Alternative HLA class-I peptide presentation in processing deficient tumors

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Introduction

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A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens

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1. CD8 T-cell immunity

1.1 CD8⁺ T cells

In this thesis we study CD8⁺ T cells. This subset of T cells recognizes and kills host cells, which are infected or that display changes related to transformation. They are also referred to as cytotoxic T-lymphocytes (CTLs). The T-cell receptor (TCR) of a CTL recognizes a small peptide fragment that is displayed in molecules called the class-I major histocompatibility complex (MHC-I), which are very specific and present in every nucleated cell. To recognize the peptide fragment presented in a MHC-I molecule, each TCR has to recognize the residues of the peptides presented, and to recognize residues of the MHC-molecule, which is displaying the peptide.

T cells originate in the thymus where they undergo selection before they migrate to the lymph nodes and circulate throughout the body. Selection in the thymus occurs via two subsequent processes called positive- and negative selection. During positive selection T cells are considered for their ability to recognize MHC-I molecules. If the TCR of the T cell recognizes a self-MHC-I molecule it is rescued from death by neglect. The negative selection occurs through deleting immature T cells, with receptors interacting to strong for self MHC-I complexes. This negative selection prevents contribution to auto-immunity and induces self-tolerance (1).

Once the T cells have survived both positive- and negative selection they migrate towards the lymph nodes where they resides or circulate throughout the body until they become activated.

1.2 The MHC-I molecule

The MHC-I molecule is expressed by virtually all nucleated somatic cells and consists out of two parts: β_2-microglobulin (β_2m), and the heavy chain. The heavy chain is further divided into three regions: α1, α2, and α3. The α3 region is conserved and contains the binding site for the T-cell co-receptor CD8. The amino-terminus of α1 and α2 form the peptide-binding groove. This groove can bind to peptides which are generally 8-10 aminoacids (aa) in length, but bulging in the middle is possible and therefore sometimes longer peptides are found (2). Peptides bind into the binding groove through so called anchor residues, which are favorable amino acids at a certain position within the presented peptide. Polymorphic residues within the α1 and α2 region cause differences in MHC-I molecules. Some of these residues contribute to variantion in the peptide-binding groove, causing differences in the preference of the type of amino acid and position for the anchor residues. This polymorphism contributes to the large variety of MHC-I molecules found in the human population, which in its turn cause differences in the ability of humans to activate their immune system upon infection or outgrowth of tumors.

MHC-I molecules are divided in two categories: the classical MHC-I molecule (MHC-Ia), and the non-classical MHC-I molecule (MHC-Ib). Both molecules are similar in the responses that they can induce. However, they are not familial: MHC-Ia molecules are extremely polymorphic whereas MHC-Ib molecules have few alleles which are highly homologous between individuals (3, 4). The limited diversity of antigens which are presented in MHC-Ib molecules places distinct constraints on their interactions and their role in T-cell biology (4).

In humans we refer to MHC-I molecules as Human Leukocyte Antigens-I (HLA-I). The classical MHC-Ia molecules in human are referred to as HLA-A, -B, and -C, and MHC-Ib molecules are referred to as HLA-E, -F, -G, MIC and CD1. In this thesis we will only focus on the classical HLA-I molecules and the non-classical molecule HLA-E.

2. Antigen processing

MHC-I molecules present peptides at the cell-surface were they can be recognized by CTLs. Peptides presented in MHC-I, which are recognized by CTLs, are referred to as epitopes. Upon recognition of an epitope the CTL becomes activated and efficiently kills the cell. The process by which the peptides and possible epitopes are generated, and presented in MHC-I molecules, is called antigen processing. There are numerous pathways and enzymes involved, which generates and facilitates the peptides for presentation in MHC-I molecules.

2.1 Conventional antigen processing.

Peptides which are presented in the MHC-I molecules are mostly acquired in the Endoplasmic Reticulum (ER) and are derived from endogenous origin (2). The most-defined pathway involves both the proteasome and the transporter molecule TAP. Upon entering the cytoplasm of the cell, proteins are cleaved by the proteasome, which degrades the protein into short peptide sequences. A majority of the short peptide sequences are then able to enter the ER via the Transporter associated with Antigen Processing (TAP) (figure 1a). Once in the ER, peptides which have a correct binding motive are loaded into the MHC-I molecule with help of the Peptide Loading Complex (PLC), including tapasin, calreticulin, and the thiol oxidoreductase ERp57 (2, 5, 6). The MHC-Ia molecule containing the peptide is then transported through the Golgi apparatus, towards the cell surface where it presents itself to CD8⁺ T cells (figure 1).

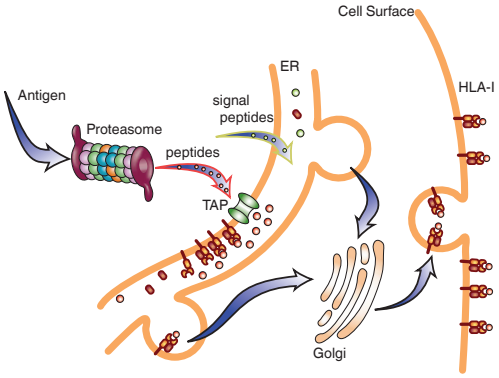

Figure 1: The classical HLA-I presentation pathway. Peptides derived from endogenous origin are transported through the HLA-I presentation pathway. The endogenously derived peptides are degraded by the proteasome and enter the endoplasmic reticulum through the TAP transporter after which they are loaded into HLA-Ia molecules. The HLA-I molecules are then transported through the Golgi towards the cell-surface where the can be recognized by CD8+ T-cells.

2.2 Enzymes involved in antigen processing

The proteasome is the primary source for generating peptides, which can be loaded into the MHC-I molecule. However, there are studies, showing the involvement of other enzymes able to generate peptides in the cytosol. One enzyme that was described to be generating pathogen-derived peptides in the cytosol in a proteasome-independent fashion is Tripeptidyl peptidase II (TPPII) (7). This enzyme generates a HIV-Nef epitope, which is presented in both HLA-A3 and HLA-A11 molecules (7). Another enzyme is Thimet oligopeptidase (TOP) is a soluble thiol-sensitive metallopeptidase that cleaves internal bonds in peptides of 6-17 aa (8). The role of TOP is debated. TOP may play a protective role for antigenic peptides (9, 10). However, another study described that TOP actually degrades class I ligands efficiently (11), suggesting that TOP plays a role in limiting peptide availability for antigen loading (11). Regardless the function of TOP the preference for binding and trimming of peptides indicates an important role for TOP in antigen processing (8).

Recently other proteolytic enzymes like caspases 5 and -10 (12, 13), Nardilysin (14) and insulin degrading enzyme (15) are implicated to play a role in proteasome-independent antigen processing.

2.3 TAP-independent presentation pathways

Although the majority of the peptides/epitopes are processed via the conventional pathway there are several processing pathways, which are independent of TAP for peptide loading of MHC-I molecules. In this paragraph three possible TAP-independent pathways will be described: the pathway involving signal peptides, the furin mediated pathway, and the liberation of peptides from the C-terminus of ER resident proteins.

2.3.1 Signal peptides

Perhaps the most well studied TAP-independent pathway is the one that involves the enzymes Signal Peptide Peptidase (SPPase) and Signal Peptidase (SPase). Their role in the MHC-I antigen processing is just one example of how peptides are able to enter the ER without the use of TAP. Newly synthesized proteins can contain a signal sequence, which plays a key role in targeting the protein from the cytosol towards the ER-membrane (16). Once the proteins are inserted in the membrane the signal sequences can be cleaved from the precursor protein by the membrane-bound SPase. The signal peptide is then further processed by SPPase (16).

The cleavage of SPase is dependent on particular amino acids, which are present on position -1 and -3 N-terminal of the cleavage site. An amino acid with a short side chain at the -1 position, and no charged amino acids at the -3 position favor SPase cleavage. Also the length and properties of other regions in the signal sequence can influence the cleavage of SPase. Once the signal sequence is cleaved from their precursor sequence further cleavage occurs by the less well defined SPPase (16).

Peptides that after cleavage of SPase and SPPase are inside the lumen of the ER, may contain epitopes which after further processing can bind to MHC-I molecules, implicating that these epitopes are processed in a TAP-independent fashion. Two examples are a peptide derived from the interferon-inducible protein IP30 and an epitope derived from calreticulin (16, 17). Recently another epitope derived from a signal sequence was described. This epitope, derived from the signal sequence of preprocalcitonin, was a result of overexpression of the *CALCA* gene in lung carcinoma, indicating a possible role for epitopes derived from signal sequences in cancer immunotherapy (18).

2.3.2 Proprotein convertases: furin mediated pathway

The proprotein convertases are part of a family consisting out of six classes of proteases. There are nine proprotein convertases and they play a role in protein processing by converting an inactive precursor to its mature form, and have a role in peptide precursors trafficking through the secretory pathway. Four of the convertases are type-I membrane-bound proteases, which are: furin, PC7, the isoform PC5/6B, and SKI-1.

Other members are either packaged into dense core granules (PC1/3, PC2, PC5/6A) and/or constitutively secreted into the extracellular milieu (PC4, PC5/6A, PACE4 and PCSK9) (19).

It is described that furin has an essential role in catalysing the maturation of a diverse collection of proprotein substrates (20-23).

Proprotein convertases can also contribute to the generation of antigenic peptides, and the role of furin in this process has been studied.

Peptides generated in the endosomes are transported into the Golgi network. In the Golgi furin can cleave these peptides and trim them at their C-terminus, making them available for loading into the MHC-I molecules. Furin is ubiquitously expressed in all vertebrates and many invertebrate. It cleaves –Arg–X–Lys/Arg–Arg↓– (Arg = Arginine, Lys = Lysine, ↓ = the cleavage site, and X = any amino-acid). The Arginines at position one and four are essential whereas the Lysine or Arginine at position three is not. Therefore -Arg-X-X-Arg↓- represents the minimal furin cleavage site and in exceptional cases even -Lys/Arg-X-X-X-Lys/Arg-Arg↓- can be cleaved by furin when favorable residues at position 2 and 6 can compensate for less favorable ones at position four (24). After peptides are generated through furin cleavage, it remains unclear how these peptides are loaded into the MHC-I molecule. MHC-I molecules normally travel to the cell-surface in their peptide-filled conformation and peptides generated in the Golgi network by furin have to be either transported back to the ER, or have to bind to empty MHC-I molecules en- route to the cell-surface (20).

2.3.3 Liberation of peptides from the C-terminus of ER resident proteins

Some proteins are upon translation directed towards the ER and fold itself in the ER-membrane. One example of such a protein is Jaw-1. Jaw-1 lacks an NH_2-terminal signal sequence and is inserted in the membrane post-translationally by a hydrophobic transmembrane region at residues 480-503. It contains a 35-residue luminal tail that can be liberated by non-proteasomal proteases in a TAP-independent manner. Further trimming occurred by carboxypeptidases or endoproteases, located inside the ER (25, 26).

2.4 Cross-presentation

Dendritic cells (DCs) play an important role in the initiation of the immune response and they initiate the clonal expansion of antigen specific T-cells. In order to have successful $CD4^+$ and $CD8^+$ T-cell priming against exogenously acquired antigens, DCs have a unique feature called cross-presentation. This leads to the presentation of exogenously derived antigens, which are mostly designated towards MHC-II molecules, in MHC-I molecules. Currently two pathways for cross-presentation are proposed which are the cytolic- and the vacuolar route. The cytolic route is a TAP-dependent process in which internalized antigens are released into the cytosol, which can then be transported to the ER by the TAP transporter and loaded into MHC-I molecules.

In the vacuolar route, antigens remain in the endocytic compartments, where they can be cleaved by proteases before being transported into MHC-I molecules. This process is independent of TAP and its underlying mechanisms are still unclear (2, 27). One question that remains is how the MHC-I molecule is transported to the endocytic compartment. A possible candidate might be the invariant chain (Ii), a chaperone molecule known for its role in the MHC-II peptide presentation pathway.

Several studies show a formation of Ii/MHC-I complexes in B-LCLs (28-30). Increasing the Ii expression through transfection also influences the MHC-I surface expression levels (31). More importantly upon Ii/MHC-I complex formation, transport towards the endocytic compartment is seen (29). The role for the Ii in the cross-presentation pathway is unconventional but recent studies show that the Ii is also responsible for transporting the TNF-related CD70 molecule towards the MHC-II compartments (32) anticipating a much broader function of the Ii than thus far described.

The association of Ii with the MHC-II is mediated via the CLIP region and it is believed that the same region is responsible for association to the MHC-I molecule (33). This matter however needs more investigation as binding towards only one MHC-I molecule has been shown (33) whereas association between Ii and MHC-I has been observed between several MHC-I molecules (28-30).

Table I: Qdm peptide sequences which bind to HLA-E (34)

Locus	Sequence	Examples of HLA-types
HLA-A	VMAPRTLLL	A01, A03
	VMAPRTLVL	A02, A24
HLA-B	VMAPRTVLL	B07, B08
HLA-C	VMAPRTLLL	Cw02, Cw15
	VMAPRTLIL	Cw03, Cw04[a]
HLA-G	VMAPRTLFL	G01

a Except the Cw0402 subtype

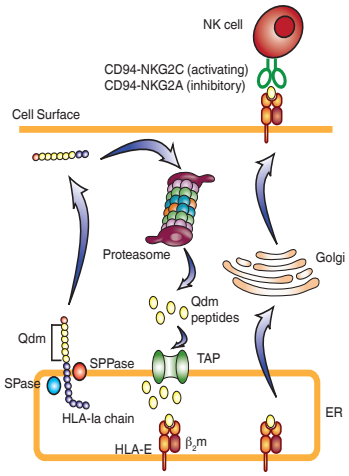

Figure 2: The non-classical presentation pathway. Peptides which are presented in HLA-E molecules are mostly derived from the leader peptide of the HLA-Ia heavy chain molecules. Once the heavy chain of the HLA-Ia is transcribed it is targeted to the ER where it is handled by SP and SPPase molecules. The leader sequence remains in the cytosol whereas the heavy chain is entering the ER where they can form the HLA-Ia molecule together with β_2m. The leader sequence is then processed through the proteasome and enters the ER through the TAP transporter. Here they are loaded on HLA-E molecules, which is then presented at the cell-surface where it can be recognized by NK cells. Figure is adapted from (4).

3. HLA-E (and its mouse homolog Qa-1)

In contrast to the extreme polymorphic nature of MHC-Ia molecules, MHC-Ib molecules have few alleles and are highly homologous between humans and other species (4).

3.1 HLA-E presentation pathway

HLA-E and its mouse homolog Qa1 are, similar to MHC-Ia molecules, expressed in most tissues. Although there is only 73% of homology between both molecules, their function and non-variable peptides that they bind are surprisingly similar (34). Both molecules can bind so called signal peptides in their hydrophobic peptide-binding groove, which are mainly derived from the leader sequence of classical MHC-Ia molecules (4, 34). This set of epitopes is also referred to as Qdm (Qa-1-determinant-modifier) peptides (table I). Qdm peptides are signal sequences located at the N-terminal regions of MHC-I molecules, serving as a hydrophilic segment to co-translationally target the newly synthesized MHC-I protein to the ER. Once in the ER-membrane, the hydrophilic domain containing a Qdm peptide is released in the cytosol by enzymes called SPase and SPPase, whereas the MHC-I heavy chain is released into the ER (see figure 2) (16). This example is in contrast with the idea of §2.3.1 were the enzymes SPase and SPPase are involved in TAP-independent peptide presentation. Upon cleavage of SPase and SPPase an epitope can be either released into the ER, and therefore processed TAP-independently, or it can be released in the cytsol where it is then processed in a TAP-dependent fashion.

The domain containing Qdm, which remained in the cytosol, is processed by the proteasome into small peptide fragments and Qdm is transported into the ER via TAP. In the ER, the Qdm peptide is loaded into HLA-E and then transported to the cell-surface were it is recognized by CD94/NKG2 receptors (35, 36) (figure 2).

3.2 Natural Killer- (NK) and T-cell recognition of HLA-E

NK cells and activated $CD8^+$ T-cells express the invariant transmembrane receptors CD94/NKG2 (34, 37). The CD94 subunit makes the most contact with the HLA-E molecule and the presented peptide, whereas the NKG2 subunit is responsible for the transduction of signals (38-40). NKG2 receptor genes are clustered in the NK-locus and there are different variants: NKG2A, -B, -C, -D, -E and -H. Of these six, only the NKG2D does not form a heterodimer with the CD94 complex and NKG2D is also clearly distinct from NKG2A, -B, -C, -E and -H; which differ by only a few amino acids (37). NKG2A and NKG2B are alternative splice products of one gene in which NKG2B appears to be in disadvantage. Also NKG2E and NKG2H are alternative splice products of one gene (41). NKG2A, -C and –E play a role during recognition of HLA-E and small differences in the NKG2A, -C, and -E receptors determines its affinity to the CD94 receptor. These differences also determine whether the CD94/NKG2 hetrodimer acts as an activating or inhibitory receptor. NKG2C and NKG2E are both activating receptors and are in general less frequently expressed than NKG2A, which provides inhibitory signals (41, 42).

NK cells are part of the so-called innate immunity, which are the first line of defense against infections or transformation. If due to these alterations the cells fail to present MHC-Ia molecules at the cell-surface the NK cells will be activated and lyse the cells. NK cells which express either CD94/NKG2A or CD94/NKG2C can engage with HLA-E and the MHC-Ia Qdm peptide and provide either an activating (NKG2C) or inhibitory (NKG2A) signal, which contributes together with other signals to determine whether the NK cell should become active.

Apart from NK-mediated recognition, TCR-mediated recognition of HLA-E or its mouse homolog Qa-1^b has previously been described (43-47). Upon cellular stress the Qdm peptide is replaced by novel much diverse repertoire of peptides (34, 43-47). Epitopes derived from Epstein bar virus (EBV) (43), *Mycobacterium Tuberculosis* (44), and mycobacteria (47) have been reported to be presented in HLA-E where then can be recognized by CD8$^+$ T cells. Romagnani *et al* reported that allogeneic T-cells might also recognize peptides in HLA-E, which are not present in the host of origin (46). This makes HLA-E a target for possible implications upon transplantation, or can be involved in anti tumor immune responses. Interestingly, Oliveira *et al* reported Qa-1^b restricted CTLs that recognized peptides presented solely on a variety of TAP-impaired tumor cells, so called TEIPPs (45). As Qa-1^b is highly homologous to HLA-E, this finding could imply that this type of CTLs can also exist in the human population thereby opening new possibilities to treat tumors with antigen processing defects (4, 45).

3.3 HLA-E expression by tumors

Almost all nucleated cells express HLA-E and a majority of tissues from healthy donors show weak to moderate staining for HLA-E. Staining of HLA-E is strong in subsets of lymphoid cells, epididymis and the adrenal gland. HLA-E expression is not detected in the liver, pancreas, myocytes and the central nerve system (CNS) (http://www.proteinatlas.org (48)).

It has been observed that in sections of melanomas variable fractions of HLA-E expression were found ranging form 30-70% on primary tumors and 10-20% on metastatic lesions (49). In colorectal cancers, and astrocytic tumors, strong HLA-E expression was observed, while its healthy counterpart showed weak staining of HLA-E (50, 51). In cervical carcinoma, a difference in HLA-E expression was observed between invasive- and non-invasive cancers; the more invasive the cancer the greater the HLA-E expression (52). A recent study reports HLA-E expression in early breast carcinoma patients (53). They observed that 50% of the breast carcinoma samples have expression of HLA-E. Remarkably, patients whose tumors had a complete loss of the classical HLA-I molecules, but coinciding HLA-E expression had a worse relapse free period. Also an association is seen between classical HLA-I processing pathway and HLA-E expression (53). The increased expression of HLA-E on tumors is not fully understood.

In vitro cultured melanoma- and colorectal cancer cell-lines display low but significant amounts of HLA-E on the cell surface. However in these cell-lines HLA-E expression is clearly detected intra-cellular. Upon IFNγ treatment an increase of HLA-E is observed at the cell-surface (49, 50, 54) resulting in a decreased susceptibility to CTL lysis of CTL expressing the inhibitory receptor CD94/NKG2a (49, 55). In one study an imbalance of MHC-Ia heavy chain/β_2m expression can modulate HLA-E surface expression, meaning that expression of HLA-E correlates inversely with the expression of MHC-Ia molecules in *in vitro* cultures (54). However, for several studies on other tumor cell lines don't observe such an inverse correlation (49, 56). It must also be noted that the general low HLA-E expression in tumor cell-lines observed *in vitro* contrasts with the in general increased HLA-E expression *in site* using immunohistochemistry.

4. Inhibition of the conventional MHC-I pathway upon viral infection.

Upon viral infection of a cell, the infected cells start to produce viral proteins. Like other proteins, the viral proteins are also processed via the antigen

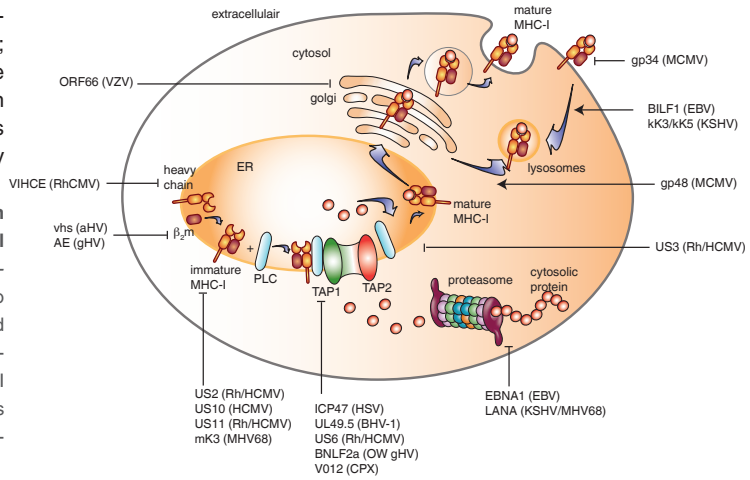

Figure 3: Inhibition of the antigen presentation pathway by viral proteins. The family of *Herpesviridae* has adapted numerous ways to avoid recognition by CTL. Depicted are the viral proteins, which are inhibiting molecules involved in MHC-I antigen processing and the virus they are derived from. Figure adapted from (57)

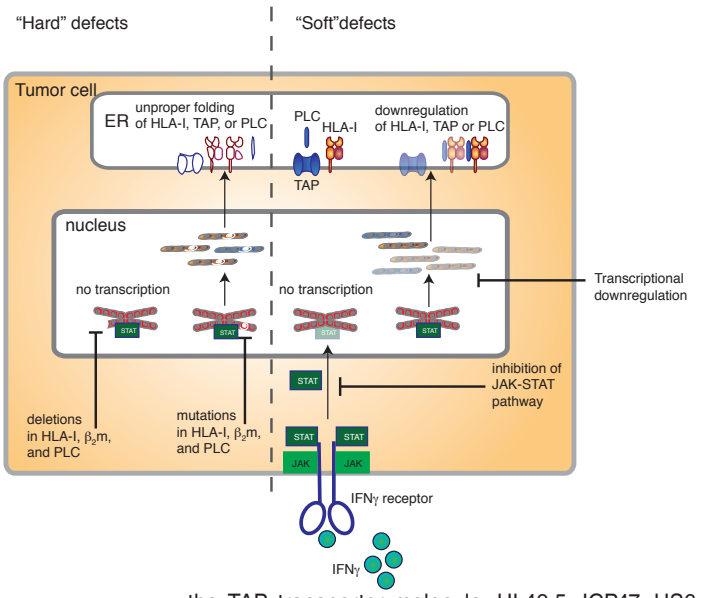

Figure 4: The "hard"- and "soft" defects in tumor tissue. Tumors can escape immune recognition through numerous ways. "Hard" defects are irreversible such as mutations and deletions of MHC-Ia heavy chain genes or the β_2m gene or LOH. Soft" defects are reversible regulatory defects such as downregulation of MHC-Ia transcription due to, hypermethylation of the promoters, oncogene activation, and or blocking of the Jack-STAT pathway.

presentation pathways and can be presented at the cell-surface. If a cell presents such viral proteins at the cell-surface they can be recognized by CTLs, becomes activated, and kills the infected cells. To avoid this recognition, most DNA viruses express so-called immune evasion proteins. The protein prohibits presentation of viral proteins in MHC-I molecules at the cell-surface, thereby avoiding recognition by the CTLs and allowing viruses to persist in the human body.

The most well studied viruses and their immune evasion proteins are from the family of *Herpesviridae* and most people are infected with one or more of the eight known Herpesviruses: herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpesviruses 6 and 7 (HHV-6 and HHV-7), Epstein-Barr virus (EBV), and Kaposi's sarcoma-associated herpesvirus (KSHV) (57). Due to their numerous strategies to prevent peptide presentation of the MHC-I molecule capability the family of *Herpesviridae* causes life-long persistent infections in humans.
For example the viral derived proteins US2, US10, US11 (HCMV), mouse cytomegalo virus (MCMC) glycoprotein (gp) 48, and murine γ-herpesvirus 68 (MHV68) mK3, target MHC-I molecules in a selective fashion thereby accelerated degradation of MHC-I molecules (57). Other mechanisms include intracellular retention (US3 (HCMV) and gp40 (MCMV)) or increased endocytosis (kK3/kK5 (KSHV) and BILF1 (EBV)) of MHC-I molecules, as well as interference with the recognition of peptide loaded MHC-I by CTLs (gp34 (MCMV)) (57). All MHC-I interfering proteins and their origin are summarized in figure 3.

Among the proteins involved in immune escape there are five viral-inhibitors known to impair the function of the TAP transporter molecule: UL49.5, ICP47, US6, BNLF2a and V012 (figure 3). For four of these TAP inhibitors their mechanism of action is unraveled. UL49.5 is a protein belonging to the genus Varicellovirus and for the bovine herpesvirus-1 (BHV-1) variant it is known to efficiently inactivate TAP by arresting it in a translocation-incompetent state; and promoting the degradation of TAP (58-60). ICP47 is found in HSV type 1 and 2 where binds to the cytosolic site of the TAP complex, thereby acting as a high-affinity competitor for peptide binding (61-64). The human HCMV protein US6 is an ER-resident protein and it prevents ATP binding to TAP, thereby blocking the peptide transport (65, 66). The viral TAP-inhibitor BNLF2a is encoded by EBV and prevents both peptide and ATP binding to TAP (67, 68). The latest identified TAP-inhibitor V012, is found in cowpox virus (CPX) and is the first TAP inhibitor outside the family of *Herpesviridae*. Its TAP-inhibiting mechanisms has not yet been identified (57, 69, 70).

5. Immune escape of tumors

Like viruses, tumors have adopted mechanisms to prevent peptide presentation by MHC-Ia. These mechanisms can be divided into two categories to which Garrido *et al* refers to as "hard" or "soft" defects (71). Both "hard" and "soft" lesions are associated with poor prognosis as it leads to failure of recognition and elimination by tumor specific CTLs, as the expression of MHC-I molecules at the cell-surface is decreased (72-74). "Hard" defects are irreversible and structural such as loss of gene copies via loss of heterozygosity (LOH), mutations and deletions of MHC-Ia heavy chain genes or the β_2m gene. These mutations and

Table II: Summary of studies on LOH

Tumor type	Studied LOH	n[a]	Percentage of LOH	Reference
HNSCC[b]	6p21.3 15q	41	49% 29%	(77)
Colorectal	6p	65	13.8%	(78)
Laryngeal	6p	34	17.6%	
Melanoma	6p	13	15.3%	
Laryngeal	6p21 15	70	36% 11%	(81)
NSCLC[b]	1p, 3p, 5q, 7q, 8q, 9p, 10q 17p, 18q	48[c]	44-76% (primary) 23-67% (metastasis)	(82)
Colorectal	33 on several chromosomes	38	26-90%	(83)
Bladder	9p21, 9q32, 17p13	79[c]	30-39% (primary) 35-48% (metastasis)	(79)
Cervical	6p	30	50%	(80)

a= n is number of samples tested
b= HNSCC is Head and Neck Sqaumous Cell Carcinoma, NSCLC is Non-Small Cell Lung Carcinoma
c= both primary and metastatic tumors

deletions are mostly found on chromosome 6, -16, and in the interferon signal transduction pathway (71, 75) (figure 4). “Soft” defects are reversible regulatory defects such as hypermethylation, oncogene activation, and blocking of the Jak-STAT pathway, and results in down-regulation of MHC-Ia surface expression (71). Similarly decreased transcription of MHC-I locus products and defects in components of the antigen-processing machinery (APM) comprising peptide transporter TAP, tapasin, and proteasome subunits, causes low MHC-Ia surface expression (76) (figure 4).

The occurrence of either LOH or alterations in the expression of the PLC and TAP has been thoroughly investigated. LOH is studied in a wide variety of tumor types and is mostly located on either the chromosomes harbouring the HLA-A/B/C genes or the β_2m gene (71). Studies that focuses on LOH in tumors are summarized in table II. In short: LOHs are present at different chromosomes and differ between 11-90% depending on which microsatellite used. Moreover, no significant difference is seen between primary and metastatic lesions (77-83).

The loss or downregulation of TAP is studied in a wide variety of tumors and its metastasis (72, 84). The alteration of TAP levels is found in all sorts of tumors but its frequency varies from 10% for Head and neck squamous cell carcinoma (HNSCC) up to 74% in renal cell carcinoma (72). Moreover the frequency of TAP loss is increased in metastatic lesions (84).

Two recent case studies show a clear association between low HLA-I expression and progression of tumors after immunotherapy (85, 86). In both studies metastatic lesions where isolated from a melanoma patient treated with autologous melanoma cell vaccine (M-VAX) and interferon-α2b. After treatment eight of the metastatic lesions regressed in the treated patient, while two lesions progressed. Studying the HLA-I surface levels it was seen that the progressive lesions had low HLA-I surface expression while the regressing lesions maintained their HLA-I surface levels (85, 86). A decrease in HLA-I surface expression prevents expression of tumor-specific antigens on the cell-surface thereby avoiding CTL recognition.

6. Tumor antigens

Tumor antigens, which are expressed at the surface of a tumor cell, are divided in different categories based the proteins they originate from. These categories are: viral epitopes, point mutations, differentiation antigens, cancer testis, and cryptic epitopes (Table III).

Viral epitopes expressed by all virus-induced tumors e.g. Burkitt lymphoma, nasopharyngeal carcinoma, and Hodgkin lymphoma, and cervical cancer in which 15-20% of tumors is virally induced. Cervical cancer is in virtually all cases caused by the Human Papilloma

Table III: Categories of human tumor antigens recognized by CTL

Category	Description	Examples
Viral antigens	Virus-induced tumors (e.g. EBV, HPV)	EBNA-1, E6, E7
Point mutations	Unique for each tumor	MUM-1, CDK-4, p53, Caspase-8
Differentiation antigens	Expressed in tissue lineage	Tyrosinase, GP100, Mart-1
Cancer testis antigens	Largely expressed during development and cancers	MAGE, NYO-ESO-1
Cryptic epitopes	Associated with aberrant transcription and translation	RU2, GnT-V, HPX42B
TEIPP	*Associated with antigen processing defects*	*CALCA*

Virus and known epitopes are derived from the E6 and E7 oncoproteins (87, 88). Burkitt lymphoma, Nasopharyngeal carcinoma, and Hodgkin lymphoma are caused by EBV, and expression of the EBV specific proteins; EBNA-1, LMP-1 and LMP2 have been observed in tumor cells (89).

The majority of cancer antigens are caused by point mutations in constitutive cellular proteins. CTL responses against e.g. Ras protein have been reported in breast carcinoma (90). Moreover, in vitro CTL responses derived from healthy donors against p53 have been reported (91) and p53 is frequently mutated and/or overexpressed in numerous cancers. Several phase I/II immunization trials have been conducted using p53 as a target (92). However, thus far therapeutic vaccination strategies targeting p53 have not shown consistent or convincing clinical efficiency (92), and therefore improvement in the efficiency of the immunogenic response against p53 is desirable.
Other mutations such as in MUM-1 and CDK4 have shown to lead to presentation of epitopes in MHC-I molecules (90). Some point mutations may reflect changes contributing to neoplastic transformation, while other mutations may reflect the genetic instability of tumors. These mutations and their effects are patient specific.

Differentiation antigens are selectively expressed in certain normal tissues and, therefore, also in tumors derived from these tissues. Most described epitopes were detected as targets for CTL in patients with melanoma and are usually not mutated (89, 90, 93). Three differentiation antigens are expressed in more than 90% of human melanoma namely MART-1, tyrosinase, and gp100 and it is known that melanoma patients have CTLs capable of recognizing these antigens (93).

Several tumors, e.g melanoma, bladder carcinoma, lung carcinoma and liver carcinoma, express genes which are normally only expressed during embryonic development (94). The epitopes representing this group are referred to as cancer/testis antigens, defined on the basis of their normal tissue specific expression in testis or ovary germ-line cells (89, 90, 93, 94). CTLs directed against these antigens have been identified and as they are expressed in a wide variety of tumors, they form an attractive group to use in tumor vaccination strategies.

The last group of tumor antigens, cryptic epitopes, is derived from aberrant gene transcripts or unusual translation products (95-97). Most of these translation products appear to be tumor-specific, are not detected in normal cells, and are caused through alternative open reading frames, translated introns or pseudogenes.

Even though there has been a large number of tumor antigens identified, T cell based immunotherapy suffers from a discrepancy between the induction of tumor-specific immune responses in experimental settings and therapeutic immunity in clinical relevant conditions (98).

A problem might arise when tumors impair their antigen presentation pathway. In this case, the presentation at the cell surface of the classical tumor antigens is decreased or even lost (99). Studies have shown that the residual peptides presented on TAP-impaired cells are dramatically different than the normal peptide repertoire (17, 100, 101). However, this alteration leads to presentation of new self-immunogenic peptides, which can be recognized by CTLs, thereby eradicating the tumor (45, 102, 103). These newly presented immunogenic peptides represent a novel category of tumor antigens, called TEIPP.

7. T-cell Epitopes associated with Impaired Peptide Processing (TEIPP)

TEIPPs are like most tumor antigens not presented at the cell-surface of healthy tissue. However, unlike the other classes of tumor antigens, TEIPPs are presented on tumors that have an impaired antigen presentation pathway. Moreover, TEIPPs are derived from housekeeping proteins and are expressed by a wide variety of cells (103, 104). CTLs recognizing TEIPP display preferential killing towards antigen-impaired tumors and it has been demonstrated that TEIPP T-cell receptors engage with epitopes that are displayed in the residual MHC-I molecules (103).

So far, studies on identifying TEIPPs and their CTLs have been limited to mouse models (45, 102-104) and only one TEIPP has been fully identified namely the Trh4-epitope (also known as Lass5), which is presented in H-2D^b molecules, a classical MHC-Ia in the mouse. There are also TEIPP-CTLs found directed against TAP-independent epitopes presented in the non-classical Qa-1, which is the mouse homolog of HLA-E (45). However, the exact epitopes have not been identified.

The Trh4 epitope is derived from the C-terminus of the Trh4-protein, which is an ER membrane spanning protein. TAP$^+$ RMA cells were not recognized by the Trh4-CTL whereas the TAP-negative counterpart RMA-S was efficiently lysed, demonstrating that the Trh4-epitope is processed in a TAP-independent fashion. As expression of the Thr4 protein was similar in both cells, it was hypothesized that the Trh4 epitope is not presented in TAP$^+$ RMA due to the large quantities of peptide transported into the ER by TAP, thereby creating an unfavorable environment for the TAP-independent Trh4-epitope to be loaded in H-2D^b molecules (103). Further study shows that overexpression of Trh4 epitope in TAP$^+$ cells resulted in recognition and lysis by the Trh4-CTL illustrating that indeed the influx of TAP-transported peptides create an efficient barrier to inhibit loading of peptides from alternative processing routes. The impairment of TAP function lowers this resistance allowing the MHC-I presentation of other peptide sources (104).

The Trh4-protein belongs to a family of fatty-acid regulators and these housekeeping proteins are expressed by a wide variety of cells (103). Indeed, tissues derived from TAP1 knockout mice were recognized by the Trh4-CTL apart from the spleen and liver. As expected, none of the tissues derived from wild-type mice were recognized (104). This shows that the Trh4-epitope can be applied to treat a wide variety of processing deficient tumors and limits possible side-reactivity as healthy TAP$^+$ cells are not recognized (103). Although the reactivity of TEIPP CTLs resembles that of natural killer cells, TEIPP antigens behave like conventional tumor antigens for CTL.

The induction of TEIPP CTLs has been investigated in a mouse model in numerous ways: vaccination with the Trh4 short peptide, cellular vaccination with TAP-deficient dendritic cells and adoptive transfer of *in vitro* expanded CTLs (102, 103). In all three cases the TEIPP-targeted immunotherapy resulted in clearance of tumors with MHC-I defects, in particular TAP deficiencies. Inducing TEIPP-specific CTLs might provide the first local immune activation and cytokine release upon recognition of antigen-impaired lesions.

8. Scope of thesis

This thesis focuses on alternative antigen presentation in the context of tumors with defects in antigen processing. In **chapter 2**, we investigate whether the human T-cell repertoire harbors a population reactive to TAP-impaired targets. The detection of such T-cell reactivity would indicate that, similar to mice, the human system contains a TEIPP-specific T-cell repertoire. To identify TEIPP antigens in humans, we set up a bioinformatics approach to predict HLA-I binding peptides with potential TEIPP characteristics in **chapter 3**. We made a start with a screening method to determine the immunogenicity of these self-peptides.
An attractive strategy towards the development of immune-therapy based on TEIPPs is to identify TEIPPs in the human HLA-E molecule. This non-classical HLA-I molecule has only two variants among the human population. As these molecules only differ by one amino acid, the presented peptides are the same among humans. Moreover, HLA-E is frequently preserved or over-expressed in tumors. To gain a more profound insight in the peptide repertoire presented by the non-classical molecule HLA-E under conditions were TAP is impaired; we performed peptide elutions in **chapter 4**. Since these peptides derived from the elution are based natural selection through antigen presentation we also determine a peptide-binding motif for HLA-E. The implications of increased HLA-E expression in ovarian- and cervical carcinoma patients are assessed in **chapter 5**, in which we also study the presence of T-cells containing CD94/NKG2 receptors which are able to interact with HLA-E.
It has been described in literature that the invariant chain interacts with HLA-I molecules, especially in the absence of TAP. This prompted us to test the idea that this protein-protein complex is implicated in the HLA-I

presentation of TEIPPs. In **chapter 6** we studied the surface expression of the CLIP peptide, which is derived from the invariant chain, in leukemia patients. We confirmed the interaction of the invariant chain with HLA-I molecules in leukemic cells, and surprisingly found that the CLIP peptide promiscuously binds HLA-I molecules, maybe as a result of this interaction. The existence and nature of human TEIPP antigens is reviewed in the **discussion** where we also give an overview on the current therapies aiming at counteracting MHC-I deficiencies in tumors.

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