al. TCR-peptide-MHC interactions in situ show accelerated kinetics and increased affinity. *Nature*. 2010;463(7283):963-967.

290. Huang J, Zarnitsyna VI, Liu B, et al. The kinetics of two-dimensional TCR and pMHC interactions determine T-cell responsiveness. *Nature*. 2010;464(7290):932-936.

291. Willcox BE, Gao GF, Wyer JR, et al. TCR binding to peptide-MHC stabilizes a flexible recognition interface. *Immunity*. 1999;10(3):357-365.

292. Boniface JJ, Reich Z, Lyons DS, Davis MM. Thermodynamics of T cell receptor binding to peptide-MHC: evidence for a general mechanism of molecular scanning. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(20):11446-11451.

293. Prabhu NV, Sharp KA. Heat capacity in proteins. *Annual review of physical chemistry*. 2005;56:521-548.

294. Murphy KP, Gill SJ. Solid model compounds and the thermodynamics of protein unfolding. *J Mol Biol*. 1991;222(3):699-709.

295. Spolar RS, Livingstone JR, Record MT, Jr. Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water. *Biochemistry*. 1992;31(16):3947-3955.

296. Henriques DA, Ladbury JE, Jackson RM. Comparison of binding energies of SrcSH2-phosphotyrosyl peptides with structure-based prediction using surface area based empirical parameterization. *Protein science : a publication of the Protein Society*. 2000;9(10):1975-1985.

297. Armstrong KM, Insaidoo FK, Baker BM. Thermodynamics of T-cell receptor-peptide/MHC interactions: progress and opportunities. *Journal of molecular recognition : JMR*. 2008;21(4):275-287.

298. Anikeeva N, Lebedeva T, Krogsgaard M, et al. Distinct molecular mechanisms account for the specificity of two different T-cell receptors. *Biochemistry*. 2003;42(16):4709-4716.

299. Garcia KC, Degano M, Stanfield RL, et al. An alphabeta T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. *Science*. 1996;274(5285):209-219.

300. Garcia KC, Degano M, Pease LR, et al. Structural basis of plasticity in T cell receptor recognition of a self peptide-MHC antigen. *Science*. 1998;279(5354):1166-1172.

301. Reiser JB, Darnault C, Gregoire C, et al. CDR3 loop flexibility contributes to the degeneracy of TCR recognition. *Nature immunology*. 2003;4(3):241-247.

302. Davis-Harrison RL, Armstrong KM, Baker BM. Two different T cell receptors use different thermodynamic strategies to recognize the same peptide/MHC ligand. *J Mol Biol*. 2005;346(2):533-550.

303. Dressel A, Chin JL, Sette A, Gausling R, Hollsberg P, Hafler DA. Autoantigen recognition by human CD8 T cell clones: enhanced agonist response induced by altered peptide ligands. *Journal of immunology*. 1997;159(10):4943-4951.

304. Gakamsky DM, Lewitzki E, Grell E, et al. Kinetic evidence for a ligand-binding-induced conformational transition in the T cell receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2007;104(42):16639-16644.

305. Garcia KC. Molecular interactions between extracellular components of the T-cell receptor signaling complex. *Immunological reviews*. 1999;172:73-85.

306. Stites WE. Proteinminus signProtein Interactions: Interface Structure, Binding Thermodynamics, and Mutational Analysis. *Chemical reviews*. 1997;97(5):1233-1250.

307. Armstrong KM, Insaidoo FK, Baker BM. Thermodynamics of T-cell receptor-peptide/MHC interactions: progress and opportunities. *J Mol Recognit*. 2008;21(4):275-287.

308. Holler PD, Kranz DM. T cell receptors: affinities, cross-reactivities, and a conformer model. *Molecular immunology*. 2004;40(14-15):1027-1031.

309. James LC, Tawfik DS. Structure and kinetics of a transient antibody binding intermediate reveal a kinetic discrimination mechanism in antigen recognition. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(36):12730-12735.

310. Lee JK, Stewart-Jones G, Dong T, et al. T cell cross-reactivity and conformational changes during TCR engagement. *The Journal of experimental medicine*. 2004;200(11):1455-1466.

311. Dessen A, Lawrence CM, Cupo S, Zaller DM, Wiley DC. X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity*. 1997;7(4):473-481.

312. Kuhns JJ, Batalia MA, Yan S, Collins EJ. Poor binding of a HER-2/neu epitope (GP2) to HLA-A2.1 is due to a lack of interactions with the center of the peptide. *The Journal of biological chemistry*. 1999;274(51):36422-36427.

313. Hillig RC, Coulie PG, Stroobant V, Saenger W, Ziegler A, Hulsmeyer M. High-resolution structure of HLA-A*0201 in complex with a tumour-specific antigenic peptide encoded by the MAGE-A4 gene. *J Mol Biol*. 2001;310(5):1167-1176.

314. Sharma AK, Kuhns JJ, Yan S, et al. Class I major histocompatibility complex anchor substitutions alter the conformation of T cell receptor contacts. *The Journal of biological chemistry*. 2001;276(24):21443-21449.

315. Fremont DH, Dai S, Chiang H, Crawford F, Marrack P, Kappler J. Structural basis of cytochrome c presentation by IE(k). *The Journal of experimental medicine*. 2002;195(8):1043-1052.

316. Hulsmeyer M, Fiorillo MT, Bettosini F, et al. Dual, HLA-B27 subtype-dependent conformation of a self-peptide. *The Journal of experimental medicine*. 2004;199(2):271-281.

317. Wucherpfennig KW. Presentation of a self-peptide in two distinct conformations by a disease-associated HLA-B27 subtype. *The Journal of experimental medicine*. 2004;199(2):151-154.

318. Gagnon SJ, Borbulevych OY, Davis-Harrison RL, et al. T cell receptor recognition via cooperative conformational plasticity. *J Mol Biol*. 2006;363(1):228-243.

319. Mason D. A very high level of crossreactivity is an essential feature of the T-cell receptor. *Immunology today*. 1998;19(9):395-404.

320. Bhardwaj V, Kumar V, Geysen HM, Sercarz EE. Degenerate recognition of a dissimilar antigenic peptide by myelin basic protein-reactive T cells. Implications for thymic education and autoimmunity. *Journal of immunology*. 1993;151(9):5000-5010.

321. Hemmer B, Fleckenstein BT, Vergelli M, et al. Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T cell clone. *The Journal of experimental medicine*. 1997;185(9):1651-1659.

322. Grogan JL, Kramer A, Nogai A, et al. Cross-reactivity of myelin basic protein-specific T cells with multiple microbial peptides: experimental autoimmune encephalomyelitis induction in TCR transgenic mice. *Journal of immunology*. 1999;163(7):3764-3770.

323. Evavold BD, Sloan-Lancaster J, Wilson KJ, Rothbard JB, Allen PM. Specific T cell recognition of minimally homologous peptides: evidence for multiple endogenous ligands. *Immunity*. 1995;2(6):655-663.

324. Hagerty DT, Allen PM. Intramolecular mimicry. Identification and analysis of two cross-reactive T cell epitopes within a single protein. *Journal of immunology*. 1995;155(6):2993-3001.

325. Loftus DJ, Castelli C, Clay TM, et al. Identification of epitope mimics recognized by CTL reactive to the melanoma/melanocyte-derived peptide MART-1(27-35). *The Journal of experimental medicine*. 1996;184(2):647-657.

326. Misko IS, Cross SM, Khanna R, et al. Crossreactive recognition of viral, self, and bacterial peptide ligands by human class I-restricted cytotoxic T lymphocyte clonotypes: implications for molecular mimicry in autoimmune disease. *Proceedings of the National Academy of Sciences of the United States of America*. 1999;96(5):2279-2284.

327. Kim SK, Brehm MA, Welsh RM, Selin LK. Dynamics of memory T cell proliferation under conditions of heterologous immunity and bystander stimulation. *Journal of immunology*. 2002;169(1):90-98.

328. Brehm MA, Pinto AK, Daniels KA, Schneck JP, Welsh RM, Selin LK. T cell immunodominance and maintenance of memory regulated by unexpectedly cross-reactive pathogens. *Nature immunology*. 2002;3(7):627-634.

329. Jameson SC, Hogquist KA, Bevan MJ. Specificity and flexibility in thymic selection. *Nature*. 1994;369(6483):750-752.

330. Sebzda E, Wallace VA, Mayer J, Yeung RS, Mak TW, Ohashi PS. Positive and negative thymocyte selection induced by different concentrations of a single peptide. *Science*. 1994;263(5153):1615-1618.

331. Alam SM, Travers PJ, Wung JL, et al. T-cell-receptor affinity and thymocyte positive selection. *Nature*. 1996;381(6583):616-620.

332. Wucherpfennig KW. T cell receptor crossreactivity as a general property of T cell recognition. *Molecular immunology*. 2004;40(14-15):1009-1017.

333. Ignatowicz L, Rees W, Pacholczyk R, et al. T cells can be activated by peptides that are unrelated in sequence to their selecting peptide. *Immunity*. 1997;7(2):179-186.

334. Jameson SC, Bevan MJ. T cell receptor antagonists and partial agonists. *Immunity*. 1995;2(1):1-11.

335. Kohm AP, Fuller KG, Miller SD. Mimicking the way to autoimmunity: an evolving theory of sequence and structural homology. *Trends in microbiology*. 2003;11(3):101-105.

336. Mariuzza RA, Poljak RJ. The basics of binding: mechanisms of antigen recognition and mimicry by antibodies. *Current opinion in immunology*. 1993;5(1):50-55.

337. Oldstone MB. Molecular mimicry and autoimmune disease. *Cell*. 1987;50(6):819-820.

338. Archbold JK, Macdonald WA, Burrows SR, Rossjohn J, McCluskey J. T-cell allorecognition: a case of mistaken identity or deja vu? *Trends in immunology*. 2008;29(5):220-226.

339. Archbold JK, Macdonald WA, Miles JJ, et al. Alloreactivity between disparate cognate and allogeneic pMHC-I complexes is the result of highly focused, peptide-dependent structural mimicry. *The Journal of biological chemistry*. 2006;281(45):34324-34332.

340. Kersh GJ, Allen PM. Essential flexibility in the T-cell recognition of antigen. *Nature*. 1996;380(6574):495-498.

341. Wucherpfennig KW, Strominger JL. Selective binding of self peptides to disease-associated major histocompatibility complex (MHC) molecules: a mechanism for MHC-linked susceptibility to human autoimmune diseases. *The Journal of experimental medicine*. 1995;181(5):1597-1601.

342. Hemmer B, Vergelli M, Gran B, et al. Predictable TCR antigen recognition based on peptide scans leads to the identification of agonist ligands with no sequence homology. *Journal of immunology*. 1998;160(8):3631-3636.

343. Kersh GJ, Allen PM. Structural basis for T cell recognition of altered peptide ligands: a single T cell receptor can productively recognize a large continuum of related ligands. *The Journal of experimental medicine*. 1996;184(4):1259-1268.

344. Ohteki T, Hessel A, Bachmann MF, et al. Identification of a cross-reactive self ligand in virus-mediated autoimmunity. *European journal of immunology*. 1999;29(9):2886-2896.

345. Fujinami RS, Oldstone MB. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science*. 1985;230(4729):1043-1045.

346. Saibil SD, Ohteki T, White FM, et al. Weak agonist self-peptides promote selection and tuning of virus-specific T cells. *European journal of immunology*. 2003;33(3):685-696.

347. Nakano T, Kobayashi K, Saito S, Fujita K, Nagatsu T. Mouse dopamine beta-hydroxylase: primary structure deduced from the cDNA sequence and exon/intron organization of the gene. *Biochemical and biophysical research communications*. 1992;189(1):590-599.

348. Sandalova T, Michaelsson J, Harris RA, et al. A structural basis for CD8+ T cell-dependent recognition of non-homologous peptide ligands: implications for molecular mimicry in autoreactivity. *The Journal of biological chemistry*. 2005;280(29):27069-27075.

349. Evavold BD, Sloan-Lancaster J, Allen PM. Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunology today*. 1993;14(12):602-609.

350. Slansky JE, Jordan KR. The Goldilocks model for TCR-too much attraction might not be best for vaccine design. *PLoS biology*. 2010;8(9).

351. Iero M, Filipazzi P, Castelli C, et al. Modified peptides in anti-cancer vaccines: are we eventually improving anti-tumour immunity? *Cancer immunology, immunotherapy : CII*. 2009;58(7):1159-1167.

352. Sprent J, Lo D, Gao EK, Ron Y. T cell selection in the thymus. *Immunological reviews*. 1988;101:173-190.

353. Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Advances in immunology*. 2000;74:181-273.

354. Marincola FM, Wang E, Herlyn M, Seliger B, Ferrone S. Tumors as elusive targets of T-cell-based active immunotherapy. *Trends in immunology*. 2003;24(6):335-342.

355. Rivoltini L, Squarcina P, Loftus DJ, et al. A superagonist variant of peptide MART1/Melan A27-35 elicits anti-melanoma CD8+ T cells with enhanced functional characteristics: implication for more effective immunotherapy. *Cancer research*. 1999;59(2):301-306.

356. Fong L, Hou Y, Rivas A, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America*. 2001;98(15):8809-8814.

357. Morse MA, Hobeika AC, Osada T, et al. Depletion of human regulatory T cells specifically enhances antigen-specific immune responses to cancer vaccines. *Blood*. 2008;112(3):610-618.

358. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. *Nature medicine*. 2004;10(9):909-915.

359. Parmiani G, Castelli C, Santinami M, Rivoltini L. Melanoma immunology: past, present and future. *Current opinion in oncology*. 2007;19(2):121-127.

360. Huber V, Fais S, Iero M, et al. Human colorectal cancer cells induce T-cell death through release of proapoptotic microvesicles: role in immune escape. *Gastroenterology*. 2005;128(7):1796-1804.

361. Rivoltini L, Canese P, Huber V, et al. Escape strategies and reasons for failure in the interaction between tumour cells and the immune system: how can we tilt the balance towards immune-mediated cancer control? *Expert opinion on biological therapy*. 2005;5(4):463-476.

362. Insaidoo FK, Borbulevych OY, Hossain M, Santhanagopolan SM, Baxter TK, Baker BM. Loss of T cell antigen recognition arising from changes in peptide and major histocompatibility complex protein flexibility: implications for vaccine design. *The Journal of biological chemistry*. 2011;286(46):40163-40173.

363. Hanisch FG, Ninkovic T. Immunology of O-glycosylated proteins: approaches to the design of a MUC1 glycopeptide-based tumor vaccine. *Current protein & peptide science*. 2006;7(4):307-315.

364. Ryan SO, Vlad AM, Islam K, Gariepy J, Finn OJ. Tumor-associated MUC1 glycopeptide epitopes are not subject to self-tolerance and improve responses to MUC1 peptide epitopes in MUC1 transgenic mice. *Biological chemistry*. 2009;390(7):611-618.

365. Ninkovic T, Kinarsky L, Engelmann K, et al. Identification of O-glycosylated decapeptides within the MUC1 repeat domain as potential MHC class I (A2) binding epitopes. *Molecular immunology*. 2009;47(1):131-140.

366. Mohammed F, Cobbold M, Zarling AL, et al. Phosphorylation-dependent interaction between antigenic peptides and MHC class I: a molecular basis for the presentation of transformed self. *Nature immunology*. 2008;9(11):1236-1243.

367. Petersen J, Wurzbacher SJ, Williamson NA, et al. Phosphorylated self-peptides alter human leukocyte antigen class I-restricted antigen presentation and generate tumor-specific epitopes. *Proceedings of the National Academy of Sciences of the United States of America*. 2009;106(8):2776-2781.

368. Zarling AL, Polefrone JM, Evans AM, et al. Identification of class I MHC-associated phosphopeptides as targets for cancer immunotherapy. *Proceedings of the National Academy of Sciences of the United States of America*. 2006;103(40):14889-14894.

369. Green RS, Stone EL, Tenno M, Lehtonen E, Farquhar MG, Marth JD. Mammalian N-glycan branching protects against innate immune self-recognition and inflammation in autoimmune disease pathogenesis. *Immunity*. 2007;27(2):308-320.

370. Hill JA, Bell DA, Brintnell W, et al. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *The Journal of experimental medicine*. 2008;205(4):967-979.

371. Harauz G, Musse AA. A tale of two citrullines--structural and functional aspects of myelin basic protein deimination in health and disease. *Neurochemical research*. 2007;32(2):137-158.

372. Mannering SI, Harrison LC, Williamson NA, et al. The insulin A-chain epitope recognized by human T cells is posttranslationally modified. *The Journal of experimental medicine*. 2005;202(9):1191-1197.

373. Arentz-Hansen H, Korner R, Molberg O, et al. The intestinal T cell response to alpha-gliadin in adult celiac disease is focused on a single deamidated glutamine targeted by tissue transglutaminase. *The Journal of experimental medicine*. 2000;191(4):603-612.

374. Henderson KN, Tye-Din JA, Reid HH, et al. A structural and immunological basis for the role of human leukocyte antigen DQ8 in celiac disease. *Immunity*. 2007;27(1):23-34.

375. Kim CY, Quarsten H, Bergseng E, Khosla C, Sollid LM. Structural basis for HLA-DQ2-mediated presentation of gluten epitopes in celiac disease. *Proceedings of the National Academy of Sciences of the United States of America*. 2004;101(12):4175-4179.

376. Tye-Din JA, Stewart JA, Dromey JA, et al. Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. *Science translational medicine*. 2010;2(41):41ra51.

377. Snir O, Rieck M, Gebe JA, et al. Identification and functional characterization of T cells reactive to citrullinated vimentin in HLA-DRB1*0401-positive humanized mice and rheumatoid arthritis patients. *Arthritis and rheumatism*. 2011;63(10):2873-2883.

378. Snir O, Backlund J, Bostrom J, et al. Multifunctional T cell reactivity with native and glycosylated type II collagen in rheumatoid arthritis. *Arthritis and rheumatism*. 2012;64(8):2482-2488.

379. Hudrisier D, Oldstone MB, Gairin JE. The signal sequence of lymphocytic choriomeningitis virus contains an immunodominant cytotoxic T cell epitope that is restricted by both H-2D(b) and H-2K(b) molecules. *Virology*. 1997;234(1):62-73.

380. Moskophidis D, Zinkernagel RM. Immunobiology of cytotoxic T-cell escape mutants of lymphocytic choriomeningitis virus. *Journal of virology*. 1995;69(4):2187-2193.

381. Huber R, Carrell RW. Implications of the three-dimensional structure of alpha 1-antitrypsin for structure and function of serpins. *Biochemistry*. 1989;28(23):8951-8966.

382. Ventura S, Vega MC, Lacroix E, Angrand I, Spagnolo L, Serrano L. Conformational strain in the hydrophobic core and its implications for protein folding and design. *Nature structural biology*. 2002;9(6):485-493.

383. Montfort WR, Weichsel A. Thymidylate synthase: structure, inhibition, and strained conformations during catalysis. *Pharmacology & therapeutics*. 1997;76(1-3):29-43.

384. Ischiropoulos H, al-Mehdi AB. Peroxynitrite-mediated oxidative protein modifications. *FEBS letters*. 1995;364(3):279-282.

385. Ischiropoulos H, Zhu L, Chen J, et al. Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase. *Archives of biochemistry and biophysics*. 1992;298(2):431-437.

386. Hudrisier D, Mazarguil H, Laval F, Oldstone MB, Gairin JE. Binding of viral antigens to major histocompatibility complex class I H-2Db molecules is controlled by dominant negative elements at peptide non-anchor residues. Implications for peptide selection and presentation. *J Biol Chem*. 1996;271(30):17829-17836.

387. Bachmann MF, Oxenius A, Speiser DE, et al. Peptide-induced T cell receptor down-regulation on naive T cells predicts agonist/partial agonist properties and strictly correlates with T cell activation. *Eur J Immunol*. 1997;27(9):2195-2203.

388. Bachmann MF, Speiser DE, Zakarian A, Ohashi PS. Inhibition of TCR triggering by a spectrum of altered peptide ligands suggests the mechanism for TCR antagonism. *European journal of immunology*. 1998;28(10):3110-3119.

389. Wang B, Sharma A, Maile R, Saad M, Collins EJ, Frelinger JA. Peptidic termini play a significant role in TCR recognition. *J Immunol*. 2002;169(6):3137-3145.

390. Pinilla-Ibarz J, May RJ, Korontsvit T, et al. Improved human T-cell responses against synthetic HLA-0201 analog peptides derived from the WT1 oncoprotein. *Leukemia*. 2006;20(11):2025-2033.

391. Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR. T cell receptor antagonist peptides induce positive selection. *Cell*. 1994;76(1):17-27.

392. Colbert RA, Rowland-Jones SL, McMichael AJ, Frelinger JA. Differences in peptide presentation between B27 subtypes: the importance of the P1 side chain in maintaining high affinity peptide binding to B*2703. *Immunity*. 1994;1(2):121-130.

393. Dressel A, Chin JL, Sette A, Gausling R, Hollsberg P, Hafler DA. Autoantigen recognition by human CD8 T cell clones: enhanced agonist response induced by altered peptide ligands. *J Immunol*. 1997;159(10):4943-4951.

394. Mazza C, Auphan-Anezin N, Gregoire C, et al. How much can a T-cell antigen receptor adapt to structurally distinct antigenic peptides? *EMBO J*. 2007;26(7):1972-1983.

395. Jones LL, Colf LA, Stone JD, Garcia KC, Kranz DM. Distinct CDR3 conformations in TCRs determine the level of cross-reactivity for diverse antigens, but not the docking orientation. *J Immunol*. 2008;181(9):6255-6264.

396. Reiser JB, Darnault C, Guimezanes A, et al. Crystal structure of a T cell receptor bound to an allogeneic MHC molecule. *Nat Immunol*. 2000;1(4):291-297.

397. Jones LL, Colf LA, Bankovich AJ, et al. Different thermodynamic binding mechanisms and peptide fine specificities associated with a panel of structurally similar high-affinity T cell receptors. *Biochemistry*. 2008;47(47):12398-12408.

398. van Stipdonk MJ, Badia-Martinez D, Sluijter M, Offringa R, van Hall T, Achour A. Design of agonistic altered peptides for the robust induction of CTL directed towards H-2Db in complex with the melanoma-associated epitope gp100. *Cancer Res*. 2009;69(19):7784-7792.

399. Martin S, Kohler H, Weltzien HU, Leipner C. Selective activation of CD8 T cell effector functions by epitope variants of lymphocytic choriomeningitis virus glycoprotein. *Journal of immunology*. 1996;157(6):2358-2365.

400. Bouhdoud L, Villain P, Merzouki A, Arella M, Couture C. T-cell receptor-mediated anergy of a human immunodeficiency virus (HIV) gp120-specific CD4(+) cytotoxic T-cell clone, induced by a natural HIV type 1 variant peptide. *Journal of virology*. 2000;74(5):2121-2130.

401. Plebanski M, Lee EA, Hannan CM, et al. Altered peptide ligands narrow the repertoire of cellular immune responses by interfering with T-cell priming. *Nature medicine*. 1999;5(5):565-571.

402. Ream RM, Sun J, Braciale TJ. Stimulation of naive CD8+ T cells by a variant viral epitope induces activation and enhanced apoptosis. *Journal of immunology*. 2010;184(5):2401-2409.

403. Puglielli MT, Zajac AJ, van der Most RG, et al. In vivo selection of a lymphocytic choriomeningitis virus variant that affects recognition of the GP33-43 epitope by H-2Db but not H-2Kb. *Journal of virology*. 2001;75(11):5099-5107.

404. Zhou F. Molecular mechanisms of viral immune evasion proteins to inhibit MHC class I antigen processing and presentation. *International reviews of immunology*. 2009;28(5):376-393.

405. Groth A, Kloss S, von Strandmann EP, Koehl U, Koch J. Mechanisms of tumor and viral immune escape from natural killer cell-mediated surveillance. *Journal of innate immunity*. 2011;3(4):344-354.

406. Garrido F, Algarra I, Garcia-Lora AM. The escape of cancer from T lymphocytes: immunoselection of MHC class I loss variants harboring structural-irreversible "hard" lesions. *Cancer immunology, immunotherapy : CII*. 2010;59(10):1601-1606.

407. Orr MT, Lanier LL. Inhibitory Ly49 receptors on mouse natural killer cells. *Current topics in microbiology and immunology*. 2011;350:67-87.

408. Sijts AJ, De Bruijn ML, Ressing ME, et al. Identification of an H-2 Kb-presented Moloney murine leukemia virus cytotoxic T-lymphocyte epitope that displays enhanced recognition in H-2 Db mutant bm13 mice. *Journal of virology*. 1994;68(9):6038-6046.

409. Zeng L, Sullivan LC, Vivian JP, et al. A structural basis for antigen presentation by the MHC class Ib molecule, Qa-1b. *Journal of immunology*. 2012;188(1):302-310.

410. Brodin P. MHC class I molecules in Natural Killer cell education and tolerance. *Karolinka Institutet Doctoral Theses*. 2011.

411. Orr MT, Lanier LL. Natural killer cell education and tolerance. *Cell*. 2010;142(6):847-856.

412. Elliott JM, Yokoyama WM. Unifying concepts of MHC-dependent natural killer cell education. *Trends in immunology*. 2011;32(8):364-372.

413. Malnati MS, Peruzzi M, Parker KC, et al. Peptide specificity in the recognition of MHC class I by natural killer cell clones. *Science*. 1995;267(5200):1016-1018.

414. Zappacosta F, Borrego F, Brooks AG, Parker KC, Coligan JE. Peptides isolated from HLA-Cw*0304 confer different degrees of protection from natural killer cell-mediated lysis. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(12):6313-6318.

415. Maenaka K, Juji T, Nakayama T, et al. Killer cell immunoglobulin receptors and T cell receptors bind peptide-major histocompatibility complex class I with distinct thermodynamic and kinetic properties. *The Journal of biological chemistry*. 1999;274(40):28329-28334.

416. Stewart CA, Laugier-Anfossi F, Vely F, et al. Recognition of peptide-MHC class I complexes by activating killer immunoglobulin-like receptors. *Proceedings of the National Academy of Sciences of the United States of America*. 2005;102(37):13224-13229.

417. Thananchai H, Gillespie G, Martin MP, et al. Cutting Edge: Allele-specific and peptide-dependent interactions between KIR3DL1 and HLA-A and HLA-B. *Journal of immunology*. 2007;178(1):33-37.

418. Fadda L, Borhis G, Ahmed P, et al. Peptide antagonism as a mechanism for NK cell activation. *Proceedings of the National Academy of Sciences of the United States of America*. 2010;107(22):10160-10165.

419. Lampen MH, Hassan C, Sluijter M, et al. Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2. *Molecular immunology*. 2012;53(1-2):126-131.

420. Ohteki T, Hessel A, Bachmann MF, et al. Identification of a cross-reactive self ligand in virus-mediated autoimmunity. *Eur J Immunol*. 1999;29(9):2886-2896.

421. Sandalova T, Michaelsson J, Harris RA, et al. A structural basis for CD8+ T cell-dependent recognition of non-homologous peptide ligands: implications for molecular mimicry in autoreactivity. *J Biol Chem*. 2005;280(29):27069-27075.

422. Salazar-Onfray F, Nakazawa T, Chhajlani V, et al. Synthetic peptides derived from the melanocyte-stimulating hormone receptor MC1R can stimulate HLA-A2-restricted cytotoxic T lymphocytes that recognize naturally processed peptides on human melanoma cells. *Cancer Res*. 1997;57(19):4348-4355.

423. Chen JL, Dunbar PR, Gileadi U, et al. Identification of NY-ESO-1 peptide analogues capable of improved stimulation of tumor-reactive CTL. *J Immunol*. 2000;165(2):948-955.

424. Gnjatic S, Nagata Y, Jager E, et al. Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc Natl Acad Sci U S A*. 2000;97(20):10917-10922.

425. Pellat-Deceunynck C, Mellerin MP, Labarriere N, et al. The cancer germ-line genes MAGE-1, MAGE-3 and PRAME are commonly expressed by human myeloma cells. *Eur J Immunol*. 2000;30(3):803-809.

426. Trojan A, Schultze JL, Witzens M, et al. Immunoglobulin framework-derived peptides function as cytotoxic T-cell epitopes commonly expressed in B-cell malignancies. *Nat Med*. 2000;6(6):667-672.

427. Celie PH, Toebes M, Rodenko B, Ovaa H, Perrakis A, Schumacher TN. UV-induced ligand exchange in MHC class I protein crystals. *J Am Chem Soc*. 2009;131(34):12298-12304.