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STRUCTURAL BASES FOR MHC CLASS I PEPTIDE SELECTION AND NEOEPITOPE FORMATION

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Här går vägen fram till Lyckolandet,
den är lång och trång och stängd av snår,
ingen knipslug mästerkatt i stövlor
finns att visa oss, hur vägen går.

-Gustav Fröding, Strövtåg i hembygden III, Stänk och Flikar, 1896

ABSTRACT

One task of the immune system is to distinguish between healthy cells and altered cells due to viruses or cancers. To achieve this CD8+ T-cells are constantly monitoring the Major histocompatibility class I (MHC-I) complexes expressed on all nucleated cells in the body. The MHC-I is basically a scaffold to hold and display a short (usually 8-10 amino acids) peptide. These peptides are selected by a complicated machinery from the total peptidome as formed by the proteolytic activity in the cells. The peptides derive from the proteome within the cells, which is degraded by the proteasome to peptides of various lengths. These are transported into the endoplasmic reticulum (ER) by the transporters associated with antigen processing (TAP). Inside the ER, the peptides can be further processed by the ER aminopeptidases (ERAP1 and ERAP 2) before being loaded into the MHC-I by the peptide loading complex (PLC). The peptide loading complex consists of tapasin, ERp57 and calreticulin. The peptide MHC-I complex (pMHC-I) is then transported to the cell surface where they can be presented to the T-Cells of the immune system. The T-cells express the highly variable T-cell Receptor (TCR) which are formed from recombining the V(D)J segment in the TCR gene. The T-cells are selected in the Thymus, where self-responding T-cells are killed and the remaining, with low affinity to self, continue into circulation. This ensures protection against autoimmunity, T-cell responses against healthy cells. Cancer-cell survival and proliferation require immune system escape and consequently many cancer cells have deficiencies in the processing system.

In this thesis four studies are presented. The first concerns the mechanism of tapasin influence on the peptide selection and loading into the peptide binding cleft of the MHC-I. In this study, a leucine on a loop of tapasin is shown to be able to bind into the F-pocket of the peptide binding cleft, and influence the peptide loading of the MHC-I.

The second study is about a neoepitope that is presented on TAP-deficient cancer cells. This peptide, Trh4, was the first identified TEIPP (T-cell epitopes associated with impaired peptide processing). As a murine H-2D^{b} restricted peptide is has an unusual sequence, containing four sulphur containing residues of the total nine. Here, we show the formation and importance of sulphur-π interaction for complex stability and the unconventional methionine at peptide position 5 for the formation of TCR interaction.

The third study is a follow-up on the second study, but here the immunogenicity of the peptide is in focus. While the wild-type Trh4 peptide displays low immunogenicity, the mutation of peptide position 3 to a proline significantly increase the immunogenicity of this epitope. However, the increased immunogenicity is surprisingly not related to increased stability of the pMHC-I complex.

In the final paper, two virus-associated epitopes are studied due to their differences in immunogenicity and binding to MHC-I. Both peptides carry a glycosylation motif and are glycosylated in the source protein. Here we show the structural basis for the discrepancy between the peptides. The glycosylated residue in one peptide (GP92) is protruding from the

peptide binding cleft free for interaction with a TCR, while for the other peptide (GP392), a glycosylation would hinder the utilisation of a dominant anchor residue of the peptide for peptide binding to the MHC-I.

LIST OF SCIENTIFIC PAPERS

I. **Successive crystal structure snapshots reveal the bases for MHC class I peptide binding and editing**

Hafstrand I, Apavaloaei A, Sayitoglu EC, Buratto J, Pellegrino S, Han X, Sun R, Nilvebrant J, Nygren P-Å, Sandalova T, Springer S, Duru AD and Achour A

Manuscript

II. **The MHC Class I Cancer-Associated Neoepitope Trh4 Linked with Impaired Peptide Processing Induces a Unique Noncanonical TCR Conformer**

Hafstrand I*, Doorduijn EM*, Duru AD, Buratto J, Oliveira CC, Sandalova T, van Hall T, Achour A

Journal of Immunology, 2016 Mar 1;196(5):2327-34. doi: 10.4049/jimmunol.1502249

*IH and EMD contributed equally

III. **The Immunogenicity of a Proline-Substituted Altered Peptide Ligand toward the Cancer-Associated TEIPP Neoepitope Trh4 Is Unrelated to Complex Stability**

Hafstrand I*, Doorduijn EM*, Sun R, Talyzina A, Sluijter M, Pellegrino S, Sandalova T, Duru AD, van Hall T, Achour A

Journal of Immunology, 2018 Mar 5. doi: 10.4049/jimmunol.1700228

*IH and EMD contributed equally

IV. **Crystal structures of H-2D^{b} in complex with the LCMV-derived peptides GP92 and GP392 explain pleiotropic effects of glycosylation on antigen presentation and immunogenicity**

Hafstrand I*, Badia-Martinez D*, Josey BJ, Norstrom M, Buratto J, Pellegrino S, Duru AD, Sandalova T, Achour A

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*IH and DBM contributed equally

CONTENTS

LIST OF ABBREVIATIONS

ABU	α-aminobutyric acid
APL	altered peptide ligand
β_2m	β2-microglobulin
CD	circular dichroism
CDR	complementarity determining region
DRiP	defect ribosomal product
ER	endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing (mouse)
ERAP	ER aminopeptidases (human)
HC	heavy chain
HLA	human leukocyte antigen (human MHC)
IRAP	insulin responsive aminopeptidase
LCMV	lymphocytic choriomeningitis virus
MHC-I	major histocompatibility complex class I
NLE	norleucine
PBC	peptide binding cleft
PLC	peptide loading complex
pMHC-I	peptide MHC-I complex
PTM	post-translational modification
SEV	Sendai virus nucleus protein peptide, sequence: FAPGNYPAL
TAA	tumour-associated antigen
TAP	transporter associated with antigen processing
TAPBPR	TAP-binding protein related
TEIPP	T-cell epitopes associated with impaired peptide processing
wt	wild-type

1 INTRODUCTION

1.1 THE MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I

All nucleated cells display on their surface the Major Histocompatibility Complex class I (MHC-I) proteins. This protein complex, key for the initiation of adaptive immune responses against pathogens and cancers, consists of three different chains (subunits); the Heavy Chain (HC), the β_2-microglobulin (β_2m) and a usually eight-to-ten amino acids long peptide. The HC is C-terminally anchored in the membrane of the cell and its extracellular parts consists of three domains, named α_1-α_3. The α_1 and α_2 domains together form the aptly named peptide binding cleft (PBC) that binds the peptides (1-3) (Figure 1A and B). The peptides are highly varied and are most often derived from proteins degraded within the cell. As such, the specific set of peptides on a cell at a single time point, the so-called epitope repertoire, mirrors the status of the cell.

The first discoveries of what later became known as MHC came when George Snell published his finding on a genetic locus he named H (for histocompatibility) in 1951. The same system was discovered almost simultaneously by Peter H. Gorer who called the antigen he studied 'II'. Both were working independently on the rejection of transplanted grafts and tumours. Snell identified the H loci through thorough genetic studies of mice while Gorer based his studies on serological characterisation. They both agreed to name the system H2, a combination between the names they used. The importance of the H2 genes were published in 1951 and marked the start of MHC research (4). In 1975, Zinkernagel and Doherty discovered that the MHC genes were the targets of T-cells, which recognised a combination of the MHC and an antigen (5-8). The identification of the antigen as being a peptide came in 1985 by Townsend *et al* (9), just two years before Bjorkman *et al* published the first structure of the protein (1). The MHC molecules used in the first structural determination were isolated from cell surfaces and in combination with the limitations of X-ray crystallography, the identity of the occupier of the cleft between the two helices flanking the antigen binding cleft was not clear, but as mentioned by Bjorkman *et al* in their following article, the density observed could indeed be representing peptides (1, 2).

Fundamental to the discovery of the H-2 genes and the properties of the MHC-I is its allelic variety, which is the cause of the graft rejection the genes were initially studied for (4). The human versions of the MHC-I, commonly referred to as the Human Leukocyte Antigens (HLA) A, B and C, together compose above 12´000 alleles according to the HLA nomenclature database. Of these, the actual resulting protein variation numbers are 2781 individual HLA-A, 3501 HLA-B and 2490 HLA-C (10). With such a variety among the human population, the task of studying this specific part of the immune system is a great one.

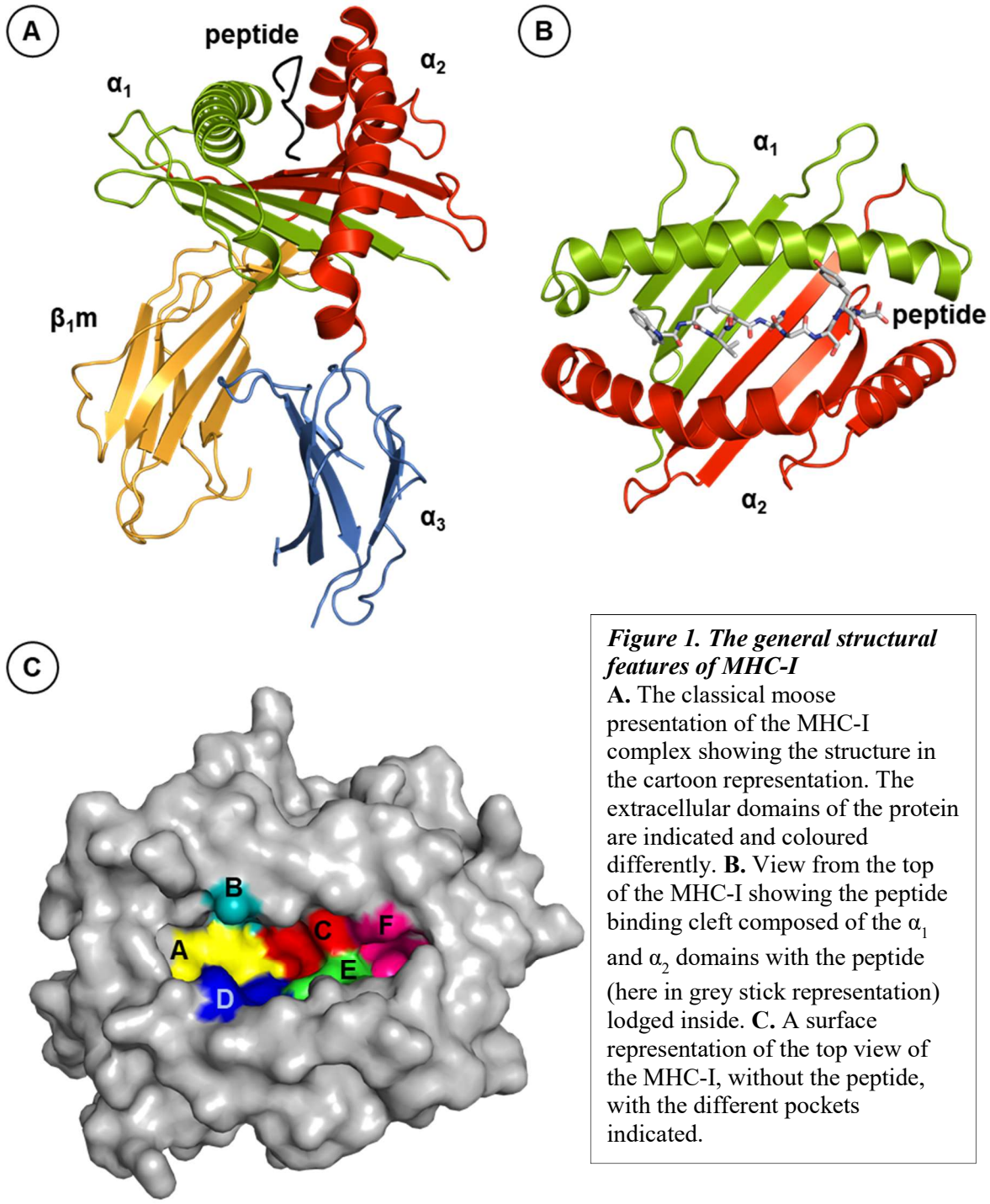


Figure 1. The general structural features of MHC-I
A. The classical moose presentation of the MHC-I complex showing the structure in the cartoon representation. The extracellular domains of the protein are indicated and coloured differently. **B.** View from the top of the MHC-I showing the peptide binding cleft composed of the α_1 and α_2 domains with the peptide (here in grey stick representation) lodged inside. **C.** A surface representation of the top view of the MHC-I, without the peptide, with the different pockets indicated.

1.2 STRUCTURAL ANALYSIS COMPOSITION OF THE MHC-I COMPLEX

As stated above, the MHC-I is a trimeric complex. The HC is the largest subunit, composed of approximately 365 amino acids. The protein consists of four domains. The α_1 and α_2 domains form the PBC, each forming half of the β-sheet floor and a lining α-helix. The α_3 domain forms an immunoglobulin-like domain under the PBC, and the final section of the HC is the membrane anchor. The β_2m is a globular protein, folding independently of the HC. Last is the peptide in the PBC, usually in an elongated conformation (1). The first structure of

a MHC-I molecule was published in 1987 (1) and was of the human allele HLA-A*0201. The protein was not recombinantly produced, as has commonly been the case later, but was produced natively on human cells and cut via trypsinisation. As such, the peptide repertoire presented within the cleft of this initial structure was not uniform, and the density within the peptide loading cleft did not allow for the identification of a specific binder (1).

During the studies of the peptide specificity and binding to the PBC, the areas in which the side chains of the peptides bind to the PBC were named ‘pockets’. These are categorised A-F, roughly aligning with the peptide from the N- to the C-terminus of the PBC (11) (Figure 1C). The peptides characterised for each allele have certain common features. The most prominent features are the anchor residues, a specific position where one or a few amino acids are required for peptide binding. The pockets these anchor residues bind to vary by allele. The first study to evaluate the motif of peptide binders for a specific allele was Falk *et al* in 1991 (12). In time, as more peptides have been characterised, the motifs have been updated. A table with the recent motifs as determined by the Internet Epitope Database (IEDB) (13), for some of the most common alleles are shown in table 1.

Position / Allele	1	2	3	4	5	6	7	8	9	10
H-2D^b		A M S	I L M		N			P	I L M	
	D E P	F G I L N Q T V			M			G	D H K P R T Y	
H-2D^d		G Q S	P		F Y					I
		D I K P R			G I R					F L M
H-2K^b	I M S V	I S	F W Y		F Y			M L V		
	D E G H N P		C I L M		H M V		I			
H-2K^d	K S Y	Y	M		M S V		Q S T		I L	
	D E P	All	D P		E F K P Y				M V	
*HLA-A*01:01*		S T	D P						Y	
		A I L M V							F	
*HLA-A*02:01*	F Y	L M	F M W		W Y		F W		I L V	
	D E P	I Q V	E K R			K R	G K R		A M	
*HLA-A*02:01*	F Y	L M	F M L Y					W	Y	V
		I Q V	K					K R		A F G I L M
*HLA-A*03:01*	K R	I L M T V	F Y						K	
	D E P	A Q S	P			D	D		R Y	
*HLA-A*03:01*	R	I L M T V	M Y							K
	D E P	A F H Q S Y	E			D E				Y
*HLA-B*07:02*	R	P	R			R		A P S	F L M	
	D E P	All							A I V	
*HLA-B*44:02*		E							F I W Y	
		D	P						D E P Q R S T	

Table 1. Preferred, accepted and deleterious residues in peptide positions for some common MHC-I alleles.
Based on data from www.iedb.org as of March 2018. The anchor positions are marked in orange. In the grey cells are preferred residues, in the white are accepted (blue) or deleterious (red) residues listed. In some cases, data is available for both nonamer and decamer peptides, but in general the number of residues noted indicates the preferred peptide length. The four top alleles are examples of murine alleles and the HLA-alleles are examples of common human alleles in Sweden.

2 PEPTIDE PROCESSING AND MHC-I FUNCTION

2.1 THE ANTIGEN PROCESSING PATHWAY

2.1.1 The proteasomes

The main source of peptides for MHC-I is the proteasome (Figure 2A), a large cytosolic complex situated in the cytosol of the cell, with the main function to degrade proteins marked for destruction. The 20S core of the proteasome is composed of four rings, with seven subunits in each ring, and is the proteolytic part of the complex. The two middle rings consist of subunits β1-7 and the two outermost of α1-7. Subunits β1, β2 and β5 possess the protease function, cleaving after acidic (caspase-like), basic (trypsin-like) and hydrophobic (chymotrypsin-like) residues respectively (14, 15).It is also these three subunits that differ significantly in the immunoproteasome, which is a variant that is induced in inflammatory processes. In the immunoproteasome, the specificity of proteolytic activity shifts and the caspase-like activity is reduced while the cleavage after small hydrophobic residues is increased. Interestingly, there is also a so-called 'intermediate proteasome' that incorporates some of the immunoproteasome subunits in a mix with the normal subunits (16-18). The main function of immunoproteasome induction is considered to be to increase the quality of the accessible peptides for antigen processing (17). This is a generalisation, however, since several immunodominant epitopes are produced by only the constitutive proteasome (19). Supporting this generalisation is that when the distribution of the different proteasome versions was quantified (18), it revealed high variability in normal tissues, but in the cancer cell-lines tested, the variability was notably diminished, with a high abundance of the constitutive proteasome and very low levels of the immunoproteasome.

The proteasome does not only degrade fully functional proteins that have been ubiquitinated and elongated, but also Defect ribosomal products (DRiPs) which also are essential to the foundations of the peptide repertoire, especially within stressed/infected cells. The DRiPs are the main reason for the rapid appearance of disease-associated epitopes prior to symptoms of infection in the cell (20, 21). One example of this is the LCMV (Lymphocytic Choriomeningitis Virus) nucleoprotein, which was not detectable in the cytoplasm of cells within a three days period after infection, while LCMV-derived MHC-restricted antigens were detectable on the surface of the infected cells within 45 minutes (22). This and other studies proved DRiPs as essential for efficient early cell responses to infection (21).

2.1.2 TAP peptide transport into the ER

The ATP-binding cassette-type Transporter associated with antigen processing (TAP) protein selectively transport peptides into the ER (Figure 2B). In 1990 the genes of the complex were identified as an ATP-dependent peptide transporter situated within the MHC-II encoding region (23). Intense work in the following years led to the discovery that the genes encoded a heterodimer consisting of TAP-1 functioning as an ATP-binding cassette and TAP-2 functioning as a peptide translocon (24-32). Interestingly, the binding of a peptide substrate to

the TAP transporter does not require ATP. The ATP hydrolysis takes place in the translocation step (33). TAP promotes the transfer of 8-16 residue-long peptides (33), with a preference for hydrophobic (and for humans also basic) C-termini (28, 34). TAP also favours hydrophobic residues in peptide position three and both hydrophobic and charged residues are preferred in position two, while N-terminal prolines in positions 1-2 (human) (33) and 1-3 (mouse) (35) inhibit transport across the membrane. The peptide C-terminus is generally not trimmed further within the ER, unlike the N-terminus (36).

2.1.3 Further peptide trimming mechanisms in the ER

The main trimming of peptides is done by the protein ERAAP (ER aminopeptidase associated with antigen processing) in mice and ERAP1 and 2 (ER aminopeptidases 1 and 2) in humans (Figure 2C). These belong to a family of aminopeptidases generally known as the oxytocinase subfamily of M1 aminopeptidases. There is an endosomal homologue called IRAP (Insulin responsive aminopeptidase) which is regulated by insulin, as opposed to IFN-γ for the ERAP proteins. IRAP is mainly involved in cross-presentation, which will be discussed further later (37). The ERAPs peptidases usually trim peptides to a length of eight or nine residues (38). In humans, ERAP1 has a specificity for hydrophobic and aromatic side chains and ERAP2 for long and positively charged side chains (39). Interestingly, the allelic variation of both ERAP1 and 2 has a significant influence on the specificity and/or processing rates. The overall importance of this trimming step can be illustrated by a 20 % observed reduction in the MHC-I cell surface expression upon knocking out ERAAP molecules in mice (40). Suggestions that ERAP1 and 2 are trimming the peptides after partial association with the MHC-I within the peptide loading complex are contradicted by structural results that revealed the distance to the active site in the amino peptidases to be equivalent to six amino acids in length from the proteins surface. Such a distance would not allow the optimal trimming to make the peptide length 8-10 amino acids, due to sterical hindrance (37).

2.1.4 The peptide loading complex

2.1.4.1 Initial folding of the MHC-I heavy chain

The initial step of MHC-I folding is facilitated by Calnexin (Figure 2D) , an abundant ER chaperone, involved in the folding of a variety of proteins (41). The unfolded HC contains a N-linked monoglucosylated glycan, the substrate for Calnexin and the related Calreticulin binding (42). There is a discrepancy here between the processes in mice and in human. Murine MHC-I does not interact with another abundant ER-chaperone, BiP, but human MHC-I do. In both cases, however, binding of the already folded β_2m causes release from these chaperones.

2.1.4.2 Components of the peptide loading complex

The MHC-I peptide loading step is facilitated by the multi-protein peptide-loading complex (PLC) (Figure 2F), which consists of Calreticulin, ERp57 and tapasin (Figure 2G) all supporting the loading and verifying the quality of peptides loaded onto the MHC-I

molecules (43). Calreticulin, an abundant ER chaperone, is thought to have a stabilising effect on the PLC. It is binds specifically to β_2m-bound MHC-I in contrast to the related Calnexin (44). Calreticulin knockout affects the repertoire of peptides displayed on the surface, both by changing the repertoire of peptides that is displayed and by reducing the number of peptide MHC-I (pMHC-I) complexes on the cell surface (45). ERp57 is a thiol oxidoreductase that usually cooperates with calnexin and/or calreticulin to assist folding of glycoproteins in the ER (46). In the PLC, the function of ERp57 differs. While it still interacts with calreticulin, it is also stably associated with tapasin through a disulphide bond formed between residue Cys57 within its catalytic site and residue Cys95 in tapasin. As a result, this disulphide bridge interaction inhibits the ERp57 oxidoreductase activity. Instead, ERp57 has a recruiting role for PLC components, combined with a protective role against disulphide bond breakage (46). It contains four domains of thioredoxin type; *a b b' a'*. The active sites of *a* and *a'* contain the redox activity, while *b* and *b'* are substrate-binding domains (47-49).

Tapasin was first discovered in an experiment investigating the interaction between TAP and partially folded MHC-I, where an unidentified 48 kDa glycoprotein co-precipitated with the proteins (50). In 1996, Sadasivan *et al* (51) named this protein tapasin (short for "TAP-associated glycoprotein") and showed it to link TAP to MHC-I before peptide binding. The protein is a type I transmembrane protein composed of 428 amino acids. It has a single N-linked glycosylation site and an ER retention signal (52, 53).

Tapasin stabilizes the open forms of peptide-deficient and peptide-filled class I molecules, thereby likely diminishing the energy barrier that bound candidate peptides must overcome in order to stable binding to in the class I binding groove (54). Interestingly, tapasin binds to TAP through interactions within the ER transmembrane regions. The N-terminal transmembrane helix from both TAP 1 and TAP 2 are interacting with tapasin (55). Tapasin association with TAP affects TAP levels in some cell types (56), with as much as a threefold increase reported for some cell lines. Soluble tapasin does not associate with TAP, but restores the surface levels of MHC-I compared to tapasin deficient cells (57), indicating that the most essential role of tapasin is the peptide loading and selection.

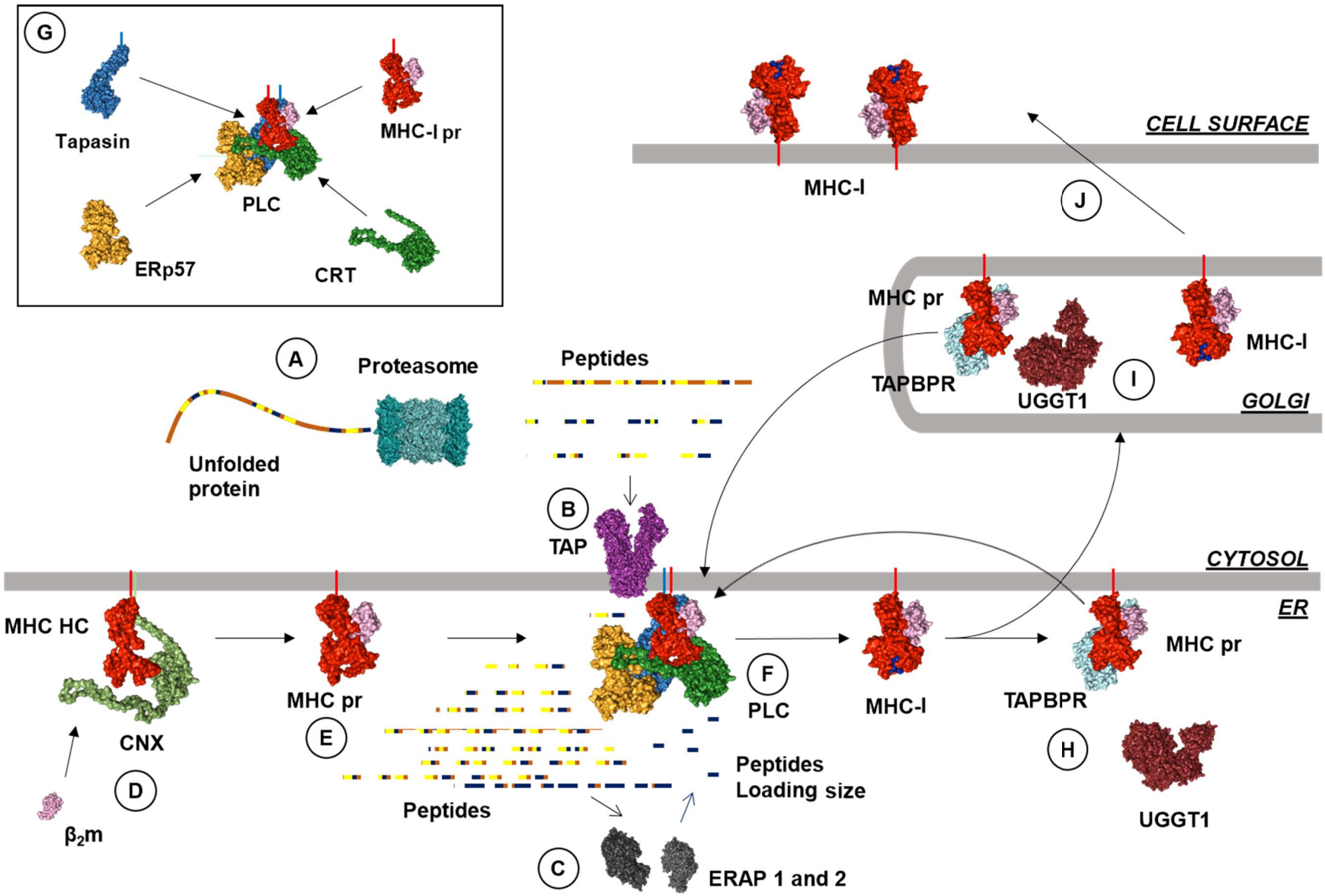
2.1.4.3 The 721.220 tapasin-deficient cell line

Many studies on the function of tapasin have been performed on the lymphoblastoid 721.220 cells. This cell line has a mutation that causes exon 2 to be spliced out and exon 1 and 3 to be annealed to each other. While this alternative splicing is detected in wild type cell lines, it is only detected at the nucleic acid level and not as a produced protein in the wt cells. Both are detected in the 721.220 cell line. The result is a 57-amino acid deletion, which abrogates MHC-I association but not TAP association (58). In 721.220 cells the surface expression of MHC-I is therefore 20-25% compared to normal cells. Since the TAP-association is still intact in these cells (59), this means that the cell surface expression levels reflect the loss of interaction between tapasin and the MHC-I, as well as possibly ERp57. Tapasin double knock-out mice display a 80-85% reduction in the amount of peripheral T-cells and the

MHC-I surface levels cells in these mice are 10-15% compared to wild-type (WT) levels, which is approximately double the levels observed on TAP double knock-out mice (56).

The first 50 N-terminal residues of tapasin are essential to the assembly of the PLC and its association with TAP (60), even though this association takes place in the C-terminal TM (transmembrane) region (55), according to a study by Bangia *et al* 1999. There is however a problem with this study of the 50 N-terminal amino acids, since there are no controls described in the paper, determining if truncation affects the structural integrity of the remaining protein (60). This means that the observed effect could be a result of full or partial misfolding of the protein and thereby a complete loss of tapasin. Furthermore, there was no positive control example of an interacting truncate, but instead the positive control was the full-length protein. Interestingly, there is a later study on a melanoma-associated tapasin mutant lacking the third exon, representing amino acids 70-156, which includes the Erp57 interacting site. This truncation did not affect the MHC-I surface levels to the same extent, as measured using the same tapasin-C-terminal restricted antibody (61). This study displayed the same issues as the previous, with no discussion or reflection of the possibility of a partially or completely misfolded tapasin. These studies are further compromised when considering that tapasin association with TAP affects TAP levels in some cell types (56), with as much as a threefold increase reported for some cell lines. This renders the results even more difficult to interpret. Are the apparent effects of the tapasin because of these deletions due to the loss of tapasin structural integrity, the loss of the amino acids described or due to a secondary effect on TAP, which is essential to peptide import? More studies will be needed to determine the answer to these questions and the use of an antibody specific for the correctly ER-lumenal domains would be essential.

Figure 2. The antigen processing pathway
A. The peptides originate from the products of the proteasomal (teal colour) degradation of the proteins from the cell. **B.** These are selectively transported by TAP (purple) into the Endoplasmic Reticulum (ER). **C.** The peptides can undergo further trimming by the ER aminopeptidases (ERAP 1 and 2, grey and dark grey) and the final peptides of an adequate length (dark blue) can compete for loading into the peptide binding cleft of the MHC-I. **D.** In the ER, the HC of the MHC (red) are stabilised by calnexin (CNX, light green) until the binding of β_2m (pink). **E.** As β_2m binds, CNX releases the peptide-receptive MHC-I (MHC-I pr). **F.** The MHC-I pr binds to the peptide loading complex (PLC). **G.** The PLC consists of tapasin (light blue), ERp57 (orange) and calreticulin (CRT, dark green) as shown in the insert. When a peptide binds, the PLC releases the loaded MHC-I. Either in the ER (**H.**) or in the Golgi (**I.**), the peptide loaded MHC-I can be further checked by TAPBPR (cyan), who in the case of suboptimal peptide loading can dislodge the peptide. The TAPBPR can also interact with the UDP-glucose:glycoprotein glucosyltransferase 1 (UGGT 1, brown) who is can reattach the glycan needed for CRT interaction and thereby recirculate the MHC-I pr back to the PLC. **J.** If the MHC-I passes the check of TAPBPR, and has an optimally loaded peptide, it can continue to the cell surface.
The structures shown in the figure are based on PDBcodes 6ENY (62) (PLC, CNX and MHC-I pr), 1PMA (63) (proteasome), 5U1D (64, 65) (TAP), 2YD0 (66) (ERAP 1), 3SE6 (67) (ERAP 2), 5OPI (68) (TAPBPR including bound MHC-I), 5JWD (69) (MHC-I). The UGGT1 structure is predicted by the RaptorX structure prediction server (70-72).


G
Tapasin
MHC-I pr
PLC
ERp57
CRT
CELL SURFACE
MHC-I
J
MHC pr
TAPBPR
UGGT1
I
MHC-I
GOLGI
A
Proteasome
Peptides
Unfolded protein
B
TAP
CYTOSOL
ER
MHC HC
CNX
D
β2m
MHC pr
E
F
PLC
MHC-I
TAPBPR
MHC pr
H
UGGT1
Peptides
Peptides Loading size
C
ERAP 1 and 2

2.1.4.4 Interactions within the peptide loading complex

The crystal structure of the tapasin-ERp57 complex has been published in 2009 combined with a site-directed mutagenesis investigation of the interactions of this complex with MHC-I. Within the PLC tapasin exists as a heterodimer with ERp57, linked via a disulphide-bridge between residues Cys95 of tapasin and Cys57 of ERp57 (73). The crystal structure revealed an L-shaped tapasin, and the interaction sites with MHC-I were determined to be close to the angle of the L. The U-shaped ERp57 is on the opposite side of tapasin, meaning that it is likely not directly interacting with the MHC-I molecule. There are several loops in the crystal structure of tapasin that are missing due to high mobility, including one loop situated near to the modelled MHC-I (73). This positioning was confirmed recently, when the first structure of the PLC was published (62). The method of structural determination was single particle electron cryo-microscopy (Cryo-EM) and the maximum resolution at 5.8Å was enough to determine the spatial positioning of the complex but provides little information on the side chains or sometimes even general main chain positioning. It did however reveal a few important surprises. While it was previously well established that each TAP molecule can interact with two tapasin molecules (74), it was not known until now that the two tapasin molecules interact with each other. Neither was it known that ERp57 and calreticulin bind across the two copies of the mega-complex (62).

2.1.5 Post PLC processing

In the year 2002, a new player in the peptide loading mechanism was discovered (75), located genetically in a region associated with MHC-I (76, 77). Named TAPBPR (for TAP-binding protein related), it had 33% similarity and only 22% sequence identity with tapasin (75). Unlike tapasin, its location is not restricted to the ER as it lacks the ER retention signal. Indeed, 10% of the expressed TAPBPR is located at the surface of the cell (75) and 50% of TAPBPR molecules show evidence of Golgi processing in a study by Boyle *et al*, meaning that they at some point have passed through or recycled from the Golgi Network (78). This was confirmed in the first follow-up study seven year later (79). It would be a total of eleven years, however, before further studies were published, when in 2013 Boyle *et al* reported TAPBPR to be implicated in peptide loading (78). It had no association with the PLC, and the binding to MHC-I was competing with tapasin, meaning that the interacting surface of these two proteins on the MHC-I overlap (80). TAPBPR is a peptide exchange catalyst (81, 82), and in absence of TAPBPR the bound peptides of MHC-I at the surface of the cells average a lower affinity than in the wild type equivalent (81). As TAPBPR associates with the low affinity peptide-loaded MHC-I it recruits the UDP-glucose:glycoprotein glycosyltransferase 1, responsible for adding a glucose to the glycan of MHC-I (and other proteins) hence halting their transport to the surface and promoting association or re-association with the PLC. UDP-glucose:glycoprotein glycosyltransferase 1 is resident in the ER and the *cis*-Golgi (83, 84). Together with the evidence mentioned earlier of TAPBPR presence in the Golgi, it seems likely that TAPBPR is a direct player in the recycling of sub-optimally loaded MHC-I between ER and Golgi (Figure 2H and 2I) (85).

2.1.6 Peptide selection and editing by tapasin and TAPBPR

Supported by the newly published structures of TAPBPR in complex with MHC-I (68, 86) and the PLC cryo-EM structure (62), in combination with paper I within this thesis, it is now possible to draw conclusions about the peptide loading mechanisms. The two structures of TAPBPR have certain things in common: 1) they are both low resolution structures at 3.4Å (Jiang et al. (86)) and 3.3Å (Thomas and Tampé (68)); 2) they both illustrate the use of the "jack hairpin" (named by Thomas and Tampé) to bind underneath the β-sheet floor of the PBC; 3) both structures show a widening of the PBC by a lateral movement of the α_2-helix. There are however still controversial issues in both structures, namely the interpretation of the positioning of the side chain of the MHC-I residue Y84, which is involved in peptide C-terminal binding. Both structures claim that the side chain of this residue flexes out from the PBC and interacts with residue E105 in TAPBPR. At this resolution interpreting the exact positioning of a side chain can be devious, especially in the Thomas and Tampé structure, where the statistics of the structure are rather poor, indicating that the actual resolution is significantly worse than 3.3Å. When calculating the omit maps for both these structures, it is revealed that the density of the Y84 sidechain is weak. A significant difference in the two structures is the visibility of a loop from TAPBPR which in Thomas and Tampé's structure are inserted into the F-pocket area of the PBC. In the Jiang structure there is some density in this area, but not enough for accurate modelling of even the main chain. The Jiang structure also includes a peptide in the N-terminal part of the PBC, bound to a mutated Cysteine on position 73 of the α_1-helix, while the PBC of the Thomas and Tampé structure has an empty PBC. In paper I new results are presented on the molecular mechanism behind the peptide editing effect of tapasin.

2.2 ATYPICAL ANTIGEN PROCESSING

2.2.1 Antigen cross-presentation

Cross-presentation of exogenous antigens on MHC-I is a process that still needs lots of investigation, since many steps are not fully understood and there are several pathways working simultaneously. The main cell type where this phenomenon has been found is the dendritic cell (DC), but other cell types such as macrophages, mast cells, epithelial cells etc… can also exhibit cross-presentation (87). There are two main pathways, the dominant phagosome-to-cytosol (P2C) and the minor vacuolar pathway (88).

2.2.1.1 The phagosome-to-cytosol pathway

In the phagosome-to-cytosol pathway the exogenous antigen source is taken up by a phagosome. As this pathway requires the use of the proteasome, the antigen must be translocalised from the phagosome into the cytosol. The pathway for this transport is yet debated, but there have been suggestions that ERAD, the ER-associated degradation pathway, is involved. ERAD proteins can be transferred into the phagosomes, together with other ER proteins such as TAP. Alternatively, phagosomal disruption, involving the collapse of the phagosomal membrane and the release of the contents into the cytosol, is a possible

mechanism (87, 88). The loading of the peptides is then expected to follow either the traditional antigen processing pathway or taking place in phagosomes. In the phagosomes, the source of the MHC-I is still not completely clear. A very popular option is that internalised MHC-I from the cell surface is re-used, which is supported by the fact that these MHC-I molecules are EndoH-resistant, meaning they have passed through Golgi and not been transported directly from the ER (87, 89). Tapasin, which contains an ER-retention signal, is not likely to be present in phagosomes (53). Recently, suggestions for TAPBPR involvement in this step have been made (83).

2.2.1.2 The vacuolar antigen pathway

The vacuolar pathway is independent from TAP and the proteasome. Instead the proteolysis and the peptide loading occur within the endocytic compartment. In this pathway the proteolysis is mainly performed by cathepsins that are induced by acidification after endosomal fusion with phagosomes. Another component of the degradation pathway is IRAP, (insulin-regulated aminopeptidase), an aminopeptidase recruited to phagosomes and that is regulated by both insulin and IgE immunoglobulin. It has a broad specificity, equal to ERAP1 and 2 combined and, as such, can mimic the function of the ERAPs in the endosomal compartment (39).

2.2.2 Post translationally modified peptides

Several types of post-translational modifications (PTMs) have been identified on MHC-I peptide epitopes. These include acetylation (90), cysteinylation (91), deamidation (92), glycosylation (93-97), methylation (98), nitrotyrosinylation (99, 100) and phosphorylation (101-103). The most well studied of these PTMs are glycosylations and phosphorylations.

Glycosylations identified in the context of MHC-I are either O-linked or N-linked. The third type of constitutive glycan, glycosylphosphatidylinositol (GPI) anchors have to my knowledge not yet been shown as a MHC-I binder. However, while potentially N-glycosylated peptides have been identified, none have yet been eluted still carrying the glycan (104). The O-linked glycans bind to either serine or threonine without any sequence specificity, unlike N-linked glycans which bind to asparagine within Asn-X-Ser/Thr where X ≠ Pro. The O-linked glycans are more varied, while the N-linked specifically consists of an initial N-acetylglucosamine (GlcNAc) moiety. O-linked glycans have been shown to be transported through TAP into the ER. N-linked glycans are still a mystery. N-glycosylations are known to affect proteasomal degradation of surrounding epitopes (105). The glycan can be cut prior to the proteasome processing by peptide N-glycanase, causing the mutation of the encoded asparagine N to a glutamate D (106), potentially causing the lack of eluted N-linked glycopeptides. Reasonably, the large glycans would not be anchoring into the PBS and indeed structures (107), predictions (97) and described glycan-restricted T-cells (93, 108) corroborate this. In some cases, however, the glycan does not protrude from the MHC-I PBC. This is the case in the binding of MUC1.-8-5GalNAc, which utilises the monoglycan as an anchor when binding to H-2K^b (109).

Phosphorylation is a fairly small PTM, but highly important in the energy homeostasis in the cell. Phosphorylated peptides are transported by TAP into the ER (110). Ser and Thr phosphorylated peptides are the most common to be identified on MHC-I (111), but phosphorylation can take place on Tyr and His also (112). A recent study (113) identifying peptides found in melanoma from patients, found 365 HLA-I restricted phosphopeptides and 25 restricted to HLA-II. Of these 78 % were phosphorylated on Ser, 19% on Threonine and 3 % on Tyrosine (113). The phosphorylation site of the peptide is usually at position P4 of the peptide (102).

2.3 THE αβ-T-CELL RECEPTOR

2.3.1 Education

T-cells are educated in the Thymus, mainly early in life from the neonatal period to puberty (114). The T-cell expresses a randomly generated T-cell Receptor (TCR) and its sequence is determined by the randomized recombination of the V, D and J gene segments for the β-chain and the V and J segments for the α-chain. The differentiation to either a $CD4^+$-expressing (MHC-II restricted) or $CD8^+$-expressing (MHC-I restricted) T-cell is done according to their affinity for either MHC class in the cortex, combined with the expression of a large ensemble of cytokines (115, 116). The first step in the T-cell education is the positive selection, which takes place in the cortex of the thymus, where cortical thymic epithelial cells are presenting peptides both on MHC-I and MHC-II (117). The selection of the peptides for both classes is aided by proteases uniquely expressed in these cell types (116). In the presentation pathway for MHC-I a third form of the proteasome, the thymoproteasome, is present. While the β1 and β2 subunits in the thymoproteasome are the same as in the immunoproteasome, the β5 subunit is unique and contains a hydrophilic residue in the middle of its hydrophobic binding site. This causes a weakened chymotrypsin activity, affecting the positive selection of T-cells during thymic education (118). The purpose and reason for this discrepancy of peptides expressed during the positive and later the negative selection is still debated (119, 120). The positively selected T-cells move thereafter on to the thymus medulla, while the cells removed from further education undergo “death by neglect” (121).

In the medulla, the negative selection occurs, with the eventual removal of self-reactive T-cells that could cause autoimmunity. In contrast to the process of positive selection, there are more than one kind of APC present, both medullar thymic epithelial cells (mTECs) and bone-marrow derived dendritic cells, and the proteasomes are the constitutive and immunoproteasome (115). The negative selection endpoint is the removal of autoreactive T-cells, which occurs upon T-cell interaction with the self-peptides expressed on the APC in the medulla. Essential to this selection is the expression of tissue specific antigens, usually restricted to a specific tissue type, meaning that the mTECs have promiscuously expressed proteins that are usually downregulated outside a specific tissue. Interestingly, only 1-3% of

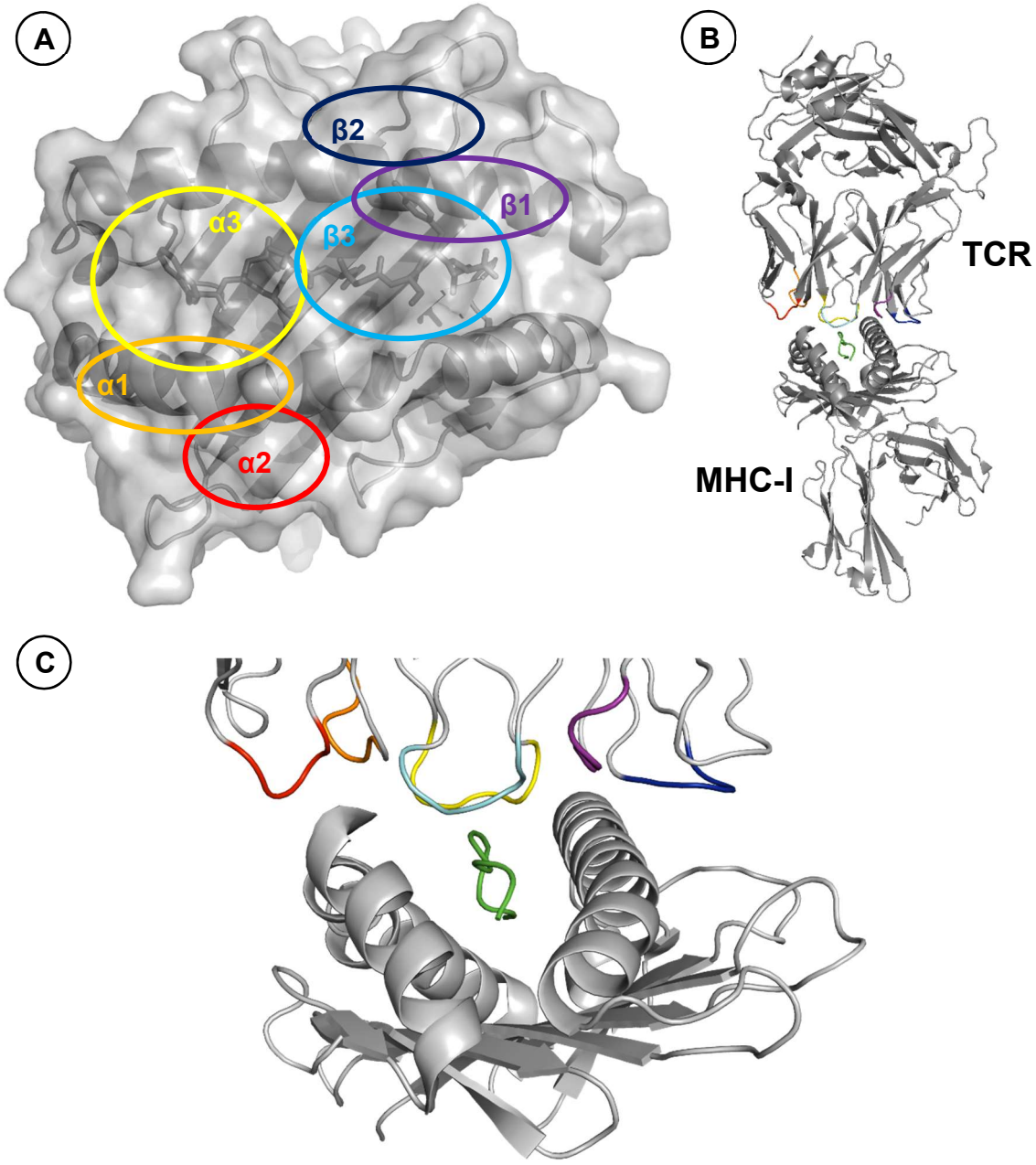


Figure 3. The classical areas for TCR CDR-loop landing on the MHC-I
A. The regions where the CDR loops most commonly land on the MHC-I are indicated in different colours. These colours are the same throughout the figure. **B.** Overall view of the TCR on top landing on the MHC-I below. The MHC-I is angled with the C-terminal region of the PBC towards the viewer. **C.** Zoom in on the CDR loops of the TCR and the PBC with the peptide in green. While this is a general pattern, based on the report by Attaf *et al* 2015 (122) there are examples of reversed polarisation where the chains change place. The structure of the TCR-MHC-I is based on PDBcode 2BNR (123).

the mTECs produce each tissue specific antigen. They do get help from DC though, by secretion into the extracellular space and normal DC uptake and presentation. Eventually, the remaining T-cells are tolerant to the self-antigens and non-immunogenic in the uninfected milieu (124).

2.3.2 Recognition on T-cell epitopes and modes of interaction

Each chain of the TCR has three MHC-I interacting loops, named complementarity determining regions (CDR) 1, 2 and 3. CDR 1 and 2 are encoded by the germline in the T cell receptor alpha and beta variable (TRAV and TRBV) regions. The CDR3 loops are the hypervariable regions encoded by the V, D and J segments as described above. The difference in the variability between the interacting loops are reflected in regions of the MHC-I where these typically interact. While there are exceptions (125), the CDR 1 and 2 loops mainly interact mainly with the α-helices of the MHC-I and the CDR3 loops with the peptide, making the hypervariability of the CDR3 mirrored by the versatility of the peptide bound in the cleft of the MHC-I (122) (Figure 3).

Interestingly, immunogenic differences in the TCR-MHC-I interactions have been difficult to correlate with major structural changes in the interaction. While this remains a mystery, the complicated system that is the *in vivo* environment of the interaction provides many different additional factors to the equation. The MHC-I specific TCR expresses a co-stimulatory molecule known as CD8 (the equivalent is CD4 for MHC-II). This complex protein stretches next to the TCR and interacts with the α_3 domain of the MHC-I heavy chain (126). Besides the TCR and the CD8, the T-cell also express the CD3 complex, with co-stimulatory functions partly responsible for initiating the immune response. It is the phosphorylations of the CD3 complex which initialises the signalling cascade inside the T cell following the activation (114). These (the MHC-I, TCR, CD8 and CD3) form the centre of what is the immunological synapse. How exactly small changes in the MHC-I due to minor changes in the peptide (such as the case in paper III), can cause marked differences in the immune response is still not completely clear. Other factors that have been suggested for fine-tuning of the immune-response is clustering of co-stimulatory molecules and/or TCRs and/or MHC-I on the cells.

Perhaps the two main models for how the TCR interacts with the MHC-I can give further clues to the difficulties in finding structural correlations to immunogenicity. These are called the “standard model” and the “tritope model”. Simply, the standard model considers the MHC-I surface as one antigenic entity, similarly to an epitope of an antibody. The TCR surface would also be considered as one interacting surface that cause differential signals depending on the quality (length, occupancy, avidity etc) of the interaction. This model is most used by structural biologists and immunologists (127, 128). The tritope model considers the MHC-I surface as three different epitopes. The first one of these is the thymic antigen site, which is selected for during positive selection, and can be considered the recognition of the MHC-I as a molecule. The second is the allogeneic epitope, recognising the allele itself, and the cause for graft-vs-host immunity responses that occur in MHC-I allele mismatched transplants. The last epitope is the peptide epitope and the cause of the specificity of the TCR-pMHC-I interaction. The idea is that the CDR 1 and 2 loops interact with either of the first two epitopes and the CDR3 loop interacts with the peptide. The interactions would then cause a conformational change that would initiate the signalling cascade for T-cell activation. The tritope models’ main advocate is Professor Melvin Cohn of the Salk institute (127).

While both these models have advantages/disadvantages, the standard model could explain the lack of conformational changes seen in some pMHC-I complexes with different immunogenicity but identical structures by instead focusing on, for example, the interaction length or occupancy. This can be difficult to explain with the tritope model, which would indicate that the peptide only is recognised by the CDR3 loops and thereby would make little differences if the conformations would change. The interacting regions of the TCR as seen in figure 3 does however support the tritope model, with the CDR loops main interaction sites agreeing with the hypothesis (with that said, it does not disagree with the standard model). Cohn seems unwilling to recognise that reality might be a compromise between these models (127, 129), but as increasing numbers of studies of specific TCR-pMHC-I interaction are published the conclusions might lead in either direction.

3 NEOEPITOPES AND IMMUNE THERAPY POTENTIAL

3.1 VIRAL IMMUNE ESCAPE STRATEGIES

Evolution has caused viruses to utilize several mechanisms to escape immune detection by interfering with the MHC-I molecule or the antigen presentation pathway. Several independent strategies are present, and it is not uncommon for the same virus to utilize several different mechanisms simultaneously (130, 131). The US3 transmembrane protein from HCMV (Human Cytomegalovirus), for example, has been shown to inhibit the peptide editing function of tapasin and bind and detain MHC-I in the ER (132, 133). Herpesviruses, the family to which HCMV belongs, have evolved various manners to inhibit the antigen presentation to T-cells. Viruses in this family have been shown to shut down cellular synthesis of MHC-I, initiate degradation of produced MHC-I complex (134) and inhibit several proteins in the peptide processing pathway in using a variety of mechanisms (130). Inhibiting proteins in the antigen processing pathway is also a common strategy for other viruses such as papilloma viruses (135) and poxviruses (134) which are interfering with TAP function which is a highly used strategy for herpesviruses. These strategies are also hinting to cancer immune escape strategies. As cancers are pathogenic transformations of cells, the need for these to escape the immune surveillance is just as necessary as viral escape for survival and progression.

3.2 CANCER ASSOCIATED EPITOPES

Cancers cause and are caused by malignant transformations of the cells. In order for a cancer to progress, the transformation needs to go under the radar of T-cells and other immune cells. There are at least five different types of peptide antigens classified, which are collectively known as tumour-associated antigens (TAA). These are mutated antigens, cancer-germline gene encoded antigens, peptides derived from oncogenic viruses, differentiation antigens and tumour over-expressed antigens (136). The names are indicative of the processes in which these antigens appear. The mutated antigens appear because of mutations within the cancer cells. Depending on the mutation, the resulting neoantigen could be the result of either a frameshift, causing new amino acid sequences to occur, or a point mutation. Even if this is a promising source of cancer-specific antigens, the mutational variability in different patients and cancer-types complicated the development of therapeutics (136, 137). Cancer-germline encoded antigens stem from genes that are turned off by methylation in all cells except male germ cells (138). As such, they are supposedly highly tumour-specific, but recently this has been questioned due to the discovery of low level expression of the cancer-germline epitope MAGE-A12 on brain cells, with lethal consequences in immunotherapy trials (139). Peptides from oncogenic viruses are promising candidates to use for T-cell based immunotherapies (140). The problem with these though, is that these viruses, such as papilloma viruses, usually are equipped with immune evasion strategies to avoid detection as discussed above. Differentiation antigens are derived from proteins that are present in a very small number of tissues. The most studied antigens in this group are the peptides derived from melanin

production in melanocytes in the skin, often present in malignant melanoma (137). Targeting these antigens have proven to give rise to side effects such as vitiligo and melanocyte death in the eyes (141). Overexpressed antigens, derived from proteins that are upregulated in the cancer cell, have the most obvious drawback in that they are present on healthy cells to some amount. This leads to two problems; Quantifying the overexpression in comparison with normal cells and the possibility of an induced T-cell response targeting both pathogenic and benign cells. While each of these antigen groups presents different challenges, two are general. At the molecular level, to overcome tolerance without modifying the epitope conformation in compared to the wild-type can be problematic, since too radical modifications might not allow cross-reactivity with the wild-type complex. It is also well established that upon breaking tolerance to one of these antigens, there is a great risk that the antigen is present on another cell type in the body and thereby any response to the tumour will also directly target healthy cells (136, 137). Thus, an ideal TAA should be a true neoepitope.

3.3 NEOEPITOPES

3.3.1 Cancer neoepitopes

Neoepitopes, neoantigens or neopeptides are all names used for peptides that are uniquely presented on cells that have some form of unwanted transformation, such as a virus or cancer. Of the TAAs described above, mutated antigens and antigens from oncogenic viruses classify as neoepitopes. These are however not the only possibilities for neoepitope formation. Mutations in cancer can affect proteins without forming a new epitope and instead cause secondary effects such as modified PTM patterns or loss of function of important proteins in the peptide processing pathway, similarity to escape-strategies by viruses.

In the case of PTM's two recent examples of identified neoepitopes are promising. Malaker *et al*. recently characterised glycosylated neopeptides from various types of leukemia. They discovered that several identified peptides were present in more than one patient, even within different types of malignancies (142), providing promising possibilities for a broad treatment based on these glycosylations. Another study identified methylated peptides (98). A portion of the identified epitopes was derived from proteins associated with cancer, and some of the peptides were derived from proteins directly involved in aggressive breast cancer.

To ensure adequate CTL recognition of cancers and/or viruses, the PLC system needs to be fully functional. Immune evasion strategies may cause defects in the peptide processing pathway and down-regulation or eradication of the involved proteins can occur, resulting in failed clearance of malignant cells. Deficiencies in the antigen presenting pathway, *i.e.* TAP-deficiency, tapasin-deficiency etc, is common in poor prognosis and metastasis-prone cancers (143). However, certain deficiencies in the antigen processing machinery have been shown to instead cause the formation of a specific kind of neoepitopes that show great promise for therapeutic use (144).

3.3.2 T-cell epitopes associated with impaired peptide processing

The existence of T-cells that react efficiently towards TAP-deficient cells was established in 1991 (145), when Aosai *et al* identified TCR clones that elicited CD8-dependent responses against both murine lymphoma cell line RMA and the TAP-deficient clone RMA-S. At the time, the authors concluded that there must be T-cell clones specific for peptide-free MHC-I, since the RMA-S clone, due to its TAP-deficiency, was suggested to be devoid of the pMHC-I complexes (146). In 1997, Wolpert *et al* (147) studied the immune response against RMA-S cells in the C57BL/6 mouse model. While these cells escaped immune recognition, the RMA-S.B7 transfectant induced responses against RMA-S targets. B7, or CD80, is a co-stimulatory T-cell activating signal molecule. These responses needed MHC-I expression and TAP-deficiency, but not until a study in 1999 was it evident that the "empty" MHC-I molecules on the RMA-S cells were occupied by low affinity peptides (148).The nature and identity of some candidates within this processing-impaired-associated peptide repertoire were not elucidated until 2006 in a breakthrough study performed by van Hall et al (149). These T-cell epitopes associated with impaired peptide processing (TEIPP) are true neoantigens that are efficiently recognized by specific T-cells, and are only presented on TAP-, tapasin- and/or proteasome-defect cells, and not on corresponding normal cells. The first identified TEIPP peptide was derived from a ceramide synthetase called Trh4, which also became the name of the peptide. Interestingly, this peptide is derived from a minor splicing product of the Trh4 gene which causes a frame shift and early stop-codon. The expressed peptide sequence is localized at the C-terminus of the Trh4 protein, which protrudes into the ER where it is cut by a signal peptide peptidase, thus a product of the less common alternate peptide processing pathways (149-151). This epitope and its binding to the murine MHC-I allele H-2D^b is studied in papers II and III. TEIPP epitopes are promising new targets for immune-based cancer therapies (143, 144). Importantly these epitopes do not participate in the thymic tolerization process. In TAP-deficient mice, the thymus selection process deletes T-cells that are specific to MHC molecules in complex with self-peptides, leading to tolerance. In TAP-proficient thymoid cells, these TEIPP-specific T-cells were indeed selected, meaning that they can clearly be used in immunotherapies (143).

3.3.3 Peptide processing in deficient cells

TAP-deficient cells were initially suggested to be void of any peptide-filled MHC-I on the surface of the cells, and hence was termed "empty" (146). It was soon discovered this was not the case and that these MHC-I complexes instead were associated with low affinity peptides (148, 152, 153). A common cause for CTL evasion is often the loss of TAP function, found in a variety of tumour cells (154) such as the RMA-s cells initially studied (146). As the peptide flow into the ER is significantly impaired, the levels of pMHC-I complexes on the surface of these cells are often reduced to less than 10 % of the normal levels (146, 155, 156). If TAP is not the source of peptides in these cells, where do they come from? Preliminary suggestions for the potential origin of these epitopes were signal peptides or TAP-independent, unidentified mechanisms of import (148, 152, 157). It is however now well-established that these peptides are derived from ubiquitously expressed house-keeping

proteins and represent a truly novel repertoire of neoantigens that appear only on antigen-processing-impaired cells (149, 150). It should also be noted that following functional knock-down of TAP, a large portion of the presented peptides are still processed by the proteasome, suggesting alternative routes for processing that involve endosomal and vesicular compartments. Many of these neoantigens also result from degradation by metalloproteases, known as endoproteases, while a relatively small number of neoantigens derive from previously known proteases in the ER and Golgi, such as furins and signal peptide peptidases (150). Until very recently, it was believed that the only protease capable of cutting the peptides C-terminally was the proteasome. This view has been challenged, and one example is a study by Kessler *et al*. (158) that shows the need of nardilysin and thimet oligopeptidase in the C-terminal trimming of the PRAME tumour antigen. Although these results provided new information on C-terminal trimming, a knock-down of the pathway gave little effect on MHC-I levels, indicating that this pathway is not alone responsible for proteasome deficient peptide production (158, 159). In a study of HLA-A2 and HLA-B51 associated peptides in TAP-deficient cells (160), 75% of the identified peptides were derived from signal sequences. Interestingly, many of these did not have the C-terminal sequence of the signal peptide, indicating further C-terminal trimming. In the same study, it was concluded that the presentation of the non-signal-sequence peptides mainly were inhibited by using proteasome inhibitors (160). There is a possibility that proteasome independent protease-processing has a different influence over different cell types. The knock-out of the abundant PSA peptidase caused a 25% increase of surface MHC-I on DCs but had no effect on B or T lymphocytes or fibroblasts (161). There are indications that endolysosomal uptake and transport of the peptides are involved in TAP-independent pathways, as blocking of endolysosomal acidification in TAP-deficient cells increased the surface expression of a TEIPP epitope (150).

Oliveira *et al* showed that inhibition of the proteasome decreases levels of H-2K^b and H-2D^b by between 40 and 55%. While they claim that their results demonstrate that most TAP-deficient epitopes derive from the proteasome (150), it should be interpreted with caution. The inhibitor used in the study, epoxomicin, is an inhibitor of the chymotrypsin-like activity (159). While the MHC-I level decrease illustrates reduction, its definite level can be misleading since the specificities of the H-2K^b and H-2D^b alleles both favour hydrophobic amino acids (12) in the C-terminal. As such, the reduction of MHC-I levels might have been less if the trypsin or caspase activity was inhibited, and the absolute level of peptides derived from the proteasome might be larger than the numbers indicate for these alleles. Proteasome independence is difficult to study. Commonly the methods of study have included the use of different proteasome inhibitors. These might give unexpected effects, as described in an excellent review by van Endert (159), inhibition of only parts of the proteasome function can cause a shift in proteasome efficiency causing an apparent independence. Also, inhibition can cause epitopes to appear that would otherwise have been destroyed by proteasome activity, making them appear to have been cut by other proteases (159). However, the peptides appearing in these cases, that would not appear with normal proteasome function, can be

thought of as neoepitopes and can be used to illustrate the changes in a proteasome with naturally occurring deficiencies.

3.4 ALTERED PEPTIDE LIGANDS

The first-time usage of the phrase “altered peptide ligand” (APL) as a term for mutated peptides was in 1993 by Evavold, Sloan-Lancaster and Allen. They suggested that the definition would be “analogues of immunogenic peptides in which the TCR contact residues have been mutated” (162). In time, the term also came to include any single modification of a MHC-I binding peptide used to influence the immunogenicity. In designing therapeutic treatments, this strategy has been very popular. The common aim is to break T-cell tolerance of a cancer- or virus-associated epitope.

APLs have been utilised frequently through the years. Commonly, the strategy has been to increase the affinity or stability of the pMHC-I complex (163-165). Harndahl claims in a study from 2012 that stability is a better predictor than affinity for prediction of immunogenicity (164, 165). This would logically make sense, since higher pMHC-I stability would lead to the pMHC-I being present longer on the cell-surface, hence giving the TCR more time to recognise it. While this strategy has worked beautifully (166-168) it is not always true. Examples of APLs that have increased affinity or stability but still not being recognised have been proposed and alternative rules suggested (169, 170). In paper III of this thesis we describe an APL with significantly increased immunogenicity, but lower stabilisation capacity (171). There have also been descriptions of large structural shifts induced by these APLs, potentially causing the altered immunogenicity, but equally so, there are examples where the conformations of TCR interacting residues remain essentially unchanged. Conclusively, even with all the studies in the area, the exact biophysical bases behind increased T-cell recognition remains a mystery to be further studied.

AIMS OF THIS THESIS

To increase the understanding of the molecular mechanisms behind peptide editing by tapasin in the peptide loading complex.

To understand the unconventional H-2D^b-restricted TEIPP epitope Trh4 at a molecular level and apply the p3P mutational approach on an Trh4 APL to increase its immunogenicity.

To investigate the contradictory effect of glycosylations on two different viral epitopes restricted to H-2D^b.

4 RESULTS AND DISCUSSION

4.1 PAPER I – SUCCESSIVE CRYSTAL STRUCTURE SNAPSHOTS REVEAL THE BASIS FOR MHC CLASS I PEPTIDE BINDING AND EDITING

This paper starts with the crystal structure of H-2K^b in complex with only a dipeptide, GL. This peptide was previously shown to stabilise the complex and promote peptide exchange of MHC-I (172, 173). The structure of H-2K^b/GL was determined using X-ray crystallography to a resolution of 1.8Å (**Paper I, S.Tab. 1**). The asymmetric unit of the crystal contained two complexes, both with the GL peptide bound to the F-pocket of the PBC. The rest of the cleft is open and contains a few waters (**Paper I, Fig. 1A and 1B**). Interestingly, the α-helices lining the PBC take the same configuration when binding GL as when binding the canonical high affinity binding peptide SEV (FAPGNYPAL) (174). Previously to this structure, the shortest peptide a MHC-I has been structurally characterised in complex with is the SEV C-terminal pentamer NYPAL (175). The NYPAL epitope was in complex with the H-2D^b allele and while the C-terminal leucine did act as an anchor, the N-terminal asparagine, did not fulfil its canonical role as an anchor. While surprising, this reveals the importance of the N-terminal to induce the observed conformation of the full-length SEV peptide. The 2.3Å structure in paper I of the H-2D^b allele in complex with the N-terminal seven residues of SEV (FP7, FAPGNYP) and the dipeptide GL (**Paper I, Fig. 2**) reveals the asparagine to be lodged in the C-pocket, in a similar conformation as in the wt H-2D^b/SEV peptide structure (95). This further illustrates the influence of the N-terminal part of the peptide in the binding of the arginine anchor.

The GL dipeptide is not the best of the peptide editing dipeptides described in the works by Saini *et al*, who describes the dipeptide GM to be more efficient in their studies (172, 173). The GM dipeptide we did not consider as interesting though since our literature review in at the start of this study revealed the leucine of the GL to be present in a very interesting mobile loop of the tapasin molecule (73). Docking the tapasin structure determined by Dong *et al*, with an MHC-I according to known interaction surfaces revealed this interesting loop to be within reach for binding into the MHC-I PBC (73, 176). To study this, we used a portion of this loop, EGAGGGGLSK ("TL10"), for refolding with the H-2D$_b$ HC and β_2m. While this first attempt yielded protein, crystallisation trails were unsuccessful, indicating an unstable complex. Adding the FP7 peptide to the mix was more successful and a structure was determined to 2.15Å (**Paper I, S.Tab. 1**).

The structure of the H-2D^b with FP7 and TL10 has four complexes in the asymmetric unit. Of these, all contain the FP7 peptide and 2 contain a density which can be modelled as a part of the TL10 peptide. The remaining two complexes are very close to symmetry related molecules, causing the F-pocket to be capped of and the TL10 loop cannot fit. In the F-pocket there is still a density, although the identity of the presence here is not known. It does not fit a glycerol (used as cryoprotection for the crystals) and it is too large to contain waters. Possibly, the crystal has a mixed occupancy in these locations making the identity difficult to elucidate. The two complexes where there is density to model parts of the peptide, reveal that

most of the TL10 peptide is mobile and cannot be modelled. This can be due to a sterical hindrance from the C-terminal of the FP7 peptide, but more likely it is due to the mobility as caused by the glycine residues. In modelling the TL10 peptide, making sure that we are satisfied it is the leucine of TL10 binding to the F-pocket was a priority. The other possibilities were the N-terminal glutamate or aspartate, the C-terminal lysine or parts of the FP7 peptide. The F-pocket of H-2D^b is hydrophobic in nature, and the anchor positions as described in table 1 of this thesis, reveal that many of the charged residues are deleterious for a peptide at the F-pocket binding C-terminal position. Accordingly, neither of the TL10 residues in the N- or C-terminal were advantageous when modelled in the F-pocket of the structure, as compared to the modelling of the leucine. Attempting to model the FP7 in the F-pocket was futile, not only due to worsened statistics but also due to sterical clashes with the other FP7, which had a clear density.

To determine the influence of this loop for peptide loading into the MHC-I, our collaborators in the Springer laboratory at Jacobs university in Bremen used a cell-based assay. They used tapasin deficient mouse ear fibroblasts transduced with different mutants of the tapasin gene. For each mutant, the levels of H-2D^b and H2K^b was measured on the cell surface by flow cytometry (**Paper I, Fig. 4**). For H-2K^b, mutating the leucine of TL10 to alanine reduced the surface expression with 50%, while the levels were not affected for H-2D^b. Other than that, the levels of MHC-I expression were not affected if the leucine was present when making more drastic reductions of the loop size. Not until a total of six amino acids were removed, including the leucine, the surface expression was significantly altered. While the effect of larger deletions could be due to the loss of structural integrity to the tapasin protein, these results further support the importance of leucine in the peptide loading. It also suggests that the tapasin affects the alleles differently. For human alleles, variations in tapasin dependence has been shown (177-179). For murine alleles, the tapasin dependence has been studied in a human cell-line expressing human tapasin. In this case, the alleles showed equal tapasin dependence. (180). This study was however done in the presence or complete removal of tapasin (180), whereas the studies on human MHC-I allele dependence was done studying single polymorphisms in the MHC-I PBC (177-179). As such, the effect of single mutations on peptide loading of tapasin in the murine setting is not elucidated and further studies of the effect of point mutations have the potential to reveal more details on the tapasin peptide editing mechanism.

Based on the results in this study and the results in recent studies of TAPBPR and the PLC, as discussed in section 2 earlier in this thesis, figure 5 in Paper I describes a model of peptide selection assisted by tapasin. In this model the tapasin keeps the MHC-I in an open peptide accessible position by binding to the F-pocket with the TL10 loop (*aka* scoop loop after the naming of Thomas and Tampé (68)) and the jack hairpin underneath the PBC. The N-terminal part of the tapasin binds into the PBC and if the affinity is good enough it competes out the tapasin and the stable MHC-I complex is formed.

4.2 PAPER II – THE MHC CLASS I CANCER-ASSOCIATED NEOEPITOPE TRH4 LINKED WITH IMPAIRED PEPTIDE PROCESSING INDUCES A UNIQUE NONCANONICAL TCR CONFORMER

This paper concerns the first identified TEIPP peptide Trh4 (149). This peptide has an unconventional sequence for a H-2D^b binding epitope (see table 1 above and (12)). The sequence (MCLRMTAVM) contains four sulphur-containing residues and two anchor positions are unconventional. Position 2, a cysteine, is usually deleterious in this position according to the statistics of iedb.org and the extremely dominant anchor in position 5 is a tolerated methionine instead of a conventional asparagine. We determined the crystal structure of the H-2D^b/Trh4 complex to 2.25Å (**Paper II, S.Tab. I**). The anchor positions are utilised as for a canonical peptide, meaning that p2C and p5M are still interacting with the B- and C-pockets (**Paper II, Fig. 1A**). The peptide binds to the PBC utilising several sulphur-π interactions (**Paper II, Fig. 1B**). Sulphur-π interactions are a group of interactions between the sulphurs in methionine or cysteine and an aromatic ring. The optimal interaction distance is 3.6-4.3Å with an angle of 30-90° from the diagonal of the plane of the aromatic ring (181-183).

In studying the effect of the sulphurs in Trh4, derivates of cysteine and methionine were used. These were α-aminobutyric acid (ABU), a cysteine where the sulphur is exchanged for -CH_3 (methyl), and norleucine (NLE), where the sulphur of methionine is exchanged for a -CH2 methyl group. These unconventional residues have the same overall size as the natural amino acids, thereby any differences should not be due to sterical differences. We used two methods to determine the stability of H-2D^b in complex with these epitopes. The first one (**Paper II, Fig. 3A and 3B**) were established in the beginning of the 1990's and makes use of the TAP deficient RMA-S cell-line (146). The method in based on the fact that the TAP-deficient cell-line, when grown in 26°C can be loaded with exogenously added peptides. In this assay, the peptide of interest is added to the RMA-S culture for 12 h after which the peptide is washed away, and the cells are incubated in 37°C for 1 hour in medium containing the Golgi transport inhibitor Brefeldin A. The cells are then sampled as certain time points after this, stained with antibodies against the H-2D^b on the surface and the expression is quantified using flow cytometry. Since the golgi transport is inhibited, the H-2D^b levels on the surface will be reduced in a manner related to the stability of the pMHC complex. From this the time it takes or the expression levels to reduce to 50% of the initial levels can be read as a stability measurement (171, 184). The second method is Circular Dichroism (CD). While the cell-surface stability measurement is a cell biology-based method, CD is a strictly biophysical method. The basis of CD is the angle of the peptide bond in the protein, which when in a circularly polarised beam of light will absorb certain angles differently for a single wavelength. Changing the wavelength will give a spectrum which is characteristic depending on the amount of α-helix and β-sheet content in the protein. When heating a protein sample, this spectrum will change as the secondary structural elements of the protein denaturates. As such, we can follow the denaturation and find a melting temperature by measuring in set intervals as the temperature of the sample is slowly increased and calculate a melting

temperature (185). These two methods have their own advantages and disadvantages. The most positive aspect of the cell-based assay, is that the measurement is performed in a more native setting. The MHC-I molecule has the natural glycosylation patterns and the surroundings that are normal on the surface of the cell. The most positive side of the CD-measurement is the controlled setting; the exact concentration, the purity and the buffer is known beyond any doubt. In the CD measurement, however, the protein is recombinantly made, meaning that any interaction in the natural environment of the cell surface is lost. But on the other hand, the cell-assay is subject to potential confounders outside the control of the experimentalist, since health of a cell-culture, exact composition of the growth media etc, are more difficult to control than a recombinant protein in solution.

The results of these assays were more pronounced in the cell-based assay than in the CD-measurements, but both assays indicated that mutated versions where the sulphur-π interactions were abrogated induce a less stable H-2D^b/peptide complex (**Paper II, Fig. 3**). Determination of the crystal structures of H-2D^b/Trh4-p2ABU and H-2D^b/Trh4-NLE (**Paper II, Fig. 4, S.Tab I and S.Tab 2**) revealed the structures of both complexes to absolutely similar to the H-2D^b/Trh4, ruling out changes in the protein structure as a reason for the observed stability differences.

Paper II, figure 5 illustrates the results in an IFN-γ release assay where peptide-loaded mouse splenocytes have been incubated with the H-2D^b/Trh4-specific T-cell LnB5. This assay is a measurement of the immunogenicity of a specific APL. Generally, mutation of methionine to norleucine does not affect the immunogenicity of the complex, unless all of them are simultaneously mutated which causes a small increase in immunogenicity (**Paper II, Fig. 5A**). Similarly, the sulphur-π interaction of peptide position 2 with the MHC heavy chain has only a small effect on immunogenicity (**Paper II, Fig 5B**). Both these effects are marginal in comparison with other APLs tested. An alanine mutation APL scan of the Trh4 peptide shows large differences in the immunogenicity of the peptides. Together with the structure of H-2D^b/Trh4 we can assess which of these are due to abrogation of direct interactions between the pMHC-I and the TCR of the LnB5 T-cells. The structure reveals that positions p2C, p3L, p5M and p9M are buried in the PBC, while the remaining residues are protruding towards the solvent. As such, we can conclude from this experiment that p5L, p6T and p8V are essential to LnB5 interactions, while the loss of the side chains of p3L, p5M and to some extent p9M are essential to the integrity of the complex (**Paper II, Fig. 5C**).

An essential observation in the paper is that the mutation of p5M to the canonical anchor asparagine is deleterious to the immunogenicity of the epitope. This is evident in figure 5D, where the IFN-γ levels are completely nulled by the p5N APL of Trh4. This is likely not due to the peptide not binding to the H-2D^b on the cell surface, since this peptide is tested in the cell-based stability assay and exhibits similar stability to the canonical dominant gp33 peptide from LCMV (**Paper II, Fig. 3A**). However, in figure 2 of this paper, the structural comparison of H-2D^b/Trh4 and H-2D^b/gp33, where the C_α-atoms of the HC of the α_1 and α_2

domains are overlaid, revealed that the only difference in the atoms of the HC is a shift in the H155 and T156 residues, which are essential to the TCR recognition.

4.3 PAPER III – THE IMMUNOGENICITY OF A PROLINE-SUBSTITUTED ALTERED PEPTIDE LIGAND TOWARD THE CANCER-ASSOCIATED TEIPP NEOEPITOPE TRH4 IS UNRELATED TO COMPLEX STABILITY

This paper also concerns the Trh4 TEIPP epitope studied in paper II. Here, however, the p3P APL strategy for increasing immunity of the epitope is utilised. This strategy was first introduced in a 2009 paper by van Stipdonk *et al*, where it was demonstrated to increase the immunogenicity of the gp100 epitope from malignant melanoma (166). The first observation in this paper is that the Trh4p3P APL peptide indeed is significantly more immunogenic than the Trh4 wt peptide (**Paper III, Fig. 1**). Mice that were injected with LnB5 T-cells (Trh4 specific) and vaccinated with the Trh4p3P epitope showed a remarkable expansion of these T-cells, and at the maximum response 80% of the T-cells present in the mouse were LnB5 T-cells. Vaccination with the Trh4 wt epitope gave a maximum expansion to 5% on the total T-cells (**Paper III, Fig. 1C**). The Trh4p3P vaccinated mice also showed higher specific killing of Trh4 wt loaded cells in an *in-vivo* killing assay (**Paper 3, Fig. 1F**).

Commonly when working with APLs the aim is to increase the pMHC-I complex stability (164, 165). However, this is not the case with the Trh4p3P peptide. The H-2D^b/Trh4p3P was determined to be significantly less stable than the H-2D^b/Trh4 in a cell-surface stability assay preformed as described for paper II. In this case, the CD-based stability assay showed a reduction in stability, but even if this reduction was very small at 1.65 °C, the change was statistically significant. Not only is the reduced stability of the H-2D^b/Trh4p3P interesting from an immunological viewpoint, but it is also interesting to note such a big difference in the results of the two methods. There is also a secondary difference in these methods that are not discussed previously for paper II. While both assays are measuring stability, the cell-based assay is measuring the spontaneous collapse and internalisation of the pMHC-I under stable conditions and the CD-based assay are measuring the collapse of the pMHC-I complex under the stress of raised temperature. The timespan of the experiments is also very different. An interesting way to determine a correlation between these two methods would be to measure the denaturation of the protein complexes by CD with a steady temperature equivalent to the cell-based assay, *i.e.* 37°C. This could be the reason for the differences observed in this paper. Determining the structure of the H-2D^b/Trh4p3P complex revealed that the mutation from leucine to proline in position 3 leads to a conformational change in the methionine at position 5. This conformation change leads the distance between the aromatic ring in HC residue Y166 and the sulphur in the methionine to become too large for efficient interaction (181-183). The structures also reveal that the surfaces of both the complexes are very similar. Unfortunately, there is not much evidence in the structures as to why these two complexes have different immunogenicity.

4.4 PAPER IV – CRYSTAL STRUCTURES OF H-2D^b IN COMPLEX WITH THE LCMV-DERIVED PEPTIDES GP92 AND GP392 EXPLAIN PLEIOTROPIC EFFECTS OF GLYCOSYLATION ON ANTIGEN PRESENTATION AND IMMUNOGENICITY

Two H-2D^b restricted epitopes from the Lymphocytic choriomeningitis virus (LCMV) were identified in 1998, GP92 (CSANNSHHYI) and GP392 (WLVTNGSYL) (186). Both contain a N-glycosylation site (Asn-X-Ser/Thr, X ≠ Pro) and which are glycosylated in the native protein (187). The glycans have opposite effects on GP92 and GP392 H-2D^b epitopes. The sub-dominant peptide GP92 and its modified forms GlcNAc-GP92 and the deglycosylated variant D-GP92 are all immunogenic (188). GP392 is immunogenic only in the unmodified form, meaning that in vaccination attempts with the peptides and its modified versions, T-cell could not be raised against GlcNAc-GP392 or D-GP392 (189).

The crystal structures of H-2D^b in complex with the unmodified versions GP92 and GP392 were determined to 2.4Å and 2.5Å respectively (**Paper IV, Tab. 1**). Both bind with the canonical asparagine as an anchor (12) and the N-terminal anchors are of the preferred spices (leucine and isoleucine). For GP92 the glycosylated residue protrudes from the surface (**Paper IV, Fig. 4**), while for the GP392, the glycosylated asparagine is lodged as the anchor in the C-pocket (**Paper IV, Fig. 5**). This does not make it impossible for the peptide to still bind as previously demonstrated for another epitope in a publication by Glithero *et al* (95). However, since this peptide is not immunogenic enough for T-cells to be raised against it, and show no binding to H-2D^b (189), it is unlikely that this possible conformation is preferred (**Paper IV, Fig. 5C**).

Another notable discussion in this paper provides insights to the possibilities and problems in the presentations of glycosylated peptides as a neoepitopes. The GlcNAc-GP92 is not presented on LCMV-infected cells (188), even though it is likely to be the major species of the protein (187). Instead, GP92 is detected, meaning that either there is a significant amount of unglycosylated protein, or providing further evidence for the DRiP hypothesis. The fact that GlcNAc-GP92 is not presented could be due to deglycosylation prior to proteasomal cleavage. This illustrates the unpredictability of glycopeptide processing and presentation.

5 FINAL REMARKS

Additional tiny pieces of the grand puzzle that is biology are added through this thesis. The mechanism of peptide editing of tapasin is far from determined, and as our results in paper I demonstrate, it is not certain that the mechanism is exactly similar for different MHC-I alleles. As tapasin deficiency is a potential immune escape mechanism for cancers, different alleles might be differently affected by a potential mutation. The formation of potentially therapy-exploitable TEIPP epitopes can potentially vary through the population. However, as shown in paper II and III, we can still be surprised by new discoveries that are not following conventional ideas of peptide motifs and immunogenicity. I believe that proceeding to study peptide selection mechanisms and unexpected peptides, thus expanding little by little the knowledgebase, will eventually lead to the discovery of the next successful therapy.

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