Alternative HLA class-I peptide presentation in processing deficient tumors

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Equipped with his five senses, man explores the universe around him and calls the adventure Science.
Edwin Powell Hubble
The Nature of Science, 1954

Introduction

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

Ursula J.E. Seidel, Claudia C. Oliveira, Margit H. Lampen, and Thorbald van Hall

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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).

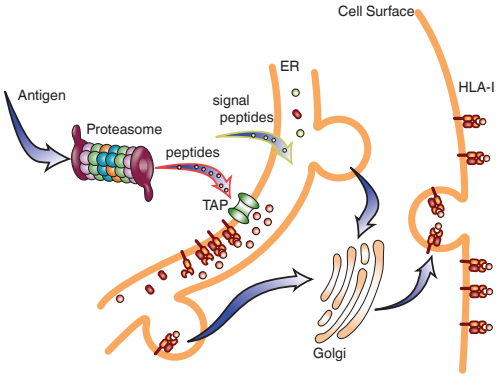

Figure 1: The classical HLA-I presentation pathway. Peptides derived from endogenous origin are transported through the HLA-I presentation pathway. The endogenously derived peptides are degraded by the proteasome and enter the endoplasmic reticulum through the TAP transporter after which they are loaded 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

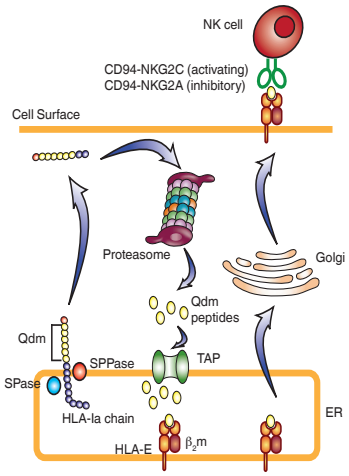

Figure 2: The non-classical presentation pathway. Peptides which are presented in HLA-E molecules are mostly derived from the leader peptide of the HLA-Ia heavy chain molecules. Once the heavy chain of the HLA-Ia is transcribed it is targeted to the ER where it is handled by SP and SPPase molecules. 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

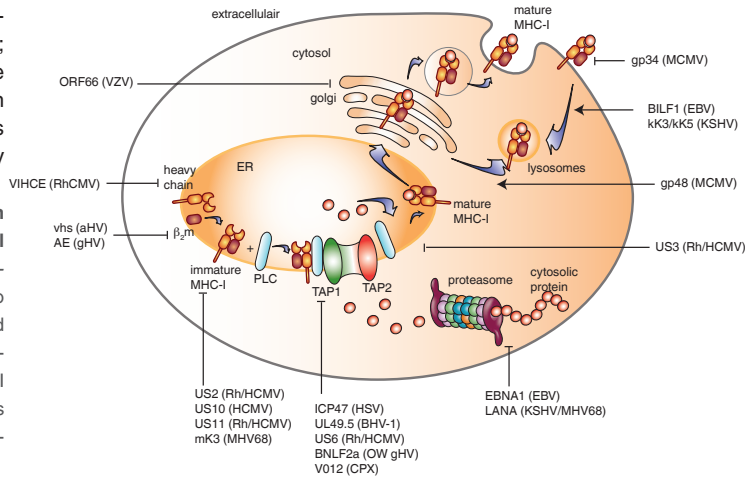

Figure 3: Inhibition of the antigen presentation pathway by viral proteins. The family of *Herpesviridae* has adapted numerous ways to avoid recognition by CTL. Depicted are the viral proteins, which are inhibiting molecules involved in MHC-I antigen processing and the virus they are derived from. Figure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

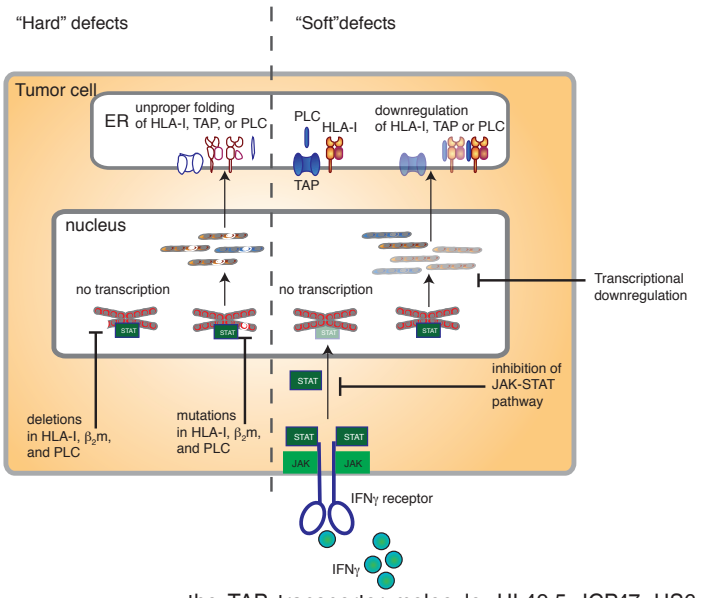

Figure 4: The "hard"- and "soft" defects in tumor tissue. Tumors can escape immune recognition through numerous ways. "Hard" defects are irreversible such as mutations and deletions of MHC-Ia heavy chain genes or the β_2m gene or LOH. Soft" defects are reversible regulatory defects such as downregulation 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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CD8 T-cell responses against TAP-inhibited cells are readily detected in the human population

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Abstract

Target cell recognition by Cytotoxic T Lymphocytes (CTL) depends on the presentation of peptides by Human Leukocyte Antigens class I (HLA-I) molecules. Tumors and herpes viruses have adopted strategies to greatly hamper this peptide presentation at the important bottleneck, the peptide transporter TAP. Previously, we have described the existence of a $CD8^+$ CTL subpopulation that selectively recognizes such TAP-deficient cells in mouse models. Here, we show that the human counterpart of this CTL subset is readily detectable in healthy subjects. Autologous PBMC cultures were initiated with dendritic cells rendered TAP-impairment by gene transfer of the viral evasion molecule UL49.5. Strikingly, specific reactivity to B-LCLs expressing one of the other viral TAP-inhibitors (US6, ICP47 or BNLF2a) was already observed after three rounds of stimulation. These short term T-cell cultures and isolated $CD8^+$ CTL clones derived thereof did not recognize the normal B-LCL, indicating that the cognate peptide-epitopes emerge at the cell surface upon an inhibition in the MHC class I processing pathway. A diverse set of T-cell receptors were used by the clones and the cellular reactivity was TCR-dependent and HLA-I-restricted, implying the involvement of a broad antigenic peptide repertoire. Our data indicate that the human $CD8^+$ T-cell pool comprises a diverse reactivity to target cells with impairments in the intracellular processing pathway and that these might be exploited for cancers that are associated with such defects and for infections with immune-evading herpesviruses.

Introduction

$CD8^+$ Cytotoxic T-Lymphocytes (CTLs) have an important role in the immune response against infections and outgrowth of tumors. They recognize antigenic peptides presented at the cell surface by class I molecules of the Major Histocompatibility complex (MHC-I). Inhibition of the intracellular MHC-I antigen presentation pathway is therefore an efficient strategy to avoid CTL recognition. Indeed, both tumors and viruses are able to escape CTL-mediated immune control through MHC-I down modulation [1-6]. Several mechanisms have been described that lead to loss of peptide presentation and these involve targeting of the MHC class I heavy chain, the β2-microglobulin chain, and components of the peptide loading complex (PLC). One common target in the PLC that results in efficient impairment of MHC-I presentation is the Transporter associated with Antigen Processing (TAP), which transports peptides generated by the proteasome from the cytosol into the Endoplasmic Reticulum (ER). Deficiency in TAP expression is frequently found in human cancers and several viral proteins have been indentified that bind to TAP and block its peptide transport function [2,7]. As the heterodimeric TAP1/TAP2 proteins constitute the bottleneck of the MHC-I processing pathway, a blockade at this step results in efficient and total decrease of antigen presentation at the cell surface, and as a consequence, resistance to antigen-specific CTL.

Within the family of *Herpesviridae* four variants of viral TAP inhibitors have been characterized. Herpesviruses cause life-long latent infections in their hosts, and occasionally reactivate. Therefore, immune evasion is an important survival strategy. UL49.5 is a protein encoded by herpesviruses belonging to the genus *Varicellovirus*. In this paper the bovine herpesvirus-1 (BHV-1) variant of UL49.5 is used, which efficiently inactivates TAP by arresting it into a translocation-incompetent state, and, moreover, promotes the degradation of TAP [8-10]. The TAP inhibitor ICP47 is found in herpes simplex virus type I and II and binds in a stable fashion to the cytosolic site of the TAP complex, thereby acting as a high-affinity competitor for peptide binding [11-14]. The human cytomegalovirus protein US6 is an ER resident protein, which prevents ATP binding to TAP and thereby blocking peptide transport [15,16]. Recently a new TAP inhibitor was indentified in Epstein-Bar Virus (EBV), BNLF2a, which prevents both peptide- and ATP binding to TAP [17,18].

TAP impairment is found in a broad range of tumor types with a frequency between 10-74%, and is regularly found in metastatic leasons [1,3]. It is the most frequent cause of decreased expression of Human Leukocyte Antigens class I (HLA-I), the MHC-I in man, and is associated with a poor prognosis [1,19,20]. Loss of antigen presentation as a result of TAP down regulation leads to failure of recognition and elimination by tumor-specific CTLs [21-24]. Interestingly, the residual peptides that are still presented at the surface of TAP-impaired cells are dramatically different from the normal peptide repertoire. In 1992, two studies described that the peptide repertoire complexity of the TAP-negative T2 cell-line was much more limited compared to that of the TAP–positive counterpart cell-line [25,26]. Weinzierl *et al* characterized these peptides in more detail using a differential tandem mass-spectrometry approach and reported a list of peptides which were much more pronounced in the repertoire of TAP-negative B-LCL [27]. These studies demonstrated that blockades in the intracellular antigen processing pathway lead to alterations in the peptide repertoire presented by HLA-I at the cell surface.

In a mouse model we recently described that some of the differentially presented peptides are immunogenic and that a subpopulation of T cells is able to target these TAP-independent peptides [28-31]. This category

of T cells eliminated tumors with a defect in MHC-I antigen processing, while cells with no processing defects were not eliminated. Such peptides were found to be presented by classical MHC-I molecules, but also by the conserved non-classical MHC-I Qa-1 [28]. The peptide-epitopes, which were recognized by the cognate T cells, were 'self' peptides which only emerge at the cell surface once there is a defect in the antigen presentation pathway. We named these new peptides TEIPP, for T cell Epitopes associated with Impaired Peptide Processing [28-31]. Because TEIPP peptides are derived from housekeeping proteins, their presentation is also induced by TAP-deficiencies in normal, non-transformed cells. Indeed, introduction of the viral TAP inhibitor UL49.5 in myeloid dendritic cells (DC), which are professional antigen presenting cells capable of activating naïve T cells, sensitized these cells for recognition by TEIPP-specific CTL [30]. These TAP-inhibited DCs were equally well recognized by TEIPP T-cells, as DCs from $TAP1^{-/-}$ mouse. Importantly, vaccination with these TAP-impaired DCs elicited strong TEIPP T-cell responses *in vivo*, which were able to prevent outgrowth of TAP-loss lymphoma and fibrosarcoma tumor variants [30].

Thus far studies on TEIPP antigens have been limited to mouse models. Here, we present the first evidence for the existence of a human T-cell subset with TEIPP-specificity. In analogy with our mouse model, we rendered monocyte-derived DC TAP-deficient by gene transfer of viral evasion molecules that block the peptide transporter TAP. These TAP-deficient DC were used as stimulator cells in cultures with autologous T cells. This approach revealed an efficient outgrowth of $CD8^+$ T-cells with selective reactivity against TAP-deficient target cells. Polyclonal cultures and T-cell clones exhibited TCR-dependent and HLA-I restricted lytic activity against TAP-inhibited targets, but not against their non-inhibited counterparts. Collectively, our data indicate that the $CD8^+$ T-cell repertoire in humans contains reactivity against peptides presented by cells with a defect or block of the MHC-I antigen-processing route.

Material and methods

Cell lines and primary cell cultures

B-LCLs modo, hodo and HD10 (see Table I) were transduced with retroviruses based on the pLZRS vector, which contains an internal ribosome entry site, followed by GFP, and one of the viral TAP inhibitors. This system is recently reported [32] and the viral TAP inhibitors UL49.5 (from BHV-1), ICP47, US6 and BNLF2a were used, which were previously described [8-18]. Cells were sorted on GFP expression by FACS to ensure homogenous and high expression of the various TAP inhibitors. All B-LCL and K562 were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS, 100 units/ml penicillin, 100µg/ml streptomycin (Life technologies, Rockerville, MD) and 2mM glutamine (Invitrogen).

We obtained blood from healthy donors (HDs), which were partly HLA-matched with Modo or Hodo B-LCLs (Table I). From these donors Peripheral Blood Mononuclear Cells (PBMCs) were isolated, and positive selection was performed on monocytes and B-cells, using respectively $CD14^+$ and $CD19^+$ MACS beads (Miltenyi Biotec, Gladbach, Germany). $CD14^+$ monocytes were MoDCs in a concentration of $2x10^6$ cells/well in a 6 wells plate (Costar, Cambridge, MA) in RPMI 1640 (Invitrogen) containing 8% heat-inactivated FCS, 100 units/ml penicillin, 100µg/ml, streptomycin and 2mM glutamine, 800 units/ml GM-GSF (Invitrogen), and 500 units/ml IL-4 (Invitrogen) at 37ºC, 5%CO_2. After electroporation 250 ng/ml LPS (Sigma-Aldrich, St Louis, MO, *Escherichia coli* 055:B5) was added for 24h to ensure maturation into mature dendritic cells, which were then washed and used as stimulators for T-cells. B-cells ($2x10^6$ cells/well) were cultured with irradiated CD40 ligand (CD40L) L-cells, $0.5x10^6$ cells/well in 6-wells plates with 500 units/ml IL-4 and ITES supplement (Lonza group) in complete IMDM medium, with 8% heat-inactivated FCS. B-cells were stimulated once a week and after electroporation the B-cells were cultured overnight in complete IMDM medium including 500 units/ml IL-4 before usage as stimulators.

RNA electroporation of MoDCs and B-cells

RNA was synthesized from the pGem4Z vector containing the UL49.5 gene from BHV-1. Vector DNA was linearized for 1h at 37ºC, with SpeI (Roche, Basel, Switzerland). RNA of UL49.5 was then synthesized with the High Yield Capped RNA transcription Kit (T7; Ambion, Austin, TX).

MoDCs cells were cultured for 6 days before electroporation with 20µg synthesized RNA. For each electroporation a maximum of $1x10^7$ cells in 100 µl Optimem media (Invitrogen) was used. Cells were collected, washed and taken up in Opti-Mem media and pulsed with a blockpulser (Biorad, Hercules, CA), in a 2mM cuvette (Biorad), at 300V, 150 µCF [33]. Afterward cells were taken up in X-vivo medium (Lonza) without phenol red for 15 min at 37ºC, 5%CO_2, and then incubated overnight in complete RPMI 1640 medium, with 8% heat inactivated FCS, 250 ng/ml LPS, 800 units/ml GM-GSF, and 500 units/ml IL-4. For electroporation of B-cells, $5x10^6$ cells were stimulated with CD40L expressing L-cells, in a concentration of $0.5x10^6$ cells/well, and 500 units/ml IL-4 the day before. A maximum of $5x10^6$ cells were electroporated with 20µg synthesized RNA with the Nucleofector II, program U-008

(Amaxa, Lonza, Cologne, Germany), using the human B-cell nucleofector kit (Amaxa). After electroporation cells were kept in X-vivo medium without phenol red for 15 min at 37ºC, 5%CO_2 and then cultured overnight in complete IMDM medium, with 8% heat-inactivated FCS containing 500 units/ml IL-4.

T-cell induction protocol

Polyclonal T-cell cultures with a concentration of 6x10^5 cells/ml were induced, in a U-bottom 96 wells plate (Costar), by co-cultures with electroporated MoDCs (week 0 and 4) or B-cells (week 2 and 6) in a concentration of 2x10^5 cells/ml, in complete IMDM medium containing 8% human AB serum (Greiner, Bio-One, Alphen aan den Rijn, the Netherlands), 10 ng/ml IL-7 (only week 0), and 60 units/ml IL-2. An alternating scheme is used as the amount of MoDCs, obtained from our healthy donors, is limited.

T-cell clones were generated by limiting dilution at week 4 or 6. Polyclonal cultures were first depleted for CD4$^+$ cells and stimulated with 1x10^5 cells/ml TAP inhibited B-LCLs (Modo or Hodo), 1x10^6 cells/ml pool PBMCs and 60 units/ml IL-2 in complete IMDM medium with 8% heat-inactivated FCS. After one week, T-cell cultures were restimulation with similar conditions. Clones were selected and restimulated every other week with 1x10^5 cells/ml a mixture of various TAP inhibited B-LCLs (Modo or Hodo), 1x10^6 cells/ml pool PBMCs, 60 units/ml IL-2, and 5 ng/ml IL-15 (Invitrogen Coroperation) in U-bottom wells.

Peptide transport assay

To ensure inhibition of TAP, a peptide transport assay was done as described previously [9,10]. In brief cells where permeabilized, and incubated with the fluoresceinconjugated synthetic peptide CVNKTERAY in the presence or absence of 10mM ATP. Peptide translocation was terminated by adding ice-cold lysis buffer, and after the removal of cell-debris incubation with ConA beads was done to isolated glycosylated peptides. The fluorescence intensity was measured and peptide transport is expressed a percentage of translocation, which is relative to the translocation in control cells.

Western blot

Cells were lysed in 0.5% Nonidet P-40 lysisbuffer (50 mM Tris, 150 mM NaCl (pH 8.0), 0.5% NP40, 25 mg/ml Leupeptin, 25 mg/ml 4-(2-amino-ethyl)-benzene-sulfonyl fluoride hydrochloride (Sigma-Aldrich, Zwijndrecht, the Netherlands. Depending on the protein, either 16.5% Tricine-PAGE (for UL49.5) or 10% SDS page gels (for β-actin and TAP1) were made [9]. The gels were loaded, and BenchMark prestained ladder (Invitrogen) was used as marker. For Tricine-PAGE gels, gels ran for 3-4h at 25mA/gel. The 10% SDS page gel ran for 1.5h at 15mA/gel. Afterwards the gel was blotted in a semi-dry manner, (Hep-1 OWL Panter; Thermo Scientific, Etten-Leur, the Netherlands) in semi-dry blot buffer (25mM Tris-Base, 192mM Glycine), for 45 min at 10V onto a Hybond-PVDF transfer membrane (GE healthcare, Zoetermeer, the Netherlands), which was activated for 1 min in pure methanol. After blotting the membrane was incubated overnight at 4ºC in either 5% elk (for UL49.5 and β-actin) or 0.6% BSA (for TAP-1) in TBST (10mM Tris-base, 150mM NaCl, 0.05% tween20, pH 7.4).

The next day the blots were stained with one of the following antibodies, H11Az (anti BHV-1 UL49.5) [34], 148.3 (anti TAP-1) [35,36], or anti-β-actin (Sigma) in either 0.6 % BSA in TBST (anti TAP1) or 1% elk in TBST (anti UL49.5 and anti-β-actin). After the staining, the membrane was washed 3 times for 10 min in TBST, followed by 3 times 10 min washing in TBS (10mM Tris-Base, 150mM NaCl, pH 7.4). HRP labeled secondary antibodies were used: anti-rabbit Ig (DakoCytomation, Carpinteria, CA) for UL49.5, and anti-mouse Ig for TAP1 and β-actin (Dako), followed by 3 times washing in TBST and 3 times TBS. The western blot was developed on film using ECL Plus Western Blotting Dectection Reagents (GE Healthcare).

Flow cytometry

For flow cytometry the following antibodies were used from BD bioscience: CD3 (SK7, FITC), CD137 (4B4-1, APC), CD8 (SK1, PE or APC), CD4 (SK3, PE) from Serotech: anti-HLA A/B/C (W6/32, APC), and from Beckman Coulter the Vβ Repetoire Analyses kit was used. Cells were analysed using a FACS Calibur, and Cellquest software (Becton Dickinson, USA), or Flowjo software (Tree star, Inc).

CD8+ T-cells clones were stained for the following NK and KIR receptors: CD226 (clone DX11), NKG2A (clone Z199PE), NKG2C (clone 134591), NKG2D (clone 149810), CD56 (clone MY-31), CD94 (clone DX22), 3DL1 (clone DX9), 2DL3 (clone GL183), 2DS1/2DL1 (clone EB6), 2DS4 (clone FES 172), 3DS1 (clone Z27), IgG1 (clone A687), 2DL4 (clone A687), 2DL5 (clone A687), all kindly provided by Dr J. van Bergen (Leiden).

T-cell reactivity assays

IFNγ production by T-cells was analysed after 24h or 48h incubation via ELISA (PeliKine compact human IFN ELISA kit, Sanquin Amsterdam). ELISA plates were developed with TMB (3, 3', 5, 5'-Tetramethylbenzidine Liquid Substrate Supersensitive, for ELISA, Sigma) and measured at 450nm. On each plate a standard curve was added to calculate the IFNγ production (pg/ml).

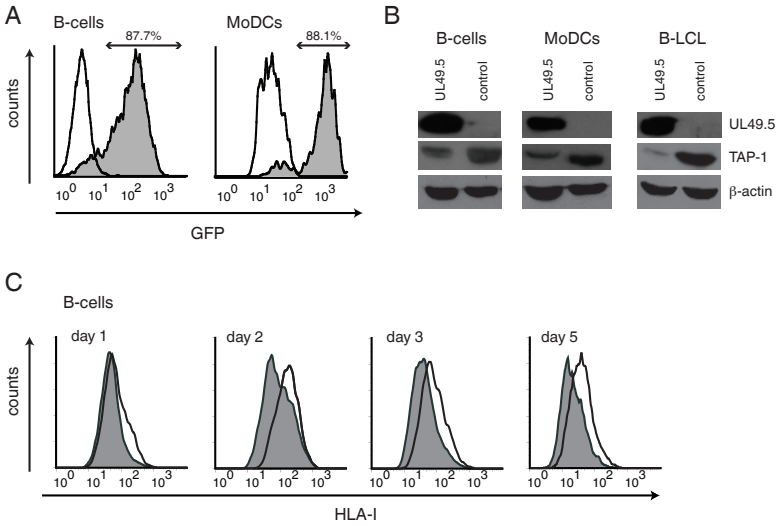

Figure 1: RNA electroporation with UL49.5 into primary B cells leads to MHC-I downregulation. (A) Primary cultures of B-cells or MoDCs were electroporated with RNA encoding GFP. Mock electroporations served as negative controls. After 24h incubation for B-cells and MoDCs ~90% of the cells where successfully transfected with GFP. (B) Primary cultures of B-cells or MoDCs were electroporated with RNA encoding the viral TAP-inhibitor UL49.5. Mock electroporations served as negative controls. After electroporation, cells were lysed and expression of UL49.5 (after 24 h) and TAP1 (48 h) was analysed by western blot. B-LCL with stable expression of the UL49.5 protein served as positive control. (C) Electroporated primary B-cells were stained with anti HLA-I antibody at different time points. HLA-I surface expression is depicted of B cells electroporated with UL49.5 RNA (filled histograms) and with control RNA (open histograms). A decrease of HLA-I at the cell-surface was seen up till day 5 after electroporation. Data are representative for three independent experiments. Monocyte-derived dendritic cells showed similar results (data not shown).

To confirm the receptor dependency of the T-cells, several antibodies were used: W6/32 (HB-95, anti HLA), BB7.2 (anti HLA-A2) and, B123.2.1 (anti HLA-B/C) (kindly provided by Dr. E. Verdegaal) and FK18 (anti CD8) (kindly provided by Dr. A. Mulder). Cells were pre-incubated at 37ºC, with 50µl of the antibody and IFNγ release was measured after overnight incubation.

Lytic activity of $CD8^+$ T-cell clones was determined in a 4h ^{51}Cr release assay as previously described [37]. $1x10^6$ target cells were labeled with 100µl ^{51}Cr for 1h at 37Cº. After labeling the cells were washed 3 times with PBS. The target cells were incubated for 4h at 37ºC, 5% CO_2, with different concentrations of $CD8^+$ T-cells, after which the chrome release was measured. The percentage of specific lysis was calculated as follows: ((specific lysis test well – spontaneous lysis)/(maximum lysis – spontaneous lysis)) x 100.

A $CD8^+$ T-cell reactive against the TAP-dependent peptide HA-1 (clone HA-1, kindly provided by Dr. E. Goulmy) was used to ensure TAP inhibition in the B-LCL modo TAP inhibited targets [38].

Results

RNA electroporation of UL49.5 leads to effective TAP inhibition

Previously, we demonstrated that inhibition of peptide transport through TAP induces the HLA-I-mediated presentation of TEIPP antigens by DCs [30]. In analogy with this approach in our mouse model, we engineered TAP-inhibition in human monocyte-derived DC (MoDC) and B cells by the introduction of the TAP inhibitor UL49.5 of the BHV-1 virus [8]. Gene transfer was accomplished through electroporation with synthetic RNA in order to prevent artificial vector sequences in these professional stimulator cells. The UL49.5 gene was chosen from the four characterized viral TAP-blocking molecules, since we previously found that this protein is the most efficient inhibitor and actively breaks down TAP proteins [32]. Introduction of synthetic RNA encoding for GFP showed that ~90% of both B-cells and MoDC were successfully transfected with GFP (fig 1a). Electroporation with synthetic RNA of UL49.5 resulted in strong expression of UL49.5 in MoDC and B-cells from healthy blood donors (fig. 1b). The high expression levels of the viral protein resulted in a specific TAP degradation, comparable to B-LCL that were stably transfected with the gene (fig 1b), and this TAP degradation was still present in B-cells 5 days after electroporation (data not shown). Importantly, the introduction of UL49.5 in the primary MoDC (12-20% reduction in intensity two days after electroporation) and B-cells (fig. 1c) led to a decrease of HLA-I molecules at the cell surface, illustrating that the inhibition of TAP function indeed limited the availability of peptides in the ER. Therefore, we concluded that

electroporation with synthetic RNA encoding the viral UL49.5 is an effective method to generate TAP-inhibited Anitgen Presenting Cells (APCs). These TAP impaired professional APCs were used for the induction of TEIPP-specific T-cells *in vitro*.

Construction of a target cell panel for the analysis of TEIPP-specific T cells

In order to determine the specificity of the T-cell cultures resulting from stimulations with TAP-inhibited APCs, we constructed target cell panels on the basis of stable B-LCL cell-lines. B-LCLs hodo and modo cell panels were used as these two cell-lines cover around 80% of the Caucasian population, including HLA-A1, -A2, -B8 and -B44 (Table I) (39). TAP was inhibited in these cell-lines via stable expression of the inhibitors UL49.5, ICP47, US6 and BNLF2a and as a control the empty pLZRS vector was used. Flow cytometry analysis was performed to determine surface HLA-I expression using an anti-HLA-A/B/C mAb. The B-LCL displayed decreased HLA-I levels after introduction of one of the TAP inhibitors, albeit to variable extent (fig 2a). Subsequently, we determined the impairment of TAP function in these B-LCLs, with a peptide transport assay. The efficiency of TAP inhibition was found to vary between 70 to 90%, with UL49.5 giving the strongest decrease in TAP transport (fig 2b). These results confirmed that all viral proteins were capable of efficiently impair TAP function, although the changes in total HLA-I surface levels were not compelling. In accordance, earlier studies indeed point at a discrepancy between TAP blockade and the effects in HLA-I expression levels at the cell surface (32), implying that alternative peptides substitute for the TAP-mediated peptide repertoire. To examine this notion, the recognition of the minor histocompatibility antigen HA-1 presented by HLA-A2 in a TAP-dependent way was determined with a previously established CD8+ CTL clone (32,38). IFNg production and cytotoxicity by this CTL clone upon incubation with the HLA-A2 positive B-LCL modo and its TAP-inhibited variants clearly indicated that this peptide-epitope is virtually absent from the TAP-inhibited B-LCLs (fig 2c and d). We

Tabel I. HLA-I serotype of cell-lines and donors

		HLA typing					
Sort	**Name**	**HLA-A**		**HLA-B**		**HLA-C**	
Cell-line	B-LCL hodo	1	11	8	60	w3	w7
	B-LCL modo	2	2	44	60	w5	w10
	B-LCL HD10	1	68	44	58	w3	w7
Donor	HD1	2	23	44	44	w5	w4
	HD2	11	31	7	44	w5	w15
	HD3	2	2	44	18	w5	w7
	HD4	1	2	8	44	w5	w7
	HD5	2	24	44	39	w5	w7
	HD6	1	11	8	40	w2	w7
	HD7	2	3	44	57	w5	w6
	HD8	1	1	8	58	w7	w7
	HD9	2	23	44	44	w5	w4

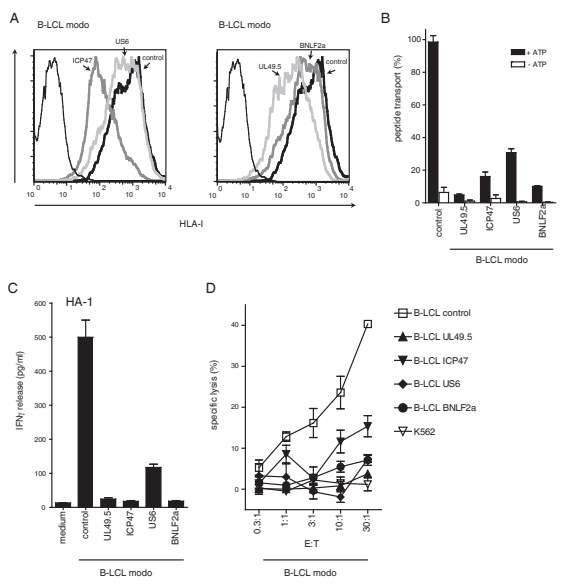

Figure 2: B-LCL panel with stable expression of each of the four known viral TAP inhibitors. (A) The genes of UL49.5, ICP47, US6 or BNLF2a were introduced into B-LCL modo using retroviral vectors. MHC-I cell-surface display was measured with a pan-HLA-I antibody. All cell lines were stained in the same experiments, but are separated in two histogram plots for clarity. Isotype control (thin lines) and modo B-LCL transfected with vector alone ('control', thick lines) are the same in both panels. Similar results were obtained in three other experiments. (B) All viral proteins efficiently inhibit TAP function, as tested in a peptide transport assay. Percentage of TAP transport was calculated against B-LCL modo control which was set at 100%. Means and standard deviations shown are representative of two assays with similar outcome. Comparable results were observed for B-LCL hodo (data not shown). (C-D) IFNγ release (C) and lytic activity (D) by an established CTL clone, specific for the HA-1 antigen, was measured to ensure that the viral TAP-inhibitors were able to block surface presentation of TAP-dependent peptide-epitopes. The HA-1 antigen is endogenously expressed by modo B-LCL and is presented by HLA-A2. Means and standard deviations are shown from one out of three experiments with similar outcome

Tabel II. IFNγ release by polyclonal T-cell cultures from different donors

	Target	Medium		Control[a]		UL49.5		ICP47		US6	
Donor	cell-line	mean[b]	SD[c]	mean	SD	mean	SD	mean	SD	mean	SD
HD1	Modo	39[d]	10	354	50	1098	176	1308	81	1407	141
HD2	Modo	44	6	68	16	145	24	198	32	164	4
	Hodo	44	6	72	3	354	76	368	78	225	16
HD3	Modo	NT[e]	NT	2	2	51	49	121	131	61	26
HD4	Modo	10	0	205	34	122	27	240	47	213	59
	Hodo	10	0	418	53	629	98	NT	NT	NT	NT
HD5	Modo	NT	NT	155	32	269	85	811	120	NT	NT
HD6	Hodo	30	4	54	1	114	24	62	1	52	19
HD7	Modo	14	0	134	28	507	49	793	215	466	187
HD8	Hodo	181	14	529	83	737	39	623	27	610	86
HD9	Modo	2	1	4	5	744	109	814	69	744	12

a B-LCLs are transduced with either an empty vector (control) or a vector containing one of the viral TAP inhibitors (UL49.5, ICP47 or US6).
b mean is calculated from triplicate samples
c SD is Standard Deviation
d IFNγ release (pg/ml)
e NT is Not Tested

therefore concluded that the control B-LCL presents a TAP-positive peptide repertoire and that the variants expressing one of the viral TAP-inhibitors predominantly present a different, TAP-independent peptide repertoire in their broad array of HLA alleles. These two cell panels constituted ideal target cells to determine TEIPP-reactivity by the T cell cultures induced with the UL49.5 RNA electroporated APCs .

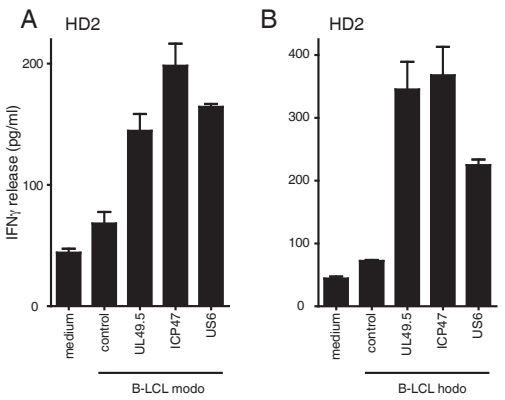

Figure 3: Polyclonal T-cell cultures exhibit TEIPP-specificity. (A-B) Short term T cell cultures from PBMC were stimulated with UL49.5 expressing autologous dendritic cells and tested four to six weeks after initial stimulation against two panels of TAP-inhibited B-LCL. Healthy subjects were selected on basis of sharing some HLA-I alleles with the modo or hodo B-LCL target cell lines (see Table I for HLA typing). IFNγ release by one T cell cultures from donor HD2 (A-B) is shown. HD2 shared HLA-I alleles with both B-LCL modo (A) and hodo (B) and was tested against both panels. Effector to target ratio was 2:1 for all experiments. (see Table II for results of all donors).

Polyclonal T-cell cultures display TEIPP-specificity

Polyclonal T-cell cultures were initiated from PBMC of healthy donors whom were selected to match HLA-I alleles with the modo or hodo B-LCL (Table I). MoDC or B-cells, with an impaired TAP function through UL49.5 RNA electroporation, were added to autologous T-cell cultures in a bi-weekly alternating scheme in order to stimulate the outgrowth of T-cells with TEIPP-specificity. Specificity of the T-cell cultures was examined after three to four *in vitro* stimulations by measuring IFNγ release upon incubation with cells from the partly HLA-matched modo and hodo B-LCL panels. Strikingly, these short term cultures already displayed selective reactivity to the TAP-inhibited variants of the B-LCL (fig 3). One donor (HD2) which shared HLA-I alleles with both B-LCL lines, also exhibited reactivity against both TAP-inhibited cell lines (fig 3), suggesting that this T-cell culture contained multiple TEIPP-specificities presented by at least two different HLA-I molecules. Complete mismatched targets showed no reactivity, and the HLA-I negative target K562 was also not recognized (data not shown). IFNγ release by the polyclonal T-cells was observed against TAP-inhibited B-LCL, irrespective of which viral TAP-targeting protein was expressed. Since the viral evasion molecules exert different mechanisms for TAP impairment [8-18], these data suggested that the T-cell recognition was induced by general TAP-impairment in the target cells rather than by a particular molecular strategy. An overview of the results from all nine polyclonal T-cell cultures is depicted in Table II. All cultures, from different blood donors, displayed selective reactivity against the TAP-inhibited B-LCL. These surprisingly broad T-cell responses from short-term cultures prompted us to

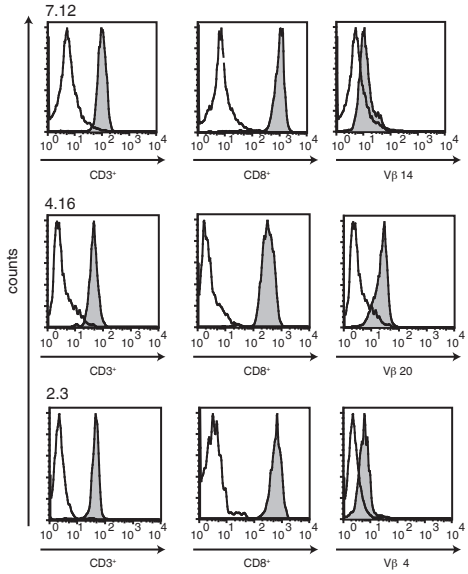

Figure 4: Clones 7.12, 4.16 and 2.3 are CD3+, CD8+ and each has their own TCR Vβ usage. All three clones were stained for CD3, CD8 and 24 different TCR Vβ. Flow cytometry analysis revealed that clone 7.12 has TCR Vβ 14, clone 4.16 Vβ 20 and clone 2.3 Vβ 4, confirming their independent origin. All clones are CD3+ and CD8+.

exclude a super-antigen like response, reflected by strong T-cell expansions of one particular TCR Vβ-positive subpopulation. Staining with a panel of Vβ-specific antibodies demonstrated a wide array of TCR Vβ usage (data not shown), indicating that the T-cell cultures were clearly polyclonal and were not the result of super-antigen mediated expansion. Together, these data suggested that TEIPP-specific T-cells can readily be detected in short-term T-cell cultures from PBMC of healthy blood donors.

Isolation of TEIPP-specific CD8+ T-cell clones

The short-term polyclonal T-cell cultures resulting from three stimulations with RNA electroporated TEIPP-presenting cells contained both CD4+ and CD8+ T cells. CD8+ T-cell clones were isolated from these cultures by limiting dilution, stimulating CD4-depleted cultures with TAP-inhibited modo or hodo B-LCL. Approximately 10-20% of the isolated clones showed higher reactivity against TAP-inhibited B-LCLs than control B-LCL. Further expansion of these T-cell clones resulted in the isolation of three stably growing T-cell clones from different donors. These three clones (clone 7.12, clone 4.16, and clone 2.3) expressed CD3, CD8 and TCR molecules from different Vβ families (fig. 4), confirming their independent origin.

Flow cytometry profiling of natural killer receptors demonstrated that the TEIPP-specific CD8+ clones did not display an aberrant expression of these receptors. Comparable to the vast majority of activated CTL, the lectin receptors CD94, NKG2A and NKG2D were expressed, but the KIR family was absent (supp. fig 1).

All three CD8+ T-cell clones recapitulated the TEIPP-specificity of the polyclonal T-cell cultures and recognized all TAP-inhibited B-LCL, but not control the B-LCL (fig. 5a-c). This indicates that all viral TAP-inhibitors present an overlapping peptide repertoire which is different than its non-inhibited counterpart, and that the viral proteins themselves were not recognized by our T-cell clones. Of note, the reactivity pattern is completely opposed to that of the HA-1-specific CTL (fig. 2c), illustrating the distinct peptide repertoires presented by TAP-proficient and TAP-impaired target cells [32]. The TAP-inhibited B-LCLs were recognized with different efficiencies by the CD8+ T-cell clones (fig 5). Interestingly, the B-LCLs inhibited with US6 had the least TAP-blocking effect (see fig 2b

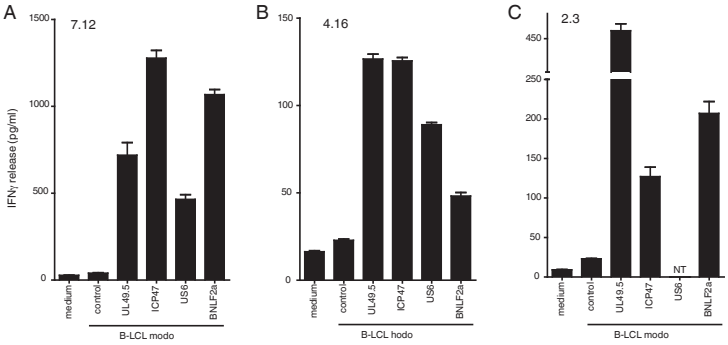

Figure 5: CD8+ T-cell clones exhibit TEIPP-specificity. (A-C) CTL clones were obtained by limiting dilution and selected on reactivity against TAP-inhibited B-LCL. Approximately 10-20% of all isolated CTL clones showed preferred reactivity against TAP-inhibited B-LCL, similar to the three CD8+ CTL clones shown here for 7.12 (A), 4.16 (B) and 2.3 (C). IFNγ release by CTL clones upon incubation with the B-LCL panels is shown. Means and standard deviations are shown from one out of four similar assays. *NT* means not tested.

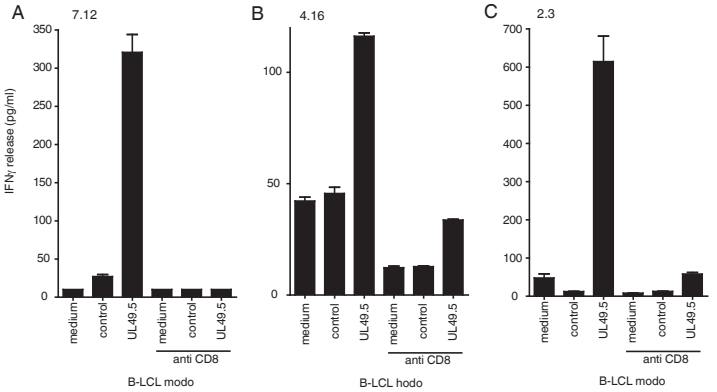

Figure 6: Recognition of TAP-inhibited target cells is TCR-mediated. (A-C) CTL clones were pre-incubated with a blocking anti-CD8 antibody before incubation with B-LCL target cells. IFNγ release for all three CTL clones is shown: 7.12 (A), 4.16 (B) and 2.3 (C). Isotype control antibodies did not alter the recognition of TAP-inhibited B-LCL (not shown).

for modo) and were also the least recognized by the T cells, suggesting that a correlation exist between the degree of TAP-inhibition and the level of HLA-I mediated TEIPP antigen presentation in HLA-I at the cell surface.

Target cell recognition is TCR-mediated and HLA-restricted

We previously reported in mouse tumor models that TEIPP-specific CTL exert their reactivity strictly via their T-cell receptors and that the TEIPP antigens can be presented on classical as well as non-classical MHC-I molecules [28,29]. Since the isolated human TEIPP-specific CD8+ T-cell clones described here displayed comparable specificity against TAP-inhibited targets, we anticipated that also the human CD8+ T-cell clones mediated target cell recognition in a TCR- and HLA-I dependent fashion. To examine this, all three CD8+ T-cell clones were incubated with a blocking antibody against CD8 during the incubation with the B-LCL panel. The anti-CD8 antibody caused a complete block of CTL-reactivity, strongly indicating that TAP-impaired targets were recognized via the TCR (fig 6). Secondly, to determine the HLA-I restriction of the human T-cell clones, we generated an additional TAP-inhibited B-LCL, which expresses a complementary set of HLA alleles (B-LCL HD10). All three CTL clones were tested for IFNγ production against B-LCL hodo, modo and HD10 (Table III). Importantly, the CTL clones did not show reactivity against completely HLA-I mismatched target cells (Table III), even when the UL49.5 TAP-inhibitor was properly expressed, clearly indicating a dependency on specific HLA class I molecules. With the use of these target cell panels we were capable to determine the shared HLA-restriction element that determined the restriction of the three CTL clones: HLA-A2, HLA-B8 and HLA-B44 (Tabel III). Some ambiguity was left for the CTL clone 7.12 concerning HLA-I restriction, since both A2 and Cw5 were still candidates (Tabel III). The HLA-A2 allele could, however, be appointed as restriction element for this CTL clone, because CTL reactivity was blocked using a HLA-A2-specific antibody, whereas an antibody against HLA-B and -C molecules did not block the recognition (supl fig 2). The fact that each T-cell clone used a different HLA-restriction element for recognition of target cells indi-

Tabel III: HLA-I restriction of CD8+ T-cell clones

		CD8+ T-cell		
B-LCL target		**clone 7.12**	**clone 4.16**	**clone 2.3**
Modo	shared HLA-I[a]	**A2**, B44, **Cw5**[b]	A2, B44, Cw5	**B44**, Cw5
	T-cell recognition[c]	654[d]	17	474
Hodo	shared HLA-I	none	A1, **B8**, Cw7	A11
	T-cell recognition	4	244	23
HD10	shared HLA-I	B44	A1, B44, Cw7	**B44**
	T-cell recognition	11	3	121

a HLA-I expressed by both the CTL of the donor and the B-LCL
b In bold are possible restriction molecules for CD8+ T-cell clones
c Specific IFNγ release (pg/ml) by the T cell clones calculated by the IFNγ release against B-LCL UL49.5 minus the IFNγ release against the B-LCL control.
d mean value of IFNγ of triplicate samples, SD < 50 pg/ml in all cases

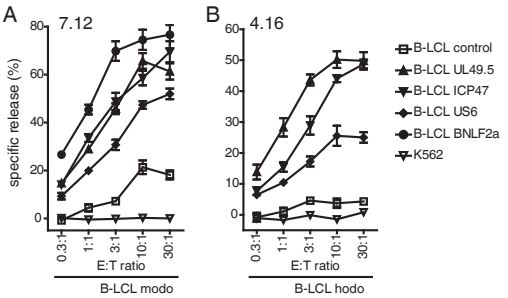

Figure 7: CD8⁺ T-cell clones lyse TAP-inhibited B-LCL. (A-B) The B-LCL cell panels were loaded with chromium and used as targets for CTL clones 7.12 (A) and 4.16 (B). K562 cells are HLA-negative target cells and were not lysed by the CTL clones, indicating the absence of natural killer-like reactivity. Different effector to target (E:T) ratios were tested and data points represent means and standard deviations from triplicate wells. Similar results were observed in two independent experiments.

cated that multiple different peptide-epitopes are involved in the TEIPP-specific response of healthy subjects. The broadness of TEIPP-specific T-cells was further supported by the finding that the polyclonal T-cell culture of donor HD2 displayed reactivity against both hodo (A11 matched) and modo (B44 matched) B-LCLs (fig. 3c), whereas the modo stimulated CTL clone derived thereof was solely restricted by the B44-allele (Tabel III). All together, these data demonstrated that the TEIPP-directed T-cell response in humans is broad and involves multiple HLA-I presented peptide-epitopes.

CD8+ T-cell clones lyse TAP-impaired B-LCL

To examine if the CD8⁺ T-cell clones were also capable to lyse TAP-deficient targets, a chromium release assays was preformed. The two tested T-cell clones 7.12 and 4.16 efficiently lysed all four TAP-inhibited target cells, whereas their TAP-proficient counterparts were not killed (fig 7a,b). The specificity pattern of the T-cell clones matched that of the IFNγ release assays (fig 5a,b). To determine the contribution of NK-like reactivity to this lysis, the K562 cell line, which is completely HLA-I negative, was included as a target. K562 cells were not killed by the T-cell clones, indicating that our cytotoxic T-cell clones did not exhibit HLA-I-independent killing activity (fig 7a,b). We speculate that our T-cell clones constitute the first examples of TEIPP-reactive CTL in humans, and that this CD8⁺ T-cell subset is well represented in PBMC of healthy subjects.

Discussion

Here we present the first evidence for the existence of TEIPP-specific CTL in humans. These CD8⁺ T cells selectively recognize TAP-inhibited target cells, which are generally resistant to detection and lysis by conventional CTL. TEIPP-specific CTL were already detectable in short term T-cell cultures that received only three rounds of stimulation, indicating that TEIPP-CTL are well represented in the PBMC of healthy subjects. All PBMC cultures exhibited reactivity against B-LCL expressing a viral TAP-inhibitor, but not against the normal B-LCL, suggesting that TEIPP-CTL are generally present in the human population and target a broad repertoire of peptides which are presented upon TAP-inhibition. Analyses of TEIPP-specific CTL clones revealed that target cell recognition depends on the T-cell receptor and that several different classical HLA-I molecules present these cognate peptides. These data are in complete analogy with our previous findings in mouse models [28-31].

TEIPP-specific T cells were isolated by stimulations of PBMC from healthy donors with autologous dendritic cells that were electroporated with RNA encoding the BHV-1 gene UL49.5. The protein from this bovine herpesvirus efficiently inhibits the peptide transporter TAP (see fig 1) through induction of a conformational arrest and proteasomal degradation [8-10]. Using this autologous culture system, we minimized the risk of inducing artificial T-cell specificities, e.g. allogenic-HLA antigens, minor histocompatibility antigens and vector-derived antigens. The generated T-cell cultures were tested against panels of B-LCL stably expressing one of four different well characterized viral TAP inhibitors (UL49.5, ICP47, US6 and BNLF2a). The polyclonal T- cell cultures, and also the isolated T-cell clones responded to all four TAP-inhibited cell lines, excluding the UL49.5 protein, which was present during the culture period, as the source of target peptides. Although RNA electroporation is only transient and not completely inhibiting TAP function (fig 1b), it is sufficient enough to alter the repertoire of peptides as such that CTLs which are specific for TAP-inhibited cells are induced. This is in line with our previous studies in which both TAP-knockout and TAP-inhibited cells where recognized [28-30].

The CTL clones isolated display different HLA-I restrictions for their TEIPP epitope. CTL 7.12 is HLA-A2 restricted, CTL 4.16 is most likely HLA-B8 restricted and CTL 2.3 is HLA-B44 restricted. HLA-I molecules associate in the peptide loading complex with TAP in order to directly load peptides which are

there pumped into the ER. The binding of HLA-I to TAP is not well understood, but it is shown that position 116 (p116) in the HLA-I molecule has a great effect on association with TAP and/or tapasin [40-42]. This is well illustrated by the two closely related alleles HLA-B4402 and B4405 [40-42]. Where B4402, with aspartic acid at p116, is highly dependent on TAP, B4405 with tyrosine at this position is completely independent of TAP [40-42]. Detailed analyses showed that peptide loading of HLA-A2, -A23, -B7, -B8, and -B4405 molecules is largely independent of TAP [25,26,43-47], while the TAP-dependent pathway is the main processing route for HLA-A1, -A3, -A11, -A24, -B15, -B27 and -B4402 [40-46]. It could be hypothesized that TEIPP peptides are predominantly presented by HLA-I molecules that are normally dependent on TAP for their peptide loading, since the transition to TAP-deficiency would then select for a completely distinct peptide repertoire. However, we isolated CTL with HLA-I restrictions from both categories (HLA-A2, HLA-B8, and HLA-B4402), suggesting that TAP associations are not predictive for presentation of TEIPP peptide-epitope, and that all alleles could display TEIPP upon inhibition of TAP.

Apart from presenting TEIPP at the cell-surface of TAP-deficient cells another explanation for reactivity against TAP-deficient targets could the involvement of the CD94/NKG2a complex which is expressed on some of the $CD8^+$ T-cells. Indeed our CTL 7.12 also expresses this complex, while it is absent on CTL 4.16 (supl fig 1). CD94/NKG2a can bind to surface HLA-E molecules loaded with peptide, which then acts as an inhibitory molecule [48]. Since TAP-deficient cells express these HLA-E molecules and TAP-deficients cells in lower amounts it could be possible that the TAP-independent peptide, which is recognized by the TCR of CTL 7.12, is already presented at TAP^+ but non-reactive due to the presence of HLA-E and NKG2a/CD94. However, in our previous studies in mice blocking the NKG2a had no effect on the reactivity of the CTLs [28], and we therefore have no reason to believe that this would be the case for our CTL 7.12 which is expressing NKG2a/CD94.

Molecular identification of human TEIPP antigens would enable immunotherapeutic approaches for the treatment of tumors harboring antigen processing defects. Since processing-deficient tumors are resistant to conventional anti-tumor CTL and represent a major percentage of all tumors, alternative T-cell based strategies, like TEIPP antigens, are more than welcome. In an attempt to indentify the nature of the involved peptide-epitopes, we tested HLA-A2 binding, TAP-independently processed peptides that were described by the group of Dr. Stevanovic (Tuebingen, Germany) [27] for recognition by our TEIPP T-cell clones. However, none of the peptides from this list stimulated our T-cells, and also the T2 cell-line from which these peptides were eluted was not recognized by our HLA-A2 specific CTL. Despite the fact that the exact identity of our human TEIPP peptides remains unknown, TAP-impaired DC could constitute an alternative in vaccination studies. RNA electroporation of MoDCs or B-cells with a viral TAP inhibitor is feasible in clinical settings. RNA electroporation has the advantage that the nucleotides are not integrated in the host genome, like viral vectors [33,49-53]. In our mouse model, TAP-deficient dendritic cells were capable to prevent the outgrow of TAP-deficient tumors [30]. Several improvements still need to be made to fully optimize dendritic cell vaccination in cancer patients, but the process will remain tailored and laborious as each patient needs their own manufactured vaccine. Therefore, we argue for the molecular identification of human TEIPP in order to enable peptide vaccination, which holds great promise for the future as a cancer treatment.

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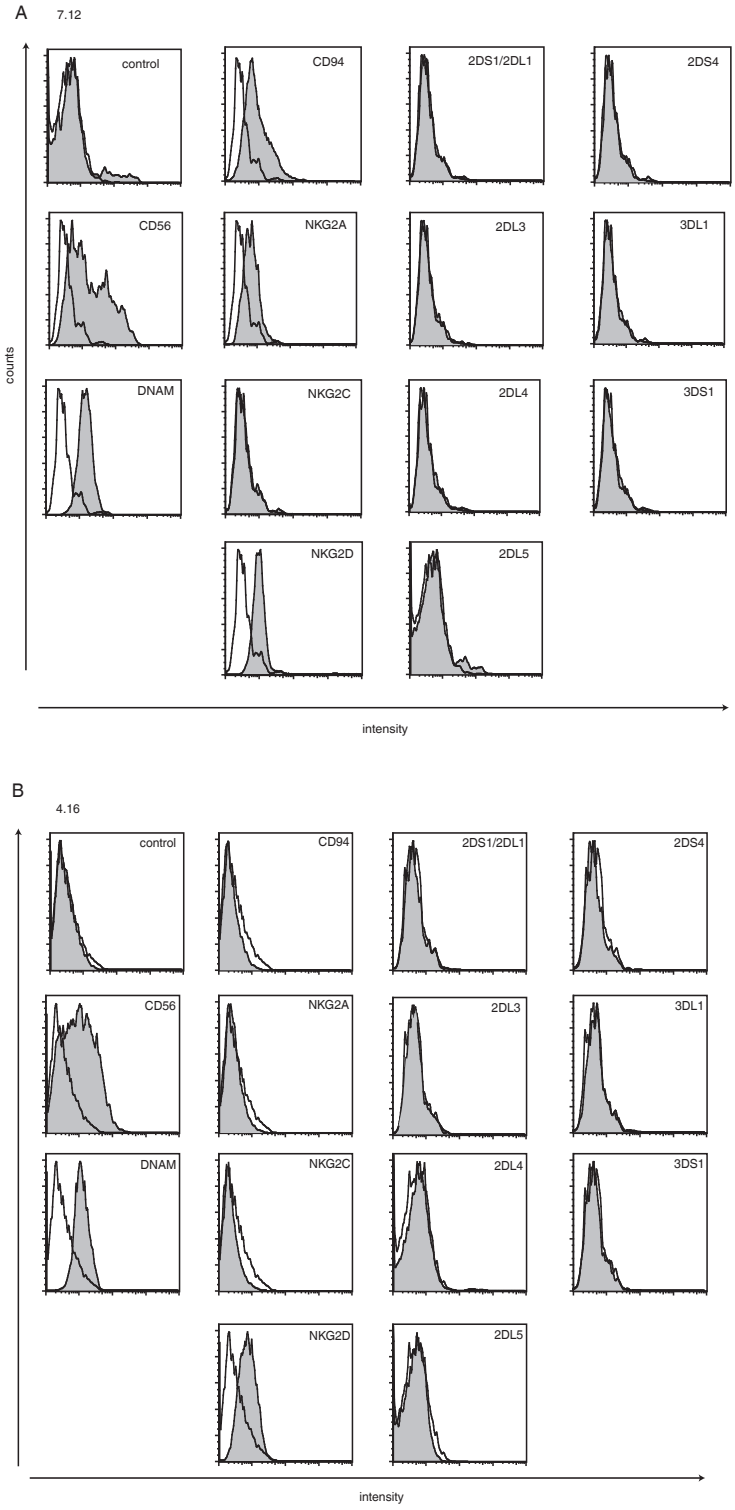
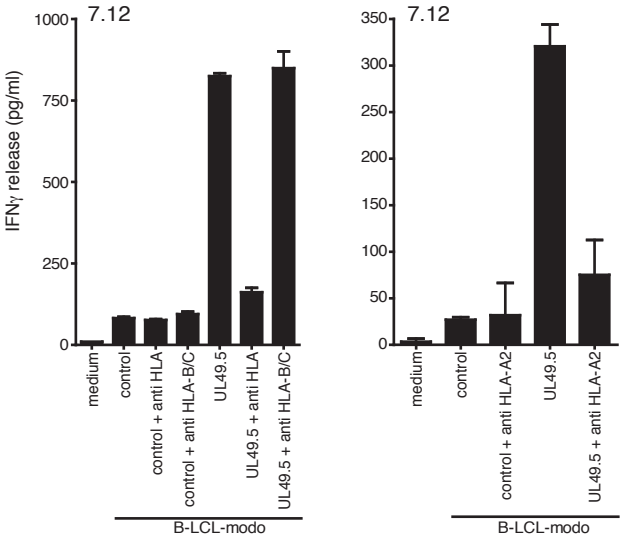


Supplementary figure 1: Clones 7.12 and 4.16 have a normal T-cell phenotype. CTL clone 7.12 (A) and clone 4.16 (B) were stained for well known T-cell, NK and KIR receptors. Clone 7.12 was positive for CD56, CD94, DNAM, NKG2A and NKG2D all markers which are expressed on a subet of T-cells. None of the KIR receptors was found at its cell surface. Clone 4.16 was positive for CD56, DNAM and NKG2D and again no KIR receptor expression was observed.

Supplementary figure 2: TEIPP-CTL clone 7.12 is HLA-A2 restricted. Target cells modo B-LCL and UL49.5 expressing modo B-LCL were pre-incubated with anti HLA-I, anti HLA-A2, and anti HLA-B/C antibodies, before clone 7.12 was added. IFNγ release by CTL was measured. Two panels represent independent experiments, which show that total HLA-I blocking and specific HLA-A2 blocking prevented CTL activation. These results corroborate those from figure 7 on the HLA-A2 restriction of TEIPP-CTL clone 7.12.



CHAPTER 3

Development of a target discovery approach for the identification of human TEIPP antigens

Margit H. Lampen, Mette Voldby Larsen, Marjolein Sluijter, Sjoerd H. van der Burg, and Thorbald van Hall

Abstract

Recently, our group identified a new category of antigens (TEIPP) that emerges at the cell surface upon impairment of the antigen presentation pathway via the TAP molecule. Two of these antigens have been identified at the molecular level, the H-2Db presented Trh4-epitope in mice and the HLA-A2 presented CALCA-epitope in humans. Moreover, in humans a large subset of T-cells was identified that recognizes target cells with a TAP-impaired antigen processing. To characterize new epitopes belonging to the category of TEIPP-peptides in humans, we developed an approach in which we predict and examine possible candidates from the human proteome. In silico prediction based on the Trh4- and CALCA-epitope resulted in 2308 candidates. Immunization of HLA transgenic mice followed by testing naïve and experienced T-cell libraries from healthy donors revealed T-cell reactivity against three out of twenty-three tested peptides binding to HLA-A*0201 molecules. It remains to be investigated whether these epitopes are indeed TEIPP-peptides and whether antigen processing results in presentation at the cell-surface.
The developed method constitutes a tool to select new TEIPP-antigens, which might be exploited in the future to elicit T-cell immunity to tumor escape variants.

Introduction

CD8+ cytolytic T-lymphocytes (CTLs) have an important role in the immune response against viruses and tumors. They recognize antigenic peptides that are presented in the molecules of the MHC class I region (MHC-I). Inhibition of the MHC-I antigen presentation pathway is therefore an attractive strategy to avoid CTL recognition, and both tumors and viruses adopted strategies to impair MHC-I antigen presentation.
Several mechanisms have been described that lead to loss of MHC-I presentation. In tumors, MHC-I antigen presentation defects are categorized by Garrido *et al* into "hard" and "soft" lesions (1). "Hard" lesions are irreversible and structural, such as loss of gene copies via loss of heterozygosity (LOH), mutations or deletions of MHC-I heavy chain genes or the β_2m gene. "Soft" lesions are reversible regulatory defects such as decreased transcription via hypermethylation or oncogene activation, and blocking of the Jak-STAT pathway. All these defects result in down-regulation of MHC-I surface expression (1-3). Similarly, decreased transcription of components of the antigen-processing machinery (APM), including peptide transporter TAP, which transports molecules from the cytosol into the Endoplasmic Reticulum (ER), results in low MHC-I surface expression (1-5). Alterations of TAP are found in a broad range of tumors varying from 10% in Head and Neck squamous cell carcinoma (HNSCC) up to 74% in renal cell carcinoma (4, 5). Moreover the frequency of TAP loss is increased in metastatic lesions. Both "hard" and "soft" lesions are associated with poor prognosis as it leads to failure of recognition and elimination by tumor specific CTLs (4-9).
Recently, our group identified a new category of tumor antigens that recognizes self-peptides emerging at the cell-surface upon impairment of the MHC-I antigen presentation pathway (10-17). We named this new category TEIPP: T-cell Epitopes Associated with Impaired Peptide Processing. TEIPP-specific T cells recognize neo-antigens and display preferential killing to cells with impairment in their antigen presentation pathway (10-16).
Two TEIPP epitopes have been identified so far at the molecular level: the Trh4-epitope in mice and the CALCA-epitope in human (14-16). The Trh4-epitope is presented in H-2Db and derives from the C'-terminus of the Trh4-protein, which is an ER membrane spanning protein (12, 14). The CALCA-epitope is derived from the signal peptide sequence of preprocalcitonin, is processed through the enzymes Signal Peptidase (SPase) and Signal Peptide Peptidase (SPPase), and presented in HLA-A2 (15, 16). Possible therapeutic applications in order to induce TEIPP CTLs have been studied in mice. Mice were vaccinated with either the short peptide Trh4, or TAP-deficient Dendritic Cells (DCs). Alternatively, in vitro expanded CTLs were transferred back in mice (10, 14). In all three cases the TEIPP-targeted immunotherapy mediated clearance of tumors with MHC-I defects, in particular TAP deficiencies (10, 14). Inducing TEIPP-specific CTLs might provide the first local immune activation and cytokine release upon recognition of these antigen-impaired lesions, and therefore addition of TEIPP-comprising antigens in vaccination could be an attractive strategy for immunotherapy.

In our search for human TEIPP epitopes, we identified a group of CD8+ T-cells, specific for TAP-impaired target cells (11). The diverse sets of T-cell Receptors (TCR) that were used by the isolated CTL clones and the confirmed TCR-dependent and different HLA-I restrictions implied the involvement of a broad TEIPP-specific repertoire (11). However the exact epitopes recognized remained unidentified. In contrast to this forward approach, we here hoped to identify TEIPP epitopes using a reverse immunology approach in which we selected predicted candidates from the human proteome, using the Trh4- and CALCA-epitopes as examples. Vaccination of HLA-A2 restricted TEIPP-candidates in HLA-A2 transgenic mice revealed five candidates out of twenty-three tested. Immunogenicity

for four of these peptide candidates was examined in human PBMCs by generating naïve and experienced T-cell libraries of healthy donors. This method revealed that T-cell reactivity against these peptides could be observed. This developed method constitutes a tool to select new TEIPP-antigens, which might be exploited in the future to elicit T-cell immunity to tumor escape variants.

Material and methods

Selection of TEIPP-candidates

Selection of possible TEIPP-comprising antigens was based the notion that the epitopes are either present in the signal sequence of the protein, like the CALCA-epitope, or at the C'-terminus of ER-resident proteins, like the Trh4 epitope. On the 1st of November in 2007, all human protein sequences from UniProtKB were downloaded (http://beta.uniprot.org/) resulting in 71.088 entries. TEIPP candidates were selected using two approaches: 1. selection of peptides within the TAP-independent part of signal sequences (method I), 2. selection of peptides from ER resident proteins (method II).

For method I we started with the SignalP-3.0 service that predicts the presence and location of signal sequences, using an algorithm model that predicts the cleavages site of SPase (http://www.cbc.dtu.dk/services/SignalP) (18-20). The SPase is determining the C-terminal amino acid of the TEIPP candidate. To take into account further trimming on the C'-terminus site of the peptide within the ER, a finding frequently observed for TAP-independent peptides, we predicted MHC-I peptide binding ligands up to four amino acids upstream of the predicted SPase cleavage site. For prediction of MHC-I peptide binding, we used NetMHC3.0 (http://www.cbc.dtu.dk/services/NetMHC) (21, 22). This sever has the best performance among 30 prediction servers for human MHC-I molecules (23). Ligands should fulfill the following criteria: 1. The ligand binds to HLA-A0101, A0201, A0301, A2402, B0702, or B4001 with a predicted binding affinity below 250 nM; 2. It has a length between 9-11 amino acids.

Method II C'-terminus: Proteins that have a subcellular location, within the UniProtKB database, in either the endoplasmic reticulum (ER) or the ER-membrane were selected. For the ER-membrane proteins the protein topology was predicted with the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) (24, 25). This program gives an indication of the organization of the protein by predicting the trans-membrane helices. Only those proteins with the C'-terminal end located inside the ER were selected. Next, MHC-I ligands were predicted by using the NetMHC 3.0 server (http://www.cbc.dtu.dk/services/NetMHC) (21, 22) according to the selection criteria as mentioned in method I.

Immunization of mice

HLA-A2 transgenic mice (B6 background HLA-A2/H2-D) (26) or control C57BL/6 mice were immunized subcutaneously with 50 µg of pools or single HLA-A2 restricted peptides and 150 µg HBV T-helper peptide (TPPAYRPPNAPIL) in PBS. As a positive control we included the HLA-A2 presented influenza epitope (GILGFVFTL). The injection site was covered with 60 mg of Aldara cream containing 5% imiquimod (3M Pharma Nederland BV). Immunization was repeated on day 7 and then combined with two intraperitoneal injections of 600.000 IU human recombinant IL-2 (Novartis) on day 7 and day 8 (27). At day 13, blood samples were taken and tested for reactivity by overnight incubation with the corresponding peptide and intracellular staining with anti-CD8-PE (clone 53-6.7, Biolegend) and anti-IFN-APC (clone XMG1.2, BD pharmingen) antibodies, as previously described (27). Samples were analyzed by flow cytometry using Cell-quest (BD bioscience) and Flowjo software (Treestar Inc). Samples were considered positive if value was higher than four times background.

Competition-based cellular peptide binding assay

To test binding affinity of predicted peptides to HLA-I, competition-based cellular peptide binding assays were performed as described earlier (28). In short, B-LCLs were treated with a mild acid (1:1 mixture of 0.263 M citric-acid and 0.126M Na2HPO4*2H2O) to remove the naturally bound peptides. After treatment, cells were incubated with 150 nM of fluorescently labeled HLA-A*0201 (FLPSDC(Fl)FPSV) reference peptide, and a serial dilution of one of the peptides of interest starting at 100 µg/ml. As a positive control, a peptide derived from influenza (GILGFVFTL) was taken, and as negative control we used a HLA-B7 binding peptide (SPSVDKARAEL) (28). Reference peptide alone was included in each experiment to determine the maximum intensity of fluorescence. Cells were measured with FACS using CellQuestPro (Calibur, BD Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA). The concentration of peptide needed to reach half-maximal binding, were defined with GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, USA).

T-cell libraries

T-cell libraries were created as previously described (29). CD14+ cells were isolated from Peripheral Blood Mononuclear Cells (PBMCs) of healthy HLA-A*0201+

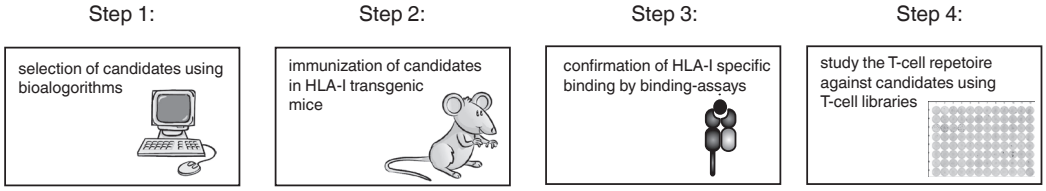


Figure 1: Selection methodology for human TEIPP T-cell epitopes. Based on the protein-location of the two TEIPP epitopes Trh4 and CALCA, a peptide candidate list was created from the human proteome using bio-informatics (step 1). The immunogenicity of these peptides was determined in corresponding HLA class I transgenic mice (step 2). Immunogenic peptides were then tested for their HLA binding affinity (step 3). Naïve and experienced T-cell libraries were created from healthy donor PBMC in order to determine if a T-cell repertoire against these peptides exist. (step 4).

donors, through positive selection using CD14+ MACS beads (Miltenyi Biotec, Gladbach Germany). Subsequently, we isolated populations of naïve and experienced CD8+ cells through FACS sorting with CD45 RA FITC (ALB11, Immunotech), CD19 PE (J3-119, Beckham Coulter), CD56 PE (MY31, BD Biosciences) , CD4 PE (HIB19, BD pharmingen), CD62L PeCy7 (DREG56, Biolegend) and CD8 APC (DK25, Dako). Naïve and experienced CD8+ cells were sorted with a FACSAria flow (BD bioscience) by selection of CD8+/CD62L+/CD45RA+ (naïve) and the remaining CD8+ (experienced) cells. After sorting, 1000 cells/well of naïve or experienced CD8+ cells were expanded with 0.5 µg/ml PHA ((HA-16) Remel Europe) in 384 wells tissue culture plates (Corning) with 50.000 irradiated pool PBMCs, and 500 units/ml IL-2 (Invitrogen Coroperation) in a volume of 50µl. Cells were cultured in IMDM medium containing 5% heat-inactivated Human Ab serum (HAb) (Greiner), 100 units/ml penicillin, 100µg/ml streptomycin (Gibco) and 2mM glutamine (Lonza), 100x Sodium Pyruvate (SP) (Gibco), and 100X Non Essential Amino Acids (NEAA) (Gibco), and incubated at 37ºC, 5% CO2.

Four days after initial stimulation, two 384 wells were pooled in one 96 well (Corning) generating mini-cultures of 2000 different specificities with a total complexity of 192,000 specificities (96 wells) of naïve T-cells, and 96,000 specificities (48 wells) of experienced T-cells. If necessary, expanding cultures were transferred to a 48 wells (Corning), or 24 wells plate (Corning) by adding medium containing 100 units/ml IL-2. Cells were tested for immunogenicity in an IFNγ ELISPOT assay at day 14 after initial stimulation.

IFNγ ELISPOT assay

ELISPOT assays were done as described before (30). Naïve or experienced CD8+ T-cell mini-cultures were co-incubated with peptide loaded (1µg/ml) autologous CD14+. Per well 100.000 T-cells were co-incubated with 10.000 peptide loaded autologous CD14+ cells overnight at 37ºC and 5% CO2. Samples were measured in duplicate and as positive control the peptide derived from influenza (GILGFVFTL), and a peptide derived from MART-1 (AAGIGILTV) were included. As a medium control, T-cells were cultured with autologous monocytes alone. ELISPOT analysis was performed using manufacturer's instructions (Mabtech Ab). Spots were visualised by SigmafastTM/BCIP®/NBT (Sigma) and the number of spots was measured with a fully automated computer-assisted video-imaging analysis system (BioSys 4000). T-cell libraries were considered positive if the number of spots exceeded the baseline, which was calculated by the mean of the medium controls plus 4 times the standard deviation (31).

Results

In silico prediction of TEIPP-candidates results in 2308 candidates

An overview of the complete methodological approach to select and test TEIPP-candidates is shown in figure 1. The two known TEIPP-epitopes are encoded at the

Table I: Number of predicted TEIPP peptide

HLA allele[a]	Representing supertype	Method I[b]	Method II
A0101	A01	3[c]	0
A0201	A02	1929	24[d]
A0301	A03	152	18
A2401	A24	52	12
B0702	B07	77	21
B4001	B44	0	20

a These alleles represent most abundant supertypes covering 99.5% of the Caucasian population
b For an overview of the selection methods see figure 2
c Number of predicted peptides. Peptides have a length of 9-11 amino acids and have a predicted HLA-binding Kd of <250nM
d These 24 peptides were tested in this study

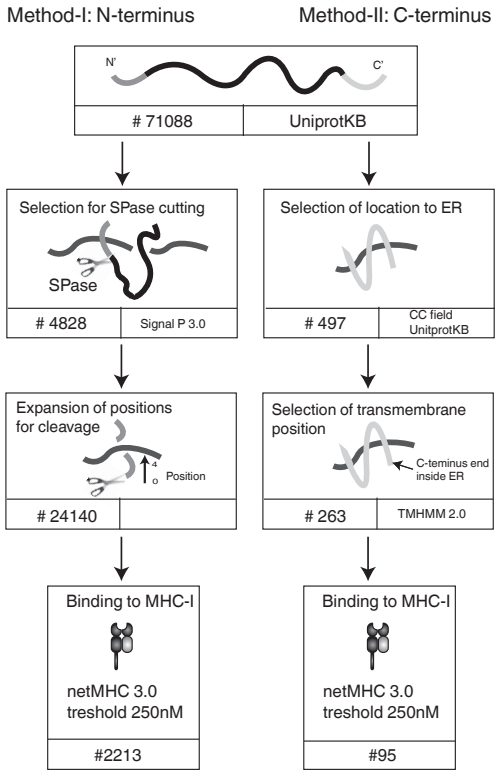


Figure 2: Overview of in silico prediction methods for the selection of peptide candidates. Two methods were used to select TEIPP candidates. Method I is based on the CALCA-epitope derived from the leader sequenced of preprocalcitonin, which is processed through the signal sequence pathway. Signal sequences from the complete human proteome were predicted with SignalP3.0 algorithm. Further trimming at the C'-terminal site of the peptide might occur within the ER and we therefore selected peptides up to four amino acids upstream of the predicted cleavage site. Binding affinity to one of the representatives of six HLA class I supertypes was then determined with netMHC 3.0.
Method II is based on the mouse Trh4-epitope, located at the C'-terminus of a ER-membrane spanning protein. All ER located proteins from the complete human proteome were selected and the transmembrane position of ER-membrane proteins was determined with the prediction program TMHMM2.0. Peptides with their C'-terminal end inside the cytosol of the ER were included. Selection of TEIPP candidates was then based on HLA class I binding predictions using netMHC 3.0

extreme ends of proteins. The human CALCA peptide is comprised at the TAP-independent segment of the leader sequence and the mouse Trh4 peptide resides at the complete C-terminus of this ER located protein (14-16). We therefore designed two in silico prediction methods that represent these two locations of TEIPP peptides. First, we selected the TAP-independent segments of all leader peptides within the human protein database, and predict their binding affinity to six supertypes, which cover 99.5% of the Caucasian population (32) (HLA-A0101, A0201, A0301, A2402, B0702, or B4001) (Figure 2; method I). The C-terminus of these peptides are predicted to be generated by the ER-resident proteolytic enzyme signal peptidase (SPase) and some imprecise determination of this C-terminus was reported for leader peptides due to cleavage variation or further trimming in the ER. Therefore, we included peptides from leader sequences up to four amino acids upstream of the predicted SPase cleavage site. Our search resulted in 2213 peptide candidates of which most were predicted to bind in HLA-A*0201 (Table I). This is not surprising as the signal peptide sequence is a hydrophobic region (20), and HLA-A*0201 prefers hydrophobic amino acids at its dominant pockets.
Second, we selected TEIPP candidates derived from the C'-terminus of ER resident or ER membrane spanning proteins. The mouse TEIPP-epitope from the Trh4 protein represents the prototype of this group. For this, we screened the human protein database for proteins with a designation of ER or ER membrane. For ER-membrane proteins we subsequently predicted their trans-membrane topology using the TMHMM-algorithm and selected those proteins that have their C'-terminal end located within the ER. These selected peptides were then subjected to prediction programs for HLA-I binding affinity to the previously described alleles (Figure 2; method II). This resulted in 95 TEIPP peptide candidates, which is considerably less than the peptides predicted in the signal sequences of proteins (Table I). However, the exact cellular localization of most peptides is not described in the SwissProt database and therefore this collection is an underrepresentation of potential TEIPP-candidates by this method.
To validate our methodology, we decided to test the immunogenicity of 24 HLA-A*0201 peptide candidates derived from ER-resident proteins, as HLA-A*0201 is the most abundant allele within the Caucasian population.

Immunization of HLA-A2 transgenic mice reveals peptide immunogenicity

To assess the immunogenicity of the selected 24 peptides, HLA-A2 transgenic mice were immunized with pools of the peptide candidates. One peptide was not analyzed because it was hard to produce due to its very hydrophobic nature. A total of 5 pools were made,

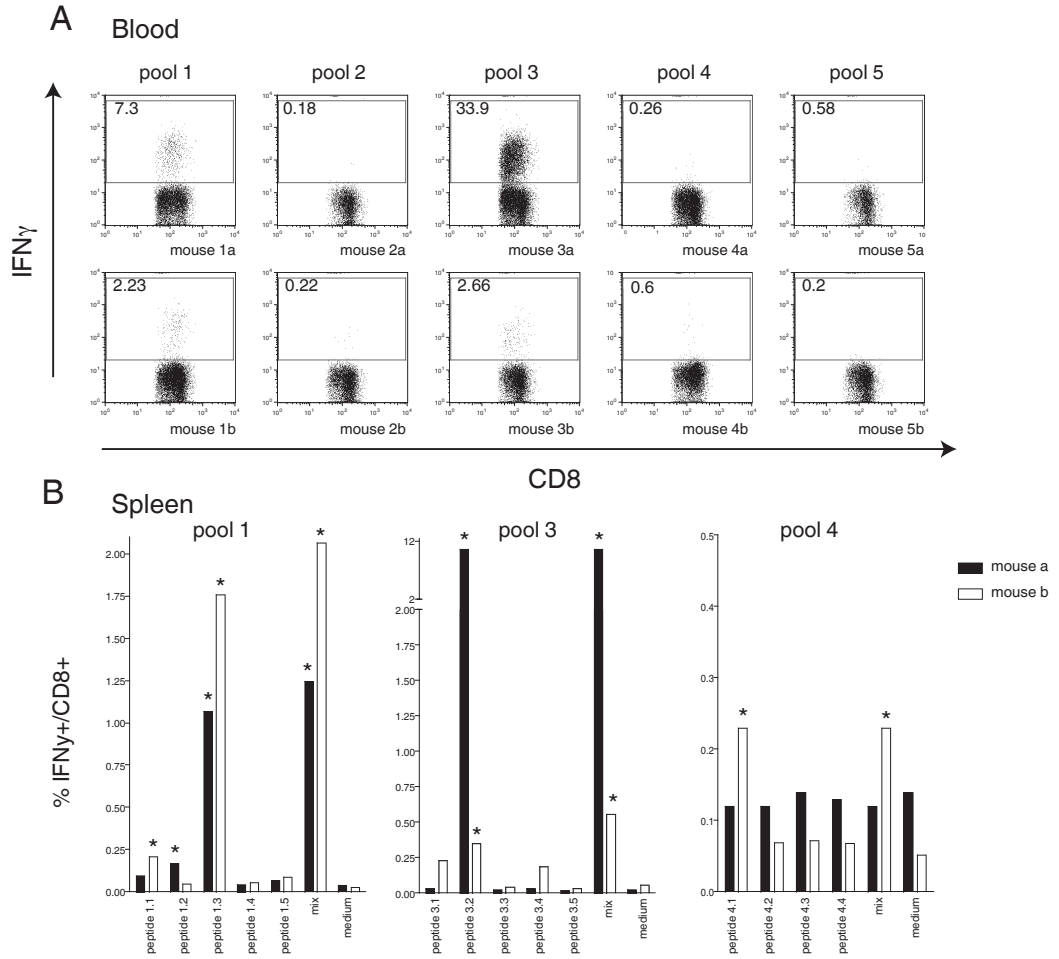


Figure 3: Immunization of HLA-A2 transgenic mice. Mice were immunized with 23 synthetic peptides from the subset of ER-resident C'-terminal ends (Method 2) that were predicted to bind to HLA-A2. These peptides were divided into 5 pools according to their predicted binding affinity (A). For each peptide pool, two mice were immunized. Intracellular flowcytometry staining of blood cells ex vivo with anti-IFNγ and anti-CD8 antibodies revealed peptide-specific CD8 T cells in pool 1, 3 and 4 (percentages are shown in upper right corner). For each pool the peptide specific response per pool is shown (e.g. pool 1 mice 1a and 1b) Average medium control of all mice was 0.18%.

(B) Spleens were isolated from IFNγ+/CD8+ mice and each peptide was tested separately for immunogenicity by means of intracellular flowcytometry. Percentage of IFNγ+/CD8+ cells after peptide stimulation is shown. In total 5 peptides showed immunogenicity in HLA-A2 transgenic mice ((indicated with a *: peptide 1.1, 1.2, 1.3, 3.2 and 4.1), exceeding the cut-off of 4 times higher than the % of the corresponding medium control. As positive control, the peptide pool was included (mix). Medium controls were non-stimulated spleen cells stained for IFNγ and CD8.

each containing five (pools 1, 2 and 3) or four peptides (pools 4 and 5), to prevent possible competition between high affinity and low affinity peptides. Pool 1 contained five peptides with the highest predicted HLA-binding affinity, and pool 5 contained four peptides with the lowest predicted binding affinity.

Mice were subcutaneously immunized and boosted with the peptide pools and stimulated with the toll-like receptor-7 ligand imiquimod and low dose IL-2. The induction of peptide-specific CD8 T cells was analyzed in blood samples five days after the last vaccination. High IFNg production was directly observed ex vivo for two peptide pools, pool 1 and 3 (Figure 3A). Comparable frequencies of peptide-specific CD8 T cells were observed for the HLA-A2 –restricted positive control peptide from influenza (GILGFVFTL) (not shown). CD8 T cells from mice immunized with pool 4 and 5 were weakly positive, and in each group one mouse exceeded the cut-off of 4 times above background (0.09% positive cells). To determine which

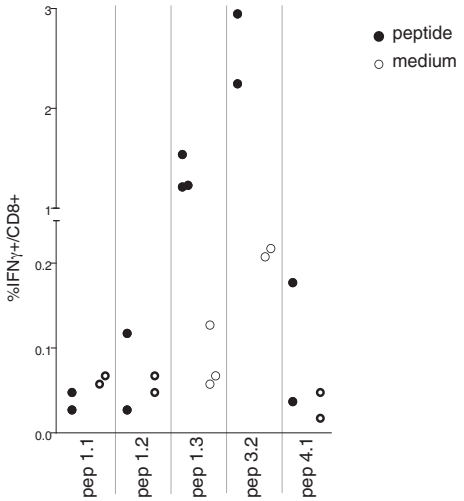


Figure 4: Single peptide immunization in HLA-A2 transgenic mice. Peptides displaying immunogenicity were selected for single peptide vaccinations in HLA-A2 transgenic mice. Mice were immunized twice with 50 µg of peptide and 150 µg HBV T-helper peptide in PBS. At the 13 after the initial stimulated blood was taken and tested for reactivity by overnight incubation with the corresponding peptide. The next day intracellular flow cytometry stainings with anti-IFNγ (APC) and anti-CD8 (PE) were performed. Peptides 1.1, 1.2 and 4.1 showed a low percentage of IFNγ+/CD8+ cells, whereas higher percentages of IFNγ+/CD8+ were observed for peptides 1.3 and 3.2. As controls, blood samples were tested in the absence of peptides and stained with anti-IFNγ and anti-CD8.

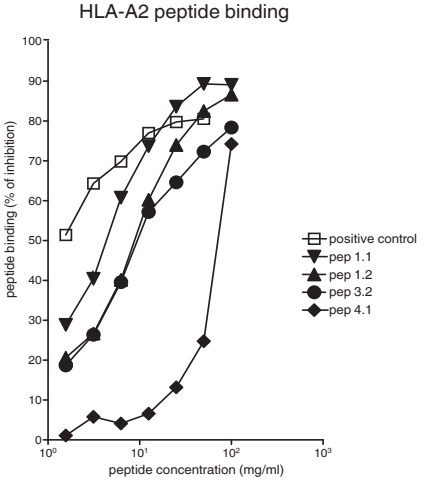


Figure 5: Peptide binding analysis for HLA-A2. Immunogenic peptides were tested for HLA-A2 binding affinity by means of a competitive-based cellular binding assay. One representative experiment is shown. Peptide binding was calculated as percentage of inhibition of the reference peptide. Peptide 1.3 showed toxicity to the cells and was therefore not included. The average IC50 values were determined based on the outcome of two independent experiments and are show in Table II.

peptides within each pool were responsible for the T cell response, spleens of the positive mice were isolated and tested for T-cell reactivity against single peptides from the peptide pools. In pool one we observed 1.4% of IFNγ+/CD8+ cells against peptide 3 (peptide 1.3, Figure 3B), whereas peptides 1 (0.15%) and 2 (0.11%) showed responses that were slightly higher than medium background (peptide 1.1 and 1.2, Figure 3B). In pool three, peptide 2 showed 8.6% of IFNγ+/CD8+ response and in pool 4 peptide number 1 (0.22%) exhibited some immunogenicity (peptide 3.2 and 4.1, Figure 3B). Reactivity against each peptide pool was confirmed from these splenocytes without further in vitro expansions (Figure 3B).

Single peptide vaccinations were then performed to test whether these peptides indeed were capable of inducing CD8+ T-cell responses. HLA-A2 transgenic-mice were immunized with peptide 1.1, 1.2, 1.3, 3.2 or 4.1. We included control wild type C57BL/6 mice to assess HLA-A2-restricted immunogenicity. We again observed high immunogenicity for peptide 1.3 and 3.2 with 1.35% and 1.92% of CD8+ cells producing IFNg (Figure 4). Peptide 1.2 (0.08%) and 4.1 (0.11%) displayed a low capacity to induce a CD8 T-cell response and no reactivity cells was seen against peptide 1.1 (0.04%) (Figure 4). Unfortunately, we observed similar results in C57BL/6 mice (data not shown) and, therefore, could not conclude that the observed T-cell reactivity was a result of HLA-A2-mediated presentation of the peptides.

Binding affinity of the selected peptides for HLA-A2

A competitive-based cellular peptide-binding assay was performed to measure the actual HLA-A2 binding affinity of the selected peptides 1.1, 1.2, 1.3, 3.2 and 4.1. Flow cytometry analysis showed that peptides 1.1, 1.2, 3.2, and 4.1 were able to bind to HLA-A2 (Figure 5). Peptide 1.1 and 1.2 showed high affinity for HLA-A2 molecules (Table II), peptide 3.2 showed intermediate affinity, and peptide 4.1 low affinity, corresponding to the predicted binding affinities by the netMHC program (Table II). No data could be generated for peptide 1.3 as high concentrations of the peptide in the cellular binding assays showed toxicity (data not shown). This prompted us to exclude this peptide in further experiments.

Table II: Peptide binding affinity to HLA-A2 of C-terminal TEIPP peptide candidates

	HLA-A*0201			
	Measured		**Predicted**	
Peptide	IC50 (mM)[a]	Affinity[b]	Kd (nM)[c]	Affinity[d]
Peptide 1.1	2.37	High	2.1	Strong
Peptide 1.2	4.08	High	6.0	Strong
Peptide 1.3	ND[e]	ND	14.6	Strong
Peptide 3.2	10.39	Intermediate	90.1	Weak
Peptide 4.1	36.31	Low	111.9	Weak
Positive control[f]	1.2	High	18	Strong
Negative control[g]	>100		24413	

a IC50 is half maximal peptide concentration in a peptide binding competition assay and represents the mean value of two independent experiments.
b Binding affinity is classified according to the following cutoff. High affinity IC50 ≤ 5 μM; intermediate affinity 5 μM < IC50 ≤ 15 μM; low affinity 15 μM < IC50 ≤ 100 μM; no binding IC50 > 100 μM.
c Kd is affinity of peptide binding according to NetMHC3.0 prediction software.
d Affinity is classified as follows: peptides with an affinity <50nM are strong binders, peptide with an affinity <500nM are classified as weak binders, no binding >500 nM
e ND= Not Determined due to cell toxicity
f Positive control is short peptide derived from influence (GILGFVFTL)
g Negative control is a HLA-B7 binding peptide (SPSVDKARAEL)

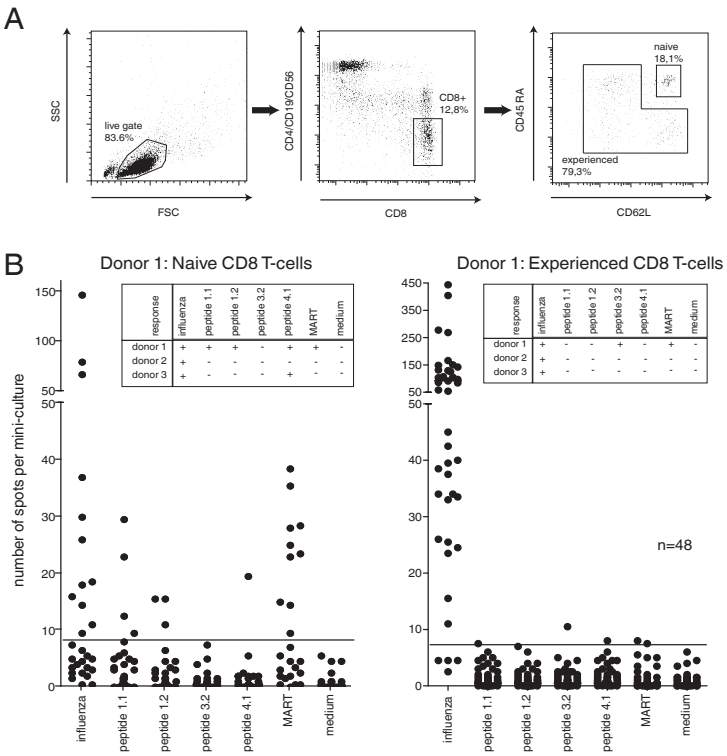


Figure 6: Naïve and experienced T-cell libraries of healthy donors. Naïve and experienced T-cell were selected by means of flowcytometry sorting (A). CD8+/CD62L+/CD45RA+ cells were considered naïve T-cells and all other CD8+ were considered experienced T-cells. Mini-cultures of 2000 cells were expanded through mitogen-driven stimulation with PHA. Two weeks after initial stimulation 96 naïve T-cell mini-cultures and 48 experienced T-cell mini-cultures were tested in ELISPOT analysis using peptide loaded autologous CD14+ cells as targets and around 105 of T-cells (B). As controls the HLA-A*0201-restricted influenza peptide and a HLA-A*0201 restricted peptide derived from the tumor antigen Melan-A/MART-1 were included. Non-peptide pulsed autologous CD14+ cells functioned as negative targets Samples were considered positive once they exceed the line. The cut-off was determined as the mean of medium plus four times its standard deviation. Tables in right corner of graphs show summarizing data for three healthy donors.

Table III: Summary of HLA-A2 restricted peptide

Experiment	Immunization HLA-A2 mice		Binding assay	T-cell library
	pool	single		
Peptide	IFNγ+/CD8+[a]	IFNγ+/CD8+	Affinity[b]	+/(no. tested)
1.1	+	-	High	1/3
1.2	+	+	High	1/3
1.3	+++	+++	ND	ND
3.2	+++	+++	Intermediate	0/3
4.1	++	+	Low	2/3

a classified as follow: - = <0.05 , + = 0.05<x<0.20, ++ <1.0, +++ >1.0
b Binding affinity is classified according to the following cutoff. High affinity IC50 ≤ 5 μM; intermediate affinity 5 μM < IC50 ≤ 15 μM; low affinity 15 μM < IC50 ≤ 100 μM; no binding IC50 > 100 μM.

Naïve T-cells mini-cultures reveal human T-cells reactive to TEIPP-candidates

To determine whether peptides 1.1, 1.2, 3.2 and 4.1 have existing cognate T-cell repertoires in humans, naïve and experienced CD8+ T-cell libraries were created. For these T-cell libraries, CD8 T were separated into naïve and experience T-cells by flow cytometry sorting and subsequently expanded in order to get mini-cultures with a maximum of 2000 different specificities. This results in a vastly increased frequency of potential peptide-specific T cells compared to their natural prevalence in the total T cell pool (29). This approach was chosen because we expected too low frequencies in the human T-cell repertoire for staining and isolation with peptide/MHC-I tetramers (29) of both naïve and experienced CD8+ T-cells. Naïve T cells from HLA-A*0201 positive healthy donor PBMCs were sorted on basis of the cell surface profile CD8+/CD45RA+/CD62L+ and the remaining CD8+ cells comprised all antigen-experienced CD8+ T-cells, including effector, effector-memory and central memory cells (Figure 6A).
Mini-cultures originating from 2000 different T-cell specificities were generated by mitogen-driven expansion, and tested for reactivity against peptides 1.1, 1.2, 3.2, and 4.1 in an ELISPOT assay. The HLA-A2-presented influenza peptide (GILGFVFTL) was included as positive control and reactivity against this peptide was indeed found in several mini-cultures of both naïve and experienced T-cells of all three tested donors (33) (Figure 6B).
For donor 1 (Figure 6B) nearly all mini-cultures from antigen-experienced T cells (45 out of 48) an influenza-specific response was detected, indicating a frequency of at least 1 in 2000 (0.05%) of the experienced T cell pool. Multiple M1-specific T cells in one mini-culture will result in multiplication of the number of spots per positive culture, a phenomenon indeed observed in the data (Figure 6B). Interestingly, we could also detect influenza M1 specific T cells in the naïve repertoire, implying an even greater potential of immune cells that have not been recruited in the anti-influenza response yet. An HLA-A2 binding peptide derived from the melanoma antigen Melan-A/MART-1 was also included, since it is known that the naïve CD8+ T-cell precursor frequency is exceptionally high against this peptide due to thymic positive selection (29, 34, 35). Indeed, MART-1 specific T cells were only detected in the population of naïve T cells (10 out of 96 cultures for donor 1; Figure 6B).
In two out of three donors tested, we observed low reactivity against peptide 4.1 in the naïve T cell repertoire, indicating the presence of T-cells against this self-peptide. One donor also showed somewhat higher reactivity against peptides 1.1, and 1.2 (Figure 6B). Further research needs to confirm the TEIPP identity of the peptides. We conclude that the approach described in this paper is suitable for identifying new T-cell epitopes.

Discussion

In this paper, we describe an in silico selection method for potential human TEIPP-epitopes. Immunogenicity and binding capacity to HLA-A*0201 of 24 peptide candidates were tested for peptides that derive from C'-termini of ER-membrane- or ER-resident proteins in vitro. Immunization of HLA transgenic mice selected five immunogenic peptides that were shown to bind to HLA-A2 molecules. Using naïve and experienced CD8+ T-cell libraries of healthy HLA-A*0201+ donors we observed T-cell reactivity in the naïve repertoire against peptide 4.1 in two out of three donors and in one donor against peptides 1.1 and 1.2. This indicates that our method is suitable for identifying new TEIPP-epitopes.

TEIPP antigens are presented by HLA class I molecules at the cell-surface of cells with impairment in the antigen presentation pathway. The transporter molecule TAP is the major target in both tumors and viruses. Since only two TEIPPs have been identified so far, the Trh4-epitope in mouse models (14) and the CALCA-epitope in human (15, 16), we conducted our predictions according to the nature of these epitopes.
In our first in silico prediction method that was based on the CALCA-epitope, we found 2213 peptide candidates for six different HLA alleles, covering 99.5% of the Caucasian population (32). Most of these peptides were predicted to bind in HLA-A*0201 molecules (Table I), which is not surprising as signal sequences and this allele both have a preference for hydrophobic residues (20). This is also substantiated by the fact that hydrophobic signal peptides were observed in HLA-A2 molecules in TAP-negative T2 cells (36, 37). HLA-A2 molecules are indeed relatively unaffected by defects in the peptide transporter and TAP-independent leader sequences emerge in HLA class I molecules on T2 cells (38).
The CALCA-epitope was not among these 2213 peptide candidates as the predicted binding affinity was weaker than the cut-off value 250nM. So far, T-cells directed against the CALCA-epitope have been identified in one lung-carcinoma patient (15, 16), and CALCA-reactive T cells were not observed in our T-cell libraries (data not shown). Furthermore, stimulation of T-cells with CALCA-loaded autologous dendritic cells did not result in peptide specific expansion (data not shown). We were also unable to detect CALCA-specific responses in PBMCs derived from the original lung-carcinoma patient ten years after treatment (data not shown). We concluded that CALCA is a weakly immunogenic epitope and this prompted us to first focus on strong HLA-binding epitopes.

Identification of TEIPP-candidates derived from the C'-terminus of ER-resident proteins, resembling the mouse TEIPP Trh4, resulted in 95 candidates (Table I). This is considerably less then the candidates derived from the N'-terminus of the whole protein, mainly due to much lower numbers of C'-terminus peptides predicted to bind in HLA-A*0201. The 24 TEIPP candidates from this category with a predicted binding to HLA-A*0201 were tested in the current study. The 23 synthesized peptides (one candidate could not be produced) were divided into 5 pools according to their predicted binding affinity and HLA-A2 transgenic mice were immunized. Five peptides showed immunogenicity in HLA-A2 transgenic mice (Figure 2 and 3), but these peptides were also shown to be reactive in C5BL7/6 mice (data not shown). We continued our approach with these five immunogenic peptides and an overview of the results is shown in Table III.
In the competitive cellular binding assay one peptide was excluded because it showed toxicity with high concentration. This is most likely due to its very high hydrophobic region at the C'-terminus of the protein (two tryptophan's at position 9 and 10 of this 11mer peptide). In two out of three donors we observed peptide reactivity against peptide 4.1, and one donor showed reactivity against peptides 1.1 and 1.2 (Figure 6). We concluded that T-cell reactivity against these self-antigens is present within the human population.

Additional experiments have to be done before we can conclude whether these three peptides are TEIPP-antigens and whether antigen processing results in presentation of these epitopes at the cell-surface. Therefore, T-cell clones specific for these peptides have to be isolated. However, the frequencies of these T-cells are low. As example only one library of the 96 tested shows a positive reaction of 30 spots. The occurrence of this T-cell specificity in this particular mini-culture is then estimated as 0.03% (=(30/100.000)*100), as each ELISPOT test well contained roughly 100.000 cells/well. This nicely corresponds to the number of one specific T cell in the original pool of 2000 other specificities. In total 96 mini-cultures were tested resulting in a final frequency of this T cell in the total naïve T-cell library of approximately 0.0003% (=0.03/96).
To successfully isolate a specific T-cell clone our approach should therefore first focus on increasing the frequency of these T-cells. Unfortunately not enough material remains after testing the mini-cultures in ELISPOT and therefore a different approach should be used after determining possible candidates by the above-described method.
One method we could use is enrichment of peptide specific T cells with a method described by Hombrink et al (39). Here they stain PBMC of healthy donors with peptide specific tetramers and then isolate these tetramer positive cells through MACS sorting (39). To further increase enrichment of the T-cell frequency we could combine this method with pre-selection of naïve T cells. Once a T-cell clone is established its reactivity should be tested against a broad panel of cell lines to determine possible cell-specific reactivity. Since the approach we used is reverse immunology it is not determined yet whether the peptides are processed and presented at the cell surface of cells. If the T cells are selectively reactive against some processing impaired cell lines, a broader panel of tumor cells should be analyzed to exclude possible autoimmune reactions. Restoration of the antigen presentation machinery could furthermore determine TEIPP specificity. If the identified epitope is indeed a TEIPP, and is expressed on a broad range of tissues, this epitope could be used

in immunotherapy against tumors frequently associated with defects in the antigen presentation pathway.

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CHAPTER 4

Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2

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in submission

Abstract

The non-classical HLA-E is a conserved class I molecule that mainly presents monomorphic leader peptides derived from other HLA class I molecules. These leader peptides comprise an optimized sequence for tight and deep binding into the HLA-E groove. Viruses and tumors have, however, adopted strategies to inhibit the peptide transporter TAP and thereby prevent the loading of classical leader peptides in HLA-E. This alternative peptide repertoire has not been revealed so far. We identified more than 500 unique peptide sequences carried by HLA-E and found that their binding motif is different from the dominant leader peptides. Hydrophobic amino acids were only found at positions 2 and 9, closely mimicking the peptide binding motif of HLA-A*0201. HLA-E-eluted peptides were indeed capable to bind this classical HLA class I molecule. Our findings suggest that the dominant leader peptides uniquely conform to HLA-E, but that in its absence a peptide pool is presented like that of HLA-A*0201.

Introduction

HLA-E molecules are encoded by the MHC class I region and belong to the non-classical antigens. They display a similar conformation as the classical HLA class I (HLA-I) molecules and also carry peptide fragments in their binding grooves. The unique feature of HLA-E is its extreme conserved nature in the human population: only two alleles exist (E*0101 and E*0103), which vary in one amino acid at a position outside the peptide-binding groove [1]. The presented peptides are also monomorphic and predominantly derive from the leader sequences of other HLA-I molecules and harbour optimal residues for deep binding in the hydrophobic pockets of HLA-E [2]. Interestingly, signal sequences from HLA-I are strikingly homologous throughout mammal species, pointing at a conserved function of this HLA-E/peptide complex [3]. Indeed, the germline encoded receptors CD94/NKG2 that engage HLA-E are found in multiple species and play an important role in natural killer cell function [4]. These heterodimer receptors interact with residues from the monomorphic leader peptides and can therefore be designated as peptide specific [4, 5].

HLA-E protein expression can be detected on almost all nucleated cells of the body and the levels on immune cells, endothelial cells and keratinocytes are particularly high [6, 7]. Increased expression has been demonstrated upon cellular stress caused by viral infection or heat shock and in cancer cells [2, 8, 9]. In these cases, HLA-E can present peptides from other sources than the signal sequences of classical HLA-I molecules. An efficient way to replace the dominant leader peptides is through inhibition of the peptide transporter TAP, which is frequently targeted by viruses and tumors. Some of the alternative peptides were able to induce CD8 T cell responses [2, 9], showing that HLA-E has a more versatile role than only serving natural killer cells by an indirect display of 'self' MHC.

So far, the peptide-binding motif of HLA-E has been based on the HLA-I leader peptide or random peptide libraries that poorly associated with HLA-E [5, 10, 11]. Here we characterized more than 550 natural peptides from TAP-deficient cells. Due to this large number of sequences, we were able to determine a clearly defined peptide-binding motif for HLA-E and surprisingly found that this motif mirrors that of HLA-A*0201. These peptides might adopt another conformation than the dominant leader peptides that are normally folded into HLA-E.

Material and methods

Cell-lines

HLA-I negative K562 cells were transfected with HLA-E*0103. K562.HLA-E.B8 cells were obtained from Dr. E. Weiss (Munchen, Germany) and contained a human b2m construct with the leader sequence of HLA-B8. The UL49.5 gene of Bovine Herpes Virus-1 was introduced via retroviral transduction [12]. The endogenous HLA-I typing of K562 is HLA-A11, -B18 and -Cw05. All cell lines were cultured in IMDM medium (Lonza group Ltd, Switzerland) containing 8% heat-inactivated FCS (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin (Gibco) and 2mM glutamine (Lonza).

Flow cytometry

The following antibodies were used: anti-HLA-A/B/C (clone W6/32) from Serotech and anti-HLA-E (clone 3D12) and anti-HLA-A2 (clone BB7.2) from BD bioscience. Cells were measured using a FACS Calibur with Cellquest software (BD bioscience), and analysed with Flowjo software (Tree star, Inc).

Peptide transport assay

Peptide transport was performed on permeabilized K562 cells and fluorescein-conjugated synthetic peptide CVNKTERAY as described previously [12]. The fluorescence intensity was measured and peptide transport calculated as a percentage of translocation relative to control cells.

Purification and sequencing of HLA-E peptides

HLA-I/peptide complexes were purified from 1011 cells by affinity chromatography using antibody W6/32. After 10 kD filtration of acetic acid eluates, the complex

peptide pool was fractionated using capillary C18-chromatography and peptide fractions were analyzed by on-line nanoHPLC mass spectrometry as described earlier [13]. Tandem mass spectra were matched against the IPIhuman v 3.72 using Mascot 2.2.04 (http://www.matrixscience.com) and sorted using Scaffold 2.2 (http://www.proteomesoftware.com). 8-13-mer peptides with a mascot ion score higher than 35 were selected.

Peptide binding assays

Peptide binding affinity to HLA-A*0201 was performed as described earlier [14]. Influenza derived peptide (GILGFVFTL) functioned as positive control and a HLA-B7 binding peptide (SPSVDKARAEL) as negative control. Binding affinity of peptides to HLA-E*0103 was determined in a competition-based refolding assay as described earlier [15]. As a positive control we used the leader peptide (VMAPRTLVL) from HLA-A2. Peptide binding of Qa-1^b eluted peptides [13] to H-2K^b and H-2D^b was assessed by cell-surface stabilization on RMA-S cells as described earlier [16]. As control peptides E1A$_{234-243}$ (SGPSNTPPEI) (for D^b) and MuLV env$_{189-196}$ (SSWFITV) (for K^b) were used.

Results and Discussion

Absence of HLA-I derived leader peptides by inhibition of TAP in K562 cells

For the characterization of the natural peptide repertoire of HLA-E that emerges at the cell surface in the absence of the dominant HLA-I leader peptides, we made use of the myeloid leukemia cells K562. This cell line is devoid of surface HLA-I molecules and transfection of HLA-E*0103 resulted in low expression levels of this non-classical HLA (Suppl Fig 1). Increased surface display was observed after introduction of a good binding leader peptide from HLA-B8 ('K562.HLA-E.B8'), confirming previous data that these leader peptides optimally stabilize HLA-E by making full use of all binding pockets [17]. Surprisingly, affinity purification of HLA-E/peptide complexes from K562.HLA-E cells without the HLA-B8 leader construct revealed the presence of leader sequences from HLA-A11 and -Cw05, the endogenous HLA genes of K562 (Suppl Table I). This indicated that transcription of HLA genes was not completely silent in our K562 cells and that the HLA-E peptide repertoire of these cells still comprised some dominant HLA-I leader peptides. To prevent these endogenous leader peptides from loading unto HLA-E, we impaired the activity of the peptide transporter TAP in K562.HLA-E cells using the small viral evasion molecule UL49.5 from a herpes virus ('K562.HLA-E.UL49.5') [12]. A peptide transport assay confirmed the impediment of TAP activity by 70% (Suppl figure 2), while the surface expression of HLA-E did not change (Suppl Fig 1). Therefore, we used the K562.HLA-E cells with the viral TAP-inhibitor to explore the alternative peptide-binding repertoire of HLA-E.

Description of the peptide binding motif of HLA-E

Biochemical purification and mass spectrometry sequencing of HLA-E bound peptides revealed 552 unique sequences (Suppl Table I). Interestingly, only eight of these peptides were also found in TAP-suffi-

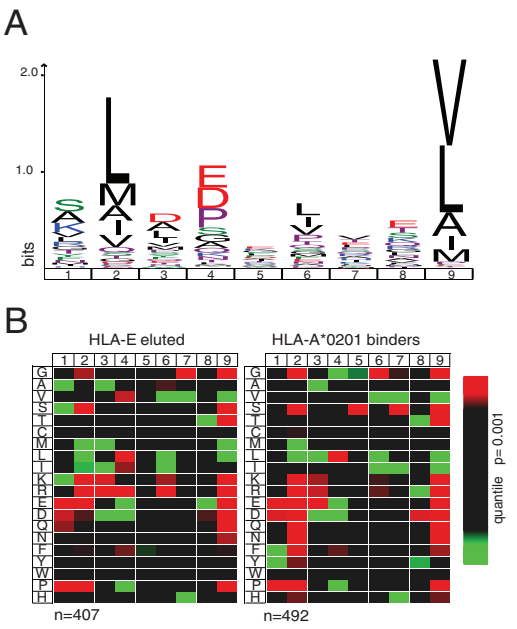

Figure 1: Peptide binding motif of HLA-E is similar to that of HLA-A*0201. (A) 9-mer peptides eluted from HLA-E and sequenced by mass spectrometry (407 hits) were subjected to a motif search algorithm [18]. The Swiss-Prot database of human proteins was set as a reference to correct for the general occurrence of amino acids. Letter sizes reflect the dominance of that amino-acid at that position. Positions 2 and 9 were assigned as major anchor positions and position 4 might represent a minor anchor. Amino acid color code: black for aliphatic or small, red for acidic, blue for basic, green for aromatic and purple all others. (B) Heat-maps of HLA-E (all 9-mers from K562.HLA-E.UL49.5 cells) and HLA-A*0201 (www.SYFPEITHI.de) are strikingly similar. Frequently occurring amino acids are indicated in green and underrepresented residues are indicated in red. Heat-maps show great similarity, indicating a shared peptide-binding motif. Statistical significance was stringently set on p=0.001. N is number of peptides of that set.

Table I: Binding affinity of some HLA-E eluted peptides

	HLA-E*0103	HLA-A*0201	
Peptide sequence	**IC50[a] (μM)**	**IC50 (μM)**	**Comment**
ALLDKLYAL	30.75	1.18	
FLMFIHQV	38.60	0.24	
LLLDVPTAAVQA	>200	1.89	Previously eluted from HLA-A*0201[b]
LLLDVPTAAV	>200	0.53	Previously eluted from HLA-A*0201[b]
KMDASLGNLFA	150	1.11	Previously eluted from HLA-A*0201[b]
ALVVQVAEA	11.15	2.58	Previously eluted from HLA-A*0201[b]
LLDRFLATV	>200	0.56	Previously eluted from HLA-A*0201[b]
ALWGPDPAAA	>200	1.62	Previously eluted from HLA-A*0201[c]
VMAPRTLVL	0.0065	10.47	Control peptide HLA-E binding
GILGFVFTL	8.58	1.2	Control peptide HLA-A2 binding
SPSVFKARAEL	>200	>100	Negative control peptide

a IC50 is peptide concentration needed for half maximal competition of reference peptide and represents the mean value of two independent experiments. Of note: HLA-E binding was determined in a cell-free protein refolding assay and HLA-A2 assays were performed with acid stripped cells.
b see reference 20.
c see reference 19.

cient K562 cells, indicating that the peptide transporter greatly impacts on the peptide repertoire of HLA-E. We recently showed the same diversity of peptides on TAP-deficient cells for the mouse homologue of HLA-E [13]. A vast majority of the natural HLA-E ligands were 9 amino acids long (73%) and less than 2% were longer than 11-mer (Suppl Table I). Of note, we did not observe an overrepresentation of signal sequences.

So far, the peptide-binding motif of HLA-E has been determined with synthetic peptides based on the classical HLA-I leader peptides or using random nonameric peptide libraries that poorly associated with HLA-E [5, 10, 11]. So the described motif was not based on the naturally presented peptide repertoire and we are the first to report sequencing of the actual natural ligands. Due to the large number of peptide sequences we identified from HLA-E, we were able to analyze preferred amino acid occurrence at each position of the total pool of 9-mers (407 peptides) using IceLogo software [18]. At amino acid position 2 and 9, the aliphatic amino acids L, M, V, I, and A were clearly preferred (Fig 1a). These positions correspond to the deep pockets B and F, respectively, of HLA-E [17]. This finding corroborates previous studies on HLA-E binding peptides that assign position 2 and 9 as major anchor positions [10]. The presence of glutamine (Q), which followed the aliphatic amino acids at position 2, was also reported before [5]. The third dominant anchor for the HLA-I leader peptide is position 7 and the crystal structure of HLA-E showed that the large hydrophobic group of leucin (L) at this site completely occupies the deep E-pocket and pulls the leader peptides down into the groove much lower than in any other class I molecule [17]. Strikingly, the TAP-independent peptide repertoire did not exhibit a preferred amino acid at position 7 nor for two shallower sockets at position 3 and 6. These additional discrepancies confirm the notion that the broad array of peptides found in HLA-E in the absence of the dominant HLA-I leader peptide displays a more central position in the groove, which is more comparable with other HLA-I molecules. The deep and firm position of HLA-I leader peptides is unique for this particular MHC/peptide complex that is engaged by CD94/NKG2 receptors on natural killer and CD8 T cells. We concluded that the main anchors of HLA-E binding peptides are positions 2 and 9 for aliphatic residues, and that position 4 might serve as a secondary minor anchor site.

*Peptides eluted from HLA-E display a high binding capacity to HLA-A*0201, revealing a shared binding motif.*

Surprisingly, evaluation of the peptide sequences obtained through mass spectrometry revealed six peptides that were described in literature to be presented

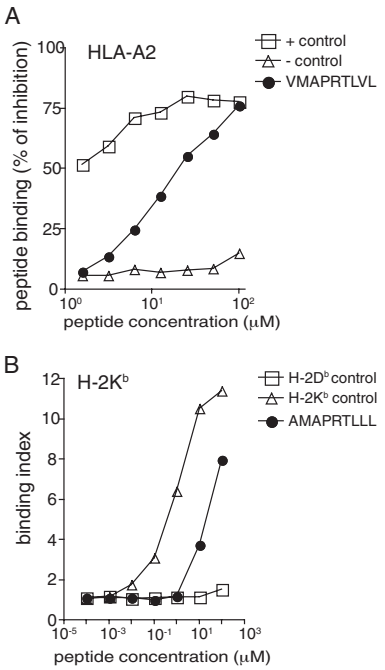

Figure 2: HLA-I derived leader peptides bind to classical class I molecules. (A) The human leader peptide VMAPRTLVL displays intermediate affinity to HLA-A2 molecules as tested in a cellular competition-based assay and (B) the mouse leader peptide AMAPRTLLL displays high affinity to H-2Kb molecules as tested in an RMA-S binding assay. Binding affinity is expressed as percentage inhibition of the reference peptide (in A) or as an index of surface stability (in B). These assays were performed three times with similar outcome.

by HLA-A*0201 (Table I) [19, 20]. Our previous peptide elution experiments confirmed that these peptides could be found in HLA-A2. To examine the actual capacity, of these six peptides, to stabilize HLA-A*0201, we preformed peptide binding affinity assays for this HLA molecule and confirmed their high affinity association (Table I). Two additional selected peptides from our HLA-E collection also bound with high affinity to this classical HLA. Importantly, K562 cells do not carry any HLA-A2 alleles in their genome, excluding the possibility that our 552 identified peptides were purified from contaminating endogenous HLA-A2 molecules. Four of the tested peptides displayed binding to HLA-E in a cell-free refolding assay (Table I).

Two other examples of peptides with dual binding capacity to both HLA-E and HLA-A*0201 were found in literature [21, 22], suggesting that this might be a more common feature. To further explore this idea of a shared binding motif, we created so-called heat-maps of all identified 9-mer peptides from HLA-E (407 hits) and compared them with a heat-map of all 9-mer HLA-A*0201 binding peptides reported in the SYFPEITHI database (492 hits) (http://www.syfpeithi.de). These heat-maps allow the visualization of preferred (in green) as well as underrepresented (in red) amino acids at all positions of the peptide repertoires. The patterns of HLA-E and HLA-A*0201 were strikingly similar (Fig 1b). Great similarities in presence and absence of amino acids were detected. Heat-maps of 8-mer or 10-mer peptides confirmed the presence of aliphatic amino acids at position 2 and 9, but numbers were too low to yield statistical significance for most positions. Main anchor positions 2 and 9 of HLA-E are indeed strikingly comparable with those of the most frequent Caucasian HLA allele HLA-A*0201, but are clearly distinct from those of all other HLA supertypes [23]. Indeed, submission of the 407 9-mer peptides to HLA prediction algorithms (www.cbc.dtu.dk/services/NetMHC) revealed that 77% yielded a high score for HLA-A*0201, but less than 2% had a high score for A*0101, A*0301, A*2402, B*0701, B*4002 and the endogenous alleles of K562 A*1101, and B*1801, illustrating the unique match between HLA-A2 and HLA-E. We concluded that the peptide binding motif of HLA-E and HLA-A*0201 are strikingly similar, suggesting that both molecules present a comparable peptide repertoire when the dominant HLA-I derived leader peptide is absent.

Finally, we obtained very similar findings for the recently identified peptides isolated from the mouse equivalent of HLA-E, the Qa-1 molecule [13] and evaluating this list with algorithms revealed that nearly 40% fulfilled the binding motif of the classical MHC-I molecules H2-Db or H2-Kb. We investigated the dual binding capacity of some of these Qa-1b-eluted mouse sequences to the classical class I molecules and found they indeed bound with high affinity (Suppl Table II). Thus, the alternative peptide repertoire of HLA-E and its equivalent Qa-1 largely display cross-binding to classical MHC-I molecules.

MHC-I derived leader peptide efficiently binds to classical class I molecules

Finally, we wondered if the dominant MHC-I derived peptides that represent the optimized ligands for HLA-E were also capable of binding to classical MHC molecules. Strikingly, the human leader peptide VMAPRTLVL also showed clear binding to HLA-A*0201 (Fig 2, Table I) and the mouse leader peptide AMAPRTLLL demonstrated affinity for H-2K^b (Fig 2, Suppl Table II). These leader peptides were indeed found in elution studies using allele-specific antibodies of these classical HLA molecules. Together, these data indicated that even the typical HLA-E/Qa-1-pre-

sented peptides, which contain full pocket occupancy in the non-classical molecules can be presented by classical MHC class I counterparts.

Concluding remarks

HLA-E is predominantly filled with conserved peptides derived from leader sequences of HLA-I molecules. However, this repertoire is greatly altered upon TAP-impairment, giving a broad diversity of alternative peptides that harbor a binding motif strikingly similar to that of HLA-A*0201. Most of these alternative peptides do not emerge in HLA-E at the cell surface under normal conditions, because the HLA-I leader peptide contains a sequence that optimally fits into the binding pockets of HLA-E, including the deep pockets at position 2, 7 and 9 and the shallower pockets at position 3 and 6 [17]. Our data suggest that the alternative repertoire presented by HLA-E adopts another conformation and docks much less deep into the groove, now primarily interacting with amino acids at positions 2 and 9 for pockets B and F. So, the absence of the dominant leaders allows peptides with an HLA-A*0201-binding motif to be presented by HLA-E. Since this alternative repertoire is normally not presented, they might constitute neo-antigens for the immune system and elicits T cell responses, like we recently showed for Qa-1 [13].

Acknowledgements

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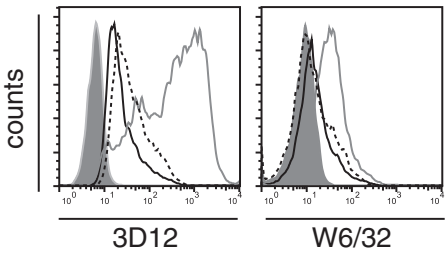
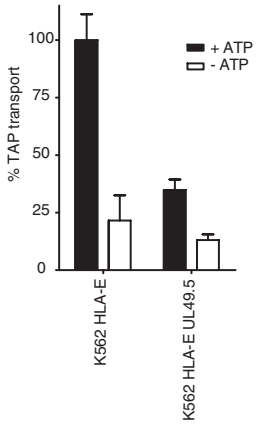


Supplementary figure 1: HLA-I negative K562 cells express HLA-E after gene transfer. The genes for HLA-E*0103 and the viral TAP inhibitor UL49.5 were introduced into HLA-I negative K562 cells. To enhance HLA-E expression, a β2m construct was transferred with the leader sequence of HLA-B8. Surface display of HLA-E was determined on K562. HLA-E cells (black-line), K562.HLA-E.UL49.5 cells (dotted line), and K562.HLA-E.B8 cells (grey line) using antibodies against HLA-E (3D12, left plot) and pan-HLA-I (W6/32, right plot). Filled histograms represent control stainings.



Supplementary figure 2: Inhibition of peptide transport via TAP by viral TAP inhibitor UL49.5. HLA-I negative K562 cells were stably transfected with HLA-E and in addition with the viral TAP-inhibitor UL49.5. A peptide-transport assay was performed using fluorescently labelled reporter peptide in the presence of ATP. As control, TAP activity is blocked by depletion of ATP. The percentage of TAP transport was calculated and compared to that of K562.HLA-E without the TAP inhibitor.

Supplementary Table I: Characteristics of the HLA-E binding peptide pool

		K562 HLA-E	K562 HLA-E UL49.5
Number of peptides[a]		104	552
Presence of leader peptides		Yes[b]	No
Peptide length (aa[c]):	8	34	19
	9	33	407
	10	18	81
	11	6	35
	12	5	9
	13	8	1
Shared peptides		DINTDGAVNF DTNADKQL DTNADKQLS DTNADKQLSF LDTNADKQL MLTELEKAL QSPSANVL VNLGGSKSISIS	

[a] peptides are included if the Mascot score ≥35 with a length between 8 and 13 amino acids
[b] peptides found: VMAPRTLIL – HLA-Cw05
VMAPRTLLL – HLA-A11
[c] amino-acids

Supplementary Table II: Peptide binding affinity of Qa-1 eluted peptides in H-2K[b] and H-2D[b]

	H-2Kb	H-2Db
Peptide sequence[a]	Affinity[b]	Affinity
FAPLPRLPTL	+	++
ASQQNSEEM	+	++
PVLAVEIEI	-	+
AAPTNANSLNSTF	-	++
AMAPRTLLL	+	-
SGPSNTPPEI	-	++
SSWDFITV	++	-

a Peptides derived from reference 13.
b Binding affinity is classified according to the following cutoff: ++ = 0.001 µM <IC50<1 µM; + = 1 µM <IC50<100 µM, - = IC50>100 µM.

HLA- E expression by gynecological cancer restrains tumor-infiltrating CD8+ T-lymphocytes

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Abstract

HLA-E is a non-classical HLA class I molecule, which differs from classical HLA molecules by its non-polymorphic, conserved nature. Expression and function of HLA-E in normal tissues and solid tumors is not fully understood. We investigated HLA-E protein expression on tissue sections of 420 ovarian and cervical cancers and found equal or higher levels than normal counterpart epithelia in 80% of the tumors. Expression was strongly associated with components of the antigen presentation pathway, e.g. TAP, ERAP, β2m, HLA class I and II, and for ovarian cancer with tumor infiltrating CTL. This argues against the idea that HLA-E would compensate for the loss of classical HLA in tumors. *In situ* detection of HLA-E interacting receptors revealed a very low infiltrate of NK cells, but up to 50% of intra-epithelial CTL expressed the inhibiting CD94/NKG2A receptor. In cervical cancer, HLA-E expression did not alter the prognostic effect of CTL, most likely due to very high infiltrating CTL numbers in this virus-induced tumor. Overall survival of ovarian cancer patients, however, was strongly influenced by HLA-E, because the beneficial effect of high CTL infiltration was completely neutralized in the subpopulation with strong HLA-E expression. Interestingly, these results indicate that CTL infiltration in ovarian cancer is only associated with better survival when HLA-E expression is low, and that intratumoral CTL are inhibited by CD94/NKG2A receptors on CTL in the tumor microenvironment.

Introduction

HLA-E is a non-classical Major Histocompatibility Complex (MHC) class I molecule that is almost non-polymorphic, in contrast to its classical class I counterparts HLA-A, -B, and -C (1, 2). There are two HLA-E subtypes, which differ by only one amino acid (3-5). This coding variation is located outside the peptide binding groove, and both HLA-E variants are indeed indistinguishable in their structure and peptide binding features (3-5). HLA-E normally presents a very limited variety of peptides, derived from signal peptide sequences of classical MHC class I. However, infections and transformation can mediate the presentation of alternative peptides (1, 2). Surface expression of HLA-E is largely dependent on β2 microglobulin (β2m) as well as transporter associated with antigen processing (TAP) and tapasin (6, 7).

HLA-E/peptide complexes are ligands of the CD94 receptor in conjunction with the inhibitory NKG2A or the stimulatory NKG2C molecule, which are expressed on the majority of natural killer (NK) cells and some activated $CD8^+$ T lymphocytes (CTL) (8, 9). The presentation of 'self' signal peptides by HLA-E enables these lymphocytes to gauge the overall MHC class I expression on the surface of target cells. Engagement of CD94/NKG2A receptors on lymphocytes reduces the reactivity of NK cells or CTL, which protects against excessive immune-mediated tissue damage, for instance during infections (10, 11). On the other hand, CD94/NKG2A expression can be induced in response to cytokines such as IL-15 (12) and transforming growth factor β (TGF-β) (13). These cytokines are frequently present in the tumor microenvironment, suggesting that CD94/NKG2A inhibitory receptors play a role in immune escape by tumor cells.

With the availability of specific antibodies to detect HLA-E in native conformation (clone 3D12; ref. (6)) or as denatured protein (clone MEM-E/02; ref. (14)), we recently investigated the normal expression of HLA-E on human tissues in collaboration with the Human Proteome Resource program (HPR at www.proteinatlas.org). Expression on blood cells, endothelium, melanocytes and intestinal epithelial cells was confirmed (6, 15-17). Furthermore, we observed a pattern of tissue staining that was very similar to that of classical MHC class I.

Here, we determined the expression of HLA-E in 150 cervical and 270 ovarian cancer samples and analyzed its association with clinical and immunological parameters. Our results indicate that HLA-E is frequently overexpressed in these tumor types and positively associated with expression patterns of antigen processing components, classical HLA molecules and immune cell infiltrate. *In situ* analysis of the interacting receptors of HLA-E, i.e. the inhibitory CD94/NKG2A and the activating CD94/NKG2C, revealed a frequent expression of the inhibitory receptor on intra-epithelial $CD8^+$ T cells. NK cells, the predominant cell type expressing CD94/NKG2A and CD94/NKG2C, were hardly found in both tumor types. Importantly, the beneficial prognostic effect of infiltrating CTL in ovarian cancer was neutralized by high expression of HLA-E, indicating that HLA-E hampers activity of anti-tumor CTL in the tumor microenvironment.

Materials and methods

Patient selection

Since 1985, the Department of Gynecological Oncology at the University Medical Center Groningen (UMCG) keeps a computerized database of patients with malignant epithelial ovarian cancer treated at this hospital at any time point during the course of their disease, prospectively collecting information on clinicopathologic characteristics and follow-up. For the current study, ovarian cancer patients were selected if primary surgery was performed by a gynecological

oncologist from the UMCG between May 1985 and June 2006, and if paraffin-embedded ovarian tumor tissue was available (N = 270). Follow-up was updated in July 2009. Patients were staged according to FIGO classification (61). Tumors were graded and classified according to WHO criteria by a gynecological pathologist. Adjuvant chemotherapy consisted of different platinum-based treatment regimens. Response to chemotherapy was evaluated according to WHO criteria. Specimens from seven patients (three premenopausal, four postmenopausal) who underwent prophylactic bilateral salpingo-oophorectomy were separately included as controls for the HLA-E staining of normal ovarian epithelium.

We included all patients with cervical cancer who underwent radical hysterectomy with complete pelvic lymphadenectomy in the Leiden University Medical Center (LUMC) from 1985 to 1999, for whom formalin-fixed, paraffin-embedded tissue available, and had not received radiotherapy or chemotherapy before surgery (N = 150). All patients had FIGO stages I and II disease, since higher stages are treated by primary chemoradiation. Tumors were HPV typed by PCR and sequencing. For all patients, a minimum of five years of follow up was available. Histologic specimens of normal cervices from women who underwent hysterectomies for benign uterine diseases with no cervical abnormalities (n = 9) were used as a control group in all section stainings.

Immunohistochemistry

Previously described ovarian (20) and cervical cancer (21) tissue microarrays (TMA) were used, which were constructed as described previously (62, 63). TMA sections were stained with mouse monoclonal antibodies recognizing HLA-E (clone MEM-E/02, Abcam ab2216). In brief, TMA sections were dewaxed in xylene and rehydrated using graded concentrations of ethanol to distilled water. After antigen retrieval using citrate buffer, endogenous peroxidase activity was blocked by submersion of sections in a 0.3% H_2O_2 solution for 30 minutes. Sections were incubated with the primary antibody for 60 minutes at room temperature and subsequently with DAKO Envision+ for 30 minutes. The antigen-antibody reactions were visualized with 3,3'-diaminobenzidine. Sections were counterstained with hematoxylin. Paraffin embedded sections were also stained with anti-CD56 (clone 1B6, Monosan, Uden, The Netherlands) and anti-NKp46 (polyclonal AF1850, R&D systems) and subsequently, rat-anti-mouse^{po} and goat-anti-rat^{po} as secondary and tertiary antibodies, respectively.

Simultaneous detection of CD3, CD94 and NKG2A was performed by three color fluorescence staining on ten cryosections of cervical carcinomas using anti-CD3 (mouse IgG1, DAKO, clone F7.2.38), anti-CD94 (mouse IgG2a, Abcam, clone ab61974) and anti-NKG2A (mouse IgG2b, Immunotech, clone Z199). Second step antibodies were all goat-anti-mouse isotype specific antibodies with Alexa fluorochromes Alexa Fluor 546, Alexa Fluor 647 and Alexa Fluor 488 (Molecular Probes). Images were captured with a confocal laser scanning microscope (LSM510, Zeiss) in a multitrack setting and analyzed as previously described (64).

Scoring

All stainings were scored independently by two observers. Observers had no prior knowledge of clinicopathological information. To obtain a high concordance rate with whole tissue slides, it was decided that minimally two cores with a minimum of 20% tumor tissue had to be present on the TMA for a sample to be entered into analysis (63). The scoring system proposed by Ruiter et al. (18) was used. The intensity of staining was scored as 0, 1, 2, or 3, indicating absent, weak, positive, or strong positive expression, respectively. The percentage of positive tumor cells was scored as 0 for 0%; 1 for 1–5%; 2 for 5–25%; 3 for 25–50%; 4 for 50–75% and 5 for 75–100%. The two scores were added up, averaged over the number of evaluable cores per tumor, and dichotomized based on the lowest quartile. This resulted in a cutoff score of 5.25 in ovarian cancer, and 5.00 in cervical cancer. We also performed analyses with previously published data on antigen processing and presentation molecules, as well as tumor infiltrating lymphocytes, using the same cutoff points as described earlier (19-22).

Flow cytometry analyses

Fresh ovarian and cervical cancer specimens were dissected in small fragments with surgical blades and passed through a cell strainer to obtain single cell suspensions. Patients had similar characteristics as those from the TMA cohort. After isolating living cells using centrifugation with ficoll, cells were washed twice in PBS/0.5% BSA buffer and incubated for 30 minutes with CD14-PeCy7 (clone M5E2, BD Bioscience), CD3-PB (clone UCHT1, Dako), CD56-Alexa700 (clone B159, BD Biosciences), CD4-PeTxRed (clone S3.5, CALTAG), CD8-PerCP (clone SK1, BD Biosciences), CD94-FITC (clone 131412, R&D systems), NKG2A-PE (clone z199, Beckman Coulter), and NKG2C-APC (clone 134591, R&D systems). After fixation in paraformaldehyde, cells were measured using an LSR II flow cytometer (BD Biosciences) and analyzed by FlowJo software.

Statistics

Associations between clinicopathological characteristics, antigen processing and presentation pathway

components, and intratumoral T-lymphocytes were tested using Pearson Chi square tests using our previously published cutoff points (19-22). Al continuous variables were tested for normality by plotting histograms and performing Kolmogorov-Smirnov tests. Since none of these variables was normally distributed, nonparametric tests were used to compare continuous variables. Mann-Whitney U tests were used to determine differences between CD8 scores in ovarian and cervical cancer. Survival was defined as date of surgery until death of cancer, or date of last follow-up. Survival was estimated using the Kaplan Meier method. The Log Rank test and Cox regression analyses were used to assess survival differences between groups. To compare CD94, NKG2A, and NKG2C expression among different T lymphocyte subsets, we used the Mann Whitney U test. For all tests, p-values <0.05 were considered significant. All p-values were two-sided. All statistical analyses were performed using SPSS 16.0 software package for Windows (SPSS Inc., Chicago, IL, USA).

Results

HLA-E expression in gynecological cancers

Two cohorts of gynecological tumor tissue were evaluated: 270 ovarian and 150 cervical cancers. Supplementary Table I summarizes clinicopathological characteristics and survival data of the two cohorts. Notably, ovarian cancer is generally diagnosed at a much later stage than cervical cancer. Hence, this cohort consists mainly of high stage tumors with an average disease-specific survival of only 3.5 years, whereas cervical cancer patients live on average 14 years after diagnosis.

We first determined the expression of HLA-E on nonmalignant ovarian and cervical tissue. For ovarian tissue, we selected seven pre- and postmenopausal samples with intact, nonmalignant ovarian epithelium. Also, we stained nine cervical sections containing normal ectocervical squamous epithelium and endocervical glands (Fig. 1). These are the structures that give rise to ovarian- and cervical cancer, respectively. The ovarian epithelium showed a weak positive staining in both pre- and postmenopausal samples, whereas ovarian stroma was negative (Fig. 1a, b). Both cervical epithelia stained negative to weak positive for HLA-E, stroma was negative (Fig. 1e, f). The endothelium of blood vessels was highly positive for HLA-E as well as resident leukocytes, in line with previous reports (15).

Next, we assessed HLA-E expression on ovarian cancer (n=270) and cervical cancer (n=150) confined in tissue micro arrays using a validated specific antibody. Examples of negative and positive staining tumors are depicted for ovarian cancer (respectively Fig. 1c, d) and cervical cancer (respectively Fig. 1g, h). Staining of HLA-E on tumor cells was scored for intensity and percentage surface area, as previously described (18), giving a range from 0 to 8.0. For both tumor types the median score was 6.0, with a range between 0 and 7.75. Based on the mean intensity score of normal epithelium (score 1 on a scale of 0 to 3), ovarian tumors and cervical tumors expressed equal or higher levels of HLA-E in 89% and 83% of the tumors, indicating that expression of HLA-E is mostly conserved in these tumors.

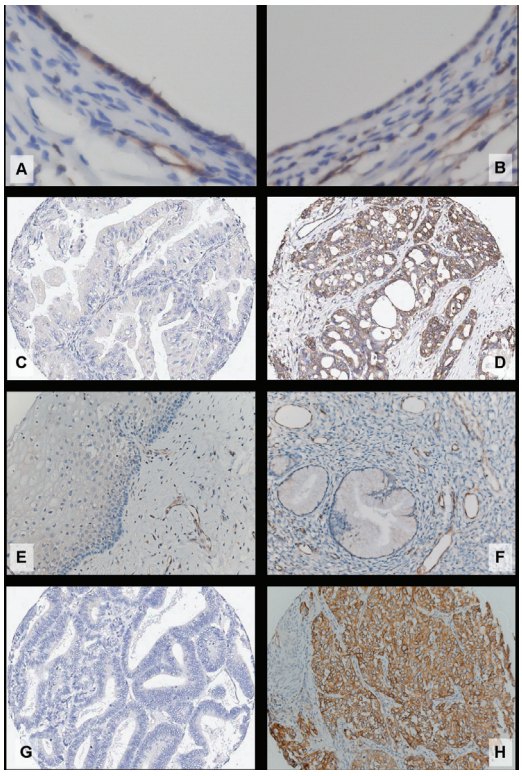

Figure 1. HLA-E expression in ovarian and cervical cancer. Paraffin embedded tissue sections were stained with the monoclonal HLA-E specific antibody MEM-E/02. (A-B) Two examples of the one layer epithelial cells of normal ovaries. No difference was observed between pre- and postmenopausal ovaries. Endothelial cells of the blood vessels in the connective tissue and resident leukocytes are known for positive HLA-E staining. (C-D) Two examples of tissue micro array spots containing ovarian cancer, one negative (C) and one positive (D) for HLA-E expression. (E-F) Examples of HLA-E staining on normal ectocervical squamous epithelium (E) and normal endocervical glands of the cervix (F). (G-H) A negative (G) and a positive (H) example of HLA-E expression from the cohort of cervical cancer.

Table I Relationship of HLA-E expression in ovarian and cervical cancer with immunological characteristics

	HLA-E ovarian cancer			HLA-E cervical cancer		
	low	high	p-value[a]	low	high	p-value[a]
LMP7						
low	17 (65.4%)	9 (34.6%)	<0.001	5 (23.8%)	16 (76.2%)	0.645
high	28 (19.6%)	115 (80.4%)		17 (19.3%)	71 (80.7%)	
TAP1						
low	11 (52.4%)	10 (47.6%)	**0.004**	9 (42.9%)	12 (57.1%)	**0.017**
high	33 (22.6%)	113 (77.4%)		16 (18.4%)	71 (81.6%)	
TAP2						
low	7 (31.8%)	15 (68.2%)	0.439	14 (31.8%)	30 (68.2%)	**0.007**
high	34 (24.1%)	107 (75.9%)		7 (10.9%)	57 (89.1%)	
ERAP						
low	13 (40.6%)	19 (59.4%)	**0.041**	4 (25.0%)	12 (75.0%)	0.524
high	32 (23.0%)	107 (77.0%)		16 (18.2%)	72 (81.8%)	
ERp57						
low	17 (27.4%)	45 (72.6%)	0.805	13 (21.7%)	47 (78.3%)	0.514
high	28 (25.7%)	81 (74.3%)		8 (16.7%)	40 (83.3%)	
HLA-A						
low	25 (46.3%)	29 (53.7%)	**<0.001**	22 (26.2%)	62 (73.8%)	0.083
high	20 (17.2%)	96 (82.8%)		8 (14.0%)	49 (86.0%)	
HLA-B/C						
low	27 (56.2%)	21 (43.8%)	**<0.001**	22 (26.2%)	62 (73.8%)	0.083
high	32 (17.7%)	149 (82.3%)		8 (14.0%)	49 (86.0%)	
HLA-DP/DQ/DR						
low	31 (39.2%)	48 (60.8%)	**<0.001**	13 (29.5%)	31 (70.5%)	**0.028**
high	14 (15.2%)	78 (84.8%)		8 (12.5%)	56 (87.5%)	
B2M						
low	26 (45.6%)	31 (54.4%)	**<0.001**	25 (26.9%)	68 (73.1%)	0.012
high	19 (16.7%)	95 (83.3%)		7 (10.6%)	59 (89.4%)	
CTL						
low	30 (39.5%)	46 (60.5%)	**0.001**	8 (23.5%)	26 (76.5%)	0.656
high	28 (18.8%)	121 (81.2%)		13 (19.7%)	53 (80.3%)	
Treg						
low	30 (32.6%)	62 (67.4%)	0.126	12 (31.6%)	26 (68.4%)	0.085
high	24 (22.9%)	81 (77.1%)		9 (16.4%)	46 (83.6%)	
CTL/Treg ratio						
low	26 (36.1%)	46 (63.9%)	**0.018**	9 (18.0%)	41 (82.0%)	0.343
high	32 (21.2%)	119 (78.8%)		11 (26.2%)	31 (73.8%)	

a p-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

Associations between HLA-E, clinicopathologic and immunologic factors

To assess whether HLA-E expression was preferentially associated with certain patient groups, we determined the relationship between HLA-E expression and well-known clinicopathologic factors. To this end, the gradual scores of HLA-E expression were dichotomized based on the lowest quartile. For ovarian cancer, there was no relationship between HLA-E expression and histology, stage, grade, or presence of residual tumor after debulking surgery (Supplementary Table II). Similarly, HLA-E expression in cervical cancer was not related to histology, stage, infiltration depth, tumor size, Human Papillomavirus (HPV) infection, lymph node positivity, and vascular invasion (Supplementary Table II).

We previously collected data from these cohorts of tumor samples describing immune cell infiltration and HLA-related molecules (19-22). We determined associations between HLA-E and components of the antigen processing machinery, using the same cutoff values as previously described for these molecules (19-22). The proteasome subunit LMP7, peptide transporter heterodimer TAP1, and the aminopeptidase ERAP (23) were associated with increased HLA-E expression in ovarian cancer (Table I). In cervical cancer, the TAP1 and TAP2 transporters were the only compo-

nents that associated with HLA-E expression. These results suggest that antigen processing components contribute to the protein expression of HLA-E.
Next, the association with classical HLA class I molecules (HLA-A, HLA-B/C and β2m) and HLA class II molecules (HLA-DP/DQ/DR) was analyzed (Table I). Induction of HLA class II molecules is observed in a majority of these cancers and can be mediated by cytokines such as interferon-γ, similar to HLA-E (24-26). This revealed a clear association with HLA-E expression, especially in ovarian cancer. This indicates that HLA-E is present in tumors with strong classical HLA expression and contrasts with the idea that HLA-E expression would compensate for loss of classical HLA molecules in cancer. In contrast, high expression of classical HLA class I promotes the stabilization of HLA-E through the delivery of leader sequences which bind to the groove of HLA-E (2, 6, 27).
Furthermore, expression of HLA-E was correlated with the presence of T cells. The degree of infiltration of CTL and regulatory T lymphocytes (Treg) was recently reported by our groups for these two cohorts (20, 21). The number of tumor-infiltrating CTL was positively correlated to HLA-E expression in ovarian cancer, but not in cervical cancer (Table I). We previously found that the ratio between CTL and Treg is predictive of clinical outcome in cervical cancer instead of the CTL counts as such (21). For the current study, we examined the relation between the CTL/Treg ratio and HLA-E expression, but these two parameters were not associated (p=0.343, Table I).
Concluding, HLA-E expression in ovarian and cervical cancers is positively associated with other components of HLA-mediated antigen presentation – indicative of a well functioning processing and presentation pathway – and the influx of T cells. These associations are especially prominent in ovarian cancer.

Intra-tumoral CTLs express HLA-E engaging receptors

The receptors for HLA-E, i.e. CD94/NKG2A and CD94/NKG2C, are predominantly expressed on natural killer (NK) cells. We therefore assessed the presence of these innate immune cells in our cohort of ovarian and cervical cancers using antibodies against the NK-associated markers CD56 and CD57, and the NK-specific marker NKp46 (28). In ovarian cancer, only 14% of the samples contained detectable NK cells, and the number of cells was very low in these tumors (less

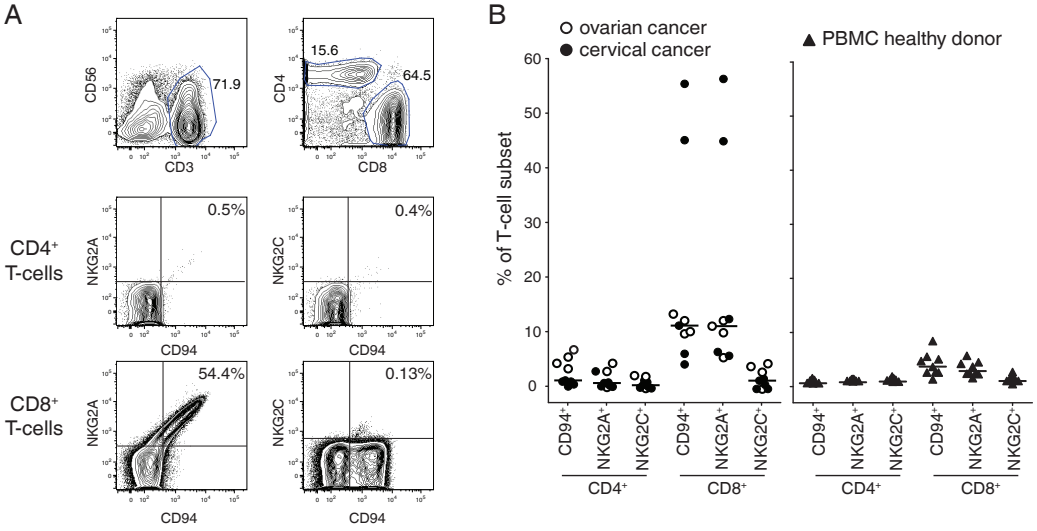

Figure 2. Flow cytometry analysis of CD94, NKG2A and NKG2C expression on T cells. Dissociated tissues from fresh tumor samples from surgery were stained with fluorescently labeled antibodies against CD14, CD56, CD3, CD4, CD8, CD94, NKG2A and NKG2C. (A) Eight color staining of dispersed cervical tumor. Leukocytes were first gated on forward and sideward scatter plot and all CD14-negative cells to exclude non-specific staining to immunoglobulin receptors on monocytes. Natural killer cells were also excluded from the analysis by removing $CD3^{-}CD56^{+}$ cells from the selecting gate. (B) Nine tumor samples and nine age-matched PBMC samples were analyzed for percentage CD4 T cells and CD8 T cells that express CD94, NKG2A or NKG2C. Ovarian cancer contained very low numbers of CD4 T cells. Percentage CD94- and NKG2A-positive CD8 T cells in tumor tissues was significantly higher than the percentage in blood (p=0.0012 for CD94, p<0.0001 for NKG2A, Mann-Whitney U test). Other comparisons were not significantly different.

than 7/mm^2). Cervical cancers also largely lacked infiltrating NK cells, and stainings with an anti-NKp46 antibody corroborated our previous results where we scored CD3$^-$CD57$^+$ cells (21). Clinicopathologic factors or HLA-E expression did not differ between tumors with or without NK cells.

Besides on NK cells, the inhibiting heterodimer CD94/NKG2A and the activating CD94/NKG2C are also expressed on a small subset of CTL (2). We hypothesized that HLA-E in cancers might serve as ligand for these receptors on intratumoral CTL. We applied 8-color flow cytometry analysis on fresh surgical samples, which were mechanically dissected to single cell suspensions (Fig. 2). Gating on CD3+CD4+ T cells and CD3+CD8+ cytotoxic T cells visualized the expression of CD94, NKG2A and NKG2C receptors on these T cell subsets (Fig. 2a). Importantly, a high frequency of the tumor-infiltrating CTL displayed the inhibiting NKG2A chain, but not the activating NKG2C chain (Fig. 2a). Nearly all NKG2A-positive CTL also co-expressed the partner CD94 (overall 98%). In contrast, CD4+ T cells were largely devoid of these HLA-E interacting receptors. The ovarian cancers contained very low numbers of CD4+ T cells, leading to seemingly high frequencies of receptor-positive subsets. Interestingly, large populations of CD4-CD8- T cells were observed in the samples of ovarian cancer and a high percentage of these cells were positive for CD94/NKG2A and these cells are currently subject of further investigation. When five cervical cancer and four ovarian cancer samples were analyzed, up to 50% of CTL were CD94/NKG2A+ with a median of 12% (Fig. 2b). The frequency of CD94/NKG2A+ CTL in age-matched normal blood was found to be around 3%, indicating that this inhibiting HLA-E binding receptor is enriched at the site of the tumor. To substantiate this finding and to analyze the localization of these CD94/NKG2A-positive CTL, we performed triple stainings on cryosections of cervical cancer using fluorescently labeled antibodies to CD3, CD94, and NKG2A (Fig. 3). Most T-cells resided in stoma areas and not within tumor nests, in line with our previous findings (20, 21). Strikingly, CD94/NKG2A expression was found on only 6% of the stoma T cells, whereas 48% of intra-epithelial T cells displayed this inhibiting receptor (SD 9% and 32%, respectively). Together, these data implied that the frequency of tumor-interacting T cells expressing

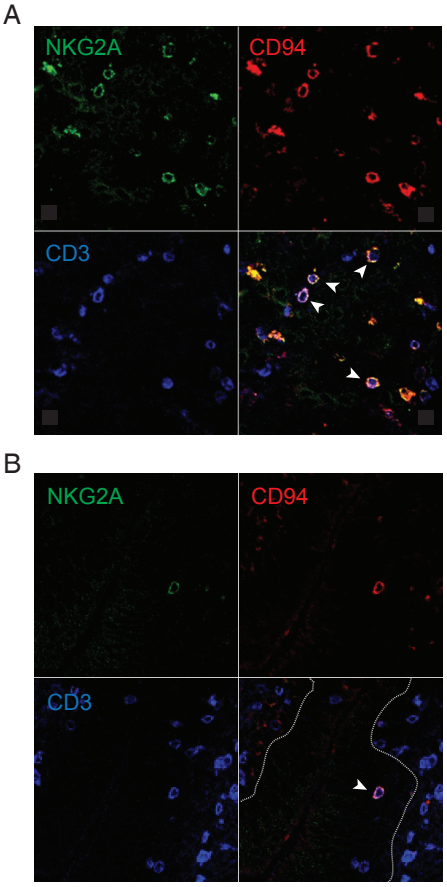

Figure 3. Triple fluorescence staining of cervical cancer detecting intra-epithelial CD94$^+$NKG2A$^+$ T cells. Immunofluorescent staining of T cells (CD3$^+$ in blue) expressing NKG2A (in green) and CD94 (in red). These two pictures of different cervical tumor samples are representative of ten tumors analyzed. Arrow heads in the merged picture (lower right quadrants) designate triple positive cells within tumor nests, whereas the surrounding single blue cells (T cells without CD94/NKG2A) are located in stroma. CD94/NKG2A expression was found on 6% (±9%) of stromal T cells, but 48% (±32%) of intra-epithelial T cells.

Table II Cox regression survival analysis

	HR	(95% C.I)	p-value
Ovarian cancer univariate analysis			
HLA-E high vs. HLA-E low	1.10	(0.74-1.64)	0.653
CD8 high vs. CD8 low	0.71	(0.50-0.99)	**0.047**
HLA-E low: CD8 high vs. CD8 low	0.53	(0.36-0.78)	**0.001**
HLA-E high: CD8 high vs. CD8 low	0.97	(0.77-1.22)	0.816
Cervical cancer univariate analysis			
HLA-E high vs. HLA-E low	0.43	(0.21-0.87)	**0.020**
CD8 high vs. CD8 low	0.94	(0.40-2.19)	0.879
HLA-E low: CD8 high vs. CD8 low	1.68	(0.34-8.36)	0.524
HLA-E high: CD8 high vs. CD8 low	0.75	(0.27-2.05)	0.569
Cervical cancer multivariate analysis			
HLA-E high vs. HLA-E low	0.58	(0.28-1.23)	0.582
Tumor size >4 cm	4.88	(2.17-11.00)	**<0.001**
Lymph node metastasis	2.73	(1.33-5.59)	**0.006**

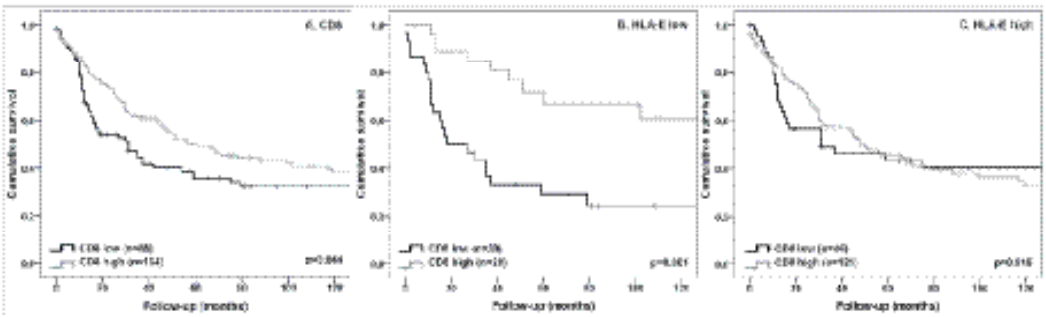

Figure 4. Kaplan-Meier survival curves of ovarian cancer. Overall survival of 249 patients with ovarian cancer for whom ≥2 cores were available in months is plotted. (A) Infiltrating CD8+ T cells were counted and stratified in two groups with a cut off on the lowest tertile. Patients with a high CTL count showed a better survival than those with low CTL counts (p=0.044, log rank test). (B-C) Subsequently, HLA-E expression was added as parameter, dividing the population into HLA-E low expression (lowest quartile) (B) and high HLA-E expression (C). The beneficial effect of high CTL counts on survival was not attributable for those cancers with high HLA-E (p=0.815, log rank). Consequently, the beneficial role of high CTL infiltration of the whole cohort was the result of a small subpopulation of patients with low expression of HLA-E.

CD94/NKG2A (Fig. 3) is much higher than anticipated on basis of the total pool of T cells in the resected tumor sample (see Fig. 2b).

Expression of HLA-E neutralizes survival benefit of infiltrating CTL in ovarian cancer

We wondered whether the observed expression of HLA-E and CD94/NKG2A in the tumor site would translate into survival differences in the context of CTL infiltration. In ovarian cancer, HLA-E expression on its own did not affect survival (Table II). We previously demonstrated (20) that high CTL counts do predict improved survival in ovarian cancer (HR 0.71, Table II and Fig. 4a). We hypothesized that, due to the presence of CD94/NKG2A on infiltrating CTL, HLA-E high tumors might resist CTL mediated lysis. To this end, we performed survival analysis for CTL infiltration stratified by HLA-E expression. Indeed, the prognostic benefit of $CD8^+$ T cells was strongly present in the stratum with low HLA-E expression (HR 0.53, p=0.001, Table II, Fig. 4b). This hazard ratio was much lower than that of the whole population, without HLA-E stratification. Strikingly, patients with high HLA-E expression, representing 75% of our cohort, completely lost the benefit of infiltrating CTL (HR 0.97, p=0.816, Table II, Fig. 4c). These data indicate that the minor subpopulation of patients with low HLA-E expression on their tumors benefits from infiltrating CTL and, moreover, that expression of HLA-E neutralizes the survival benefit of ovarian cancers with high numbers of CTL.

In cervical cancer, we observed a decreased risk of death associated with high HLA-E expression in univariate analysis. However, HLA-E expression was not an independent predictor of death in multivariate analysis (Table II). We previously reported that infiltrating CTL frequency is not an independent predictive survival factor (p=0.879, Table II) (21). Stratified analysis of CTL infiltration based on HLA-E expression did not affect these results. When repeating these analyses for disease free survival, similar results were obtained.

A notable difference between ovarian and cervical cancer is the number of intratumoral CTL, as cervical cancers are infiltrated with at least three times more CTL (median 95.3 ± 221.6/mm^2; ovarian cancer: 28.3 ± 120/mm^2; p<0.001), suggesting that the virus-positive cervical cancers are relatively overloaded with infiltrating CTL. When we repeated the stratified analysis in the subpopulation of cervical cancer with CTL counts comparable to ovarian cancer, HLA-E expression seemed to have the same impact as in ovarian cancer. However, the numbers of cervical cancer with such low numbers of CTL were insufficient for proper statistical analysis. We are currently further evaluating the differences between CTL numbers in several tumor types.

In conclusion, HLA-E is regularly expressed in ovarian and cervical cancer, often concurrently with classical MHC molecules. Instead of inhibiting NK cells, which are hardly present in these tumor types, the main role of HLA-E seems to be the inhibition of infiltrating $CD8^+$ CTL. This effect translates into survival differences in ovarian cancer, which contains fewer CTL and might therefore be more affected by a decrease of CTL below a certain threshold.

Discussion

In the current study, we determined the clinical and immunological relevance of HLA-E expression in ovarian and cervical cancer. Knowledge on the expression of HLA-E in these two cancer types was limited to small cohorts, and here we show that 89.4% of ovarian cancers and 83.7% of cervical cancers display higher levels compared to their normal epithelial counterparts. Total lack of HLA-E is rare in these tumors. Importantly, HLA-E protein expression was strongly associated with expression of classical HLA molecules (class I and class II) and components of the antigen processing machinery (immunoproteasome, peptide transporter TAP, trimming enzyme ERAP and chaperone Erp57) (Table I). This implies that tumor expression of HLA-E is regulated in a comparable fashion to classical HLA and that its presence on tumors is not a defense mechanism against NK cell mediated lysis in classical class I-negative tumors, as sometimes suggested in literature (26, 29-31). Instead, our data argue that HLA-E expression arises in the setting of an intact antigen processing apparatus and, in ovarian cancer, abundant CTL infiltration. A positive association between classical and non-classical HLA expression has recently also been reported for a large cohort of breast cancers (32), and is moreover anticipated on basis of the stabilization of HLA-E by leader peptides derived from classical HLA molecules (2, 33).

Traditionally, interaction with NK cells via receptors CD94/NKG2A and CD94/NKG2C was considered the main purpose of HLA-E. The presence of infiltrating NK cells in ovarian and cervical cancers was previously reported by several groups (34-39). Detection of NK cells in tumor samples has predominantly been performed with antibodies against CD56 and CD57, whereas these molecules can also be found on T lymphocytes. We carefully analyzed NK infiltration by inclusion of the CD3-specific T lymphocyte marker, or using the really specific molecule NKp46, which is not expressed on T lymphocytes (28). Our data reveal that NK cells hardly infiltrate ovarian and cervical cancers, in line with the general impression in solid tumors (40), in contrast to leukemias, where NK cell responses have been connected to better survival (41).
In addition to NK cells, the inhibiting receptor CD94/NKG2A and activating receptor CD94/NKG2C are expressed by minor populations of $CD8^+$ T cells. Although this subset is generally very scarce in PBMC of healthy subjects (approximately 4%) (Fig. 2) (32, 42), the frequency of CD94/NKG2A expressing $CD8^+$ T cells is much higher in tumor infiltrating lymphocytes, as shown in our study and by others (43, 44).
Interestingly, the immunosuppressive cytokine TGF-b, which is regularly detected in ovarian and cervical cancer (45-47), seems to induce this inhibiting receptor on T cells (44). Several studies have shown that the inhibiting receptor CD94/NKG2A dampens the incoming activation signals of T cells by recruitment of phosphatases like SHP-1 to the signal transducing synaps, resulting in decreased effector functions (1, 44, 48). Strikingly, the activating receptor CD94/NKG2C was absent on tumor infiltrating T cells (Fig. 2), whereas it is expressed in other inflammatory situations (49-51). This implies that expression of the NKG2 chains is differentially and independently regulated and that NKG2A is selectively upregulated in tumors.
Protein expression of HLA-E was previously analyzed on cultured cancer cell lines and small cohorts of surgical specimen of some cancer types (16, 26, 52-54). HLA-E expression was correlated with increased infiltration of CD8+ CTL in glioblastoma (53), and decreased infiltration of NK cells as well as a worse progression free survival in colorectal cancer (26). In cervical cancer, HLA-E expression seemed to gradually increase from cervical intraepithelial neoplasia (CIN) I to invasive cervical cancer (54). Intriguingly, we and others (55) found no associations with tumor stage or grade. We have to note, however, that our cervical cancer cohort represented early stage patients with relatively highly differentiated tumors, whereas the ovarian cancer cohort consisted of mostly late stage, high grade tumors. The expression pattern and frequency of HLA-E was quite similar in our two studied cancer types as well as its positive association with antigen presenting molecules. The effect on survival was however clearly different. High HLA-E expression in ovarian cancer appeared to neutralize the beneficial effect of CTL infiltration. These results are in line with the in vitro data by Malmberg et al. (56), who demonstrated that HLA-E on freshly isolated ovarian cancer cells was upregulated by IFN-γ treatment, resulting in a CD94/NKG2A-mediated resistance to CTL lysis. However, in cervical cancer HLA-E did not influence the prognostic effects of CTL nor the CTL/Treg ratio. This difference might be explained by the significantly higher numbers of infiltrating CTL in cervical cancer. At least three times more intratumoral CTL can be found in this tumor type (20, 21, 57), which is most likely the result of the presence of viral antigens from HPV and an active inflammatory response.

In conclusion, our results suggest that HLA-E expression in ovarian and cervical cancer is the result of a smoldering inflammatory response. This emerging concept (58) entails the presence of an inflammatory milieu which can either promote tumor progression or antitumor activity. The inhibiting impact of HLA-E in cervical cancer is limited due to beneficial signs of in-

flammation such as high CTL infiltrate, strong viral antigens and stimulating HLA ligands (MICA and classical HLA). In ovarian cancer, the presence of HLA-E is able to neutralize the protective role of the relatively scarce intratumoral CTL (19-21, 59, 60).

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Supplementary Table I Clinicopathological characteristics and survival data

Ovarian cancer (n=270)			Cervical cancer (n=153)		
Age (years)			Age (years)		
Mean (SD)	57.2	(13.8)	Mean (SD)	47.8	(14.3)
DSS (months)			DSS (months)		
Median (95% C.I.)	42.0	(31.3-52.7)	Median (95% C.I.)	176	(160.5-192.1)
FIGO Stage			FIGO Stage		
Early stage	93	(34.6%)	Stage Ib1	64	(45.4%)
Late stage	176	(65.4%)	Stage Ib2/II	77	(54.6%)
Missing	1		Missing	12	
Tumor type			Tumor type		
Serous	147	(52.1%)	Squamous	123	(82.6%)
Other	123	(43.6%)	Adeno(squamous)	26	(17.4%)
			Missing	4	
Tumor Grade			Lymph nodes		
Grade I/II	133	(47.2%)	Negative	106	(70.2%)
Grade III/ undiff	149	(52.8%)	Positive	45	(29.8%)
Missing	18		Missing	2	
Residual disease			Tumor size		
< 2 cm	155	(62.2%)	<40 mm	82	(57.7%)
>= 2 cm	94	(37.8%)	>=40 mm	60	(42.3%)
Missing	21		Missing	11	

DSS = disease specific survival; FIGO = International Federation of Gynaecology and Obstetrics.

Supplementary Table II HLA-E expression and clinicopathological factors

	HLA-E ovarian cancer				HLA-E cervical cancer		
	low	high	p-value[a]		low	high	p-value[a]
Histology				**Histology**			
Serous	27	98	0.203	Squamous	29	94	0.361
Non-serous	26	63		Adeno(squamous)	4	22	
Tumor stage				**Tumor stage**			
FIGO I/II	19	64	0.580	FIGO Ib1	16	48	0.325
FIGO III/IV	39	110		FIGO Ib2 / II	14	63	
Tumor grade				**Infiltration depth**			
Grade I/II	30	78	0.435	<15 mm	14	64	0.125
Grade III/IV	25	83		>= 15 mm	20	50	
Residual tumor				**Tumor size**			
<2 cm	17	42	0.219	<40 mm	21	61	0.587
>=2 cm	15	61		>= 40 mm	13	47	
				HPV infection			
				HPV 16 or 18	27	86	0.787
				HPV negative	4	15	
				Lymph nodes			
				Negative	23	83	0.508
				Positive	12	33	
				Vascular invasion			
				No	12	51	0.588
				Yes	18	61	

a p-values were calculated using Pearson Chi square-test. Bold signifies p<0.05.

Promiscuous Binding of Invariant Chain-Derived CLIP Peptide to Distinct HLA-I Molecules Revealed in Leukemic Cells

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in press
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Abstract

Antigen presentation by HLA class I (HLA-I) and HLA class II (HLA-II) complexes is achieved by proteins that are specific for their respective processing pathway. The invariant chain (Ii)-derived peptide CLIP is required for HLA-II-mediated antigen presentation by stabilizing HLA-II molecules before antigen loading through transient and promiscuous binding to different HLA-II peptide grooves. Here, we demonstrate alternative binding of CLIP to surface HLA-I molecules on leukemic cells. In HLA-II-negative AML cells, we found plasma membrane display of the CLIP peptide. Silencing Ii in AML cells resulted in reduced HLA-I cell surface display, which indicated a direct role of CLIP in the HLA-I antigen presentation pathway. In HLA-I-specific peptide eluates from B-LCLs, five Ii-derived peptides were identified, of which two were from the CLIP region. *In vitro* peptide binding assays strikingly revealed that the eluted CLIP peptide **RMATPLLMQA**-LPM efficiently bound to four distinct HLA-I supertypes (-A2, -B7, -A3, -B40). Furthermore, shorter length variants of this CLIP peptide also bound to these four supertypes, although *in silico* algorithms only predicted binding to HLA-A2 or -B7. Immunization of HLA-A2 transgenic mice with these peptides did not induce CTL responses. Together these data show a remarkable promiscuity of CLIP for binding to a wide variety of HLA-I molecules. The found participation of CLIP in the HLA-I antigen presentation pathway could reflect an aberrant mechanism in leukemic cells, but might also lead to elucidation of novel processing pathways or immune escape mechanisms.

Introduction

In immune surveillance against invading pathogens and tumor cells, antigen processing and presentation by HLA molecules is essential for induction of potent T cell-mediated immunity.

Classically, exogenously derived antigens, such as bacterial components, are processed in the endosomal/lysosomal system for loading onto HLA class II (HLA-II) complexes. After synthesis in the endoplasmic reticulum (ER), the HLA-II heterodimer binds to the invariant chain (Ii) for transport to late endosomes [1]. Here, Ii is cleaved until only a small fragment, the class II-associated invariant chain peptide (CLIP) remains bound to the class II peptide-binding groove [2]. In MHC class II loading compartments (MIICs), CLIP is exchanged for an antigenic peptide with aid of HLA-DM [3,4], and HLA-II/peptide complexes are exported to the plasma membrane and presented to $CD4^+$ T cells. In tumor cells that have APC function, efficient processing of endogenous, potentially tumor-associated antigens (TAAs) is pivotal for T cell priming of and/or recognition by specific effector T cells. We and others previously showed that such endogenous antigen presentation can also involve HLA-II complexes [5,6]. Ii silencing in certain tumor cells downmodulates CLIP, but not HLA-II expression levels [7] and results in increased presentation of endogenous antigens and tumor-specific $CD4^+$ T cell activation [5,8]. These studies contradict with the proposed requirement of Ii for HLA-II stabilization and transport [9], but agree with its function in preventing binding of endogenous peptides to HLA-II complexes in the ER [10].

For HLA-I antigen presentation, endogenous proteins, *e.g.* tumor- and virus-associated proteins, are normally degraded by the cytoplasmic proteasome followed by translocation of peptides into the ER via the transporter associated with antigen processing (TAP) molecule. Here, peptides with the appropriate binding motif associate with newly formed HLA class I (HLA-I) heavy chain/β2m heterodimers and are transported to the plasma membrane for presentation to CTLs (reviewed in ref. 11). Professional APCs, including macrophages, dendritic cells (DCs) and B cells, have well-equipped machinery to detect, internalize and process exogenous antigens. These antigens are processed for HLA-II-mediated presentation, but can also be routed for presentation by HLA-I, resulting in cross-priming of CTLs (reviewed in ref. 12). Two general routes for this so-called cross-presentation have been described: exogenous antigens are degraded and directly loaded onto HLA-I molecules in the endo-lysosomal pathway [13] or, alternatively, gain access to the cytoplasm for proteasome-dependent processing and are directed either back into endosomes or the ER via TAP [14,15]. The precise mechanism by which HLA-I molecules may enter the endo-lysosomal pathway is poorly defined.

Ii is a type II transmembrane protein that exists in different isoforms and contains one or more internal targeting signals for specific transport of newly synthesized HLA-II complexes to the MIICs [16,17]. In addition to its role in HLA-II transport, the role of Ii as chaperone seems to be more versatile. Ii binds to the actin-based motor protein myosin-II to negatively affect DC migration [18], to adhesion molecule CD44 to activate T cells [19] as well as to costimulatory molecule CD70 for targeting to the MIICs [20]. In the present study, we show an accessory role for Ii and CLIP in HLA-I processing and antigen presentation by leukemic cells.

Methods

Patient material

Bone marrow samples from nine newly diagnosed acute myeloid leukemia (AML) patients were collected after obtaining written informed consent and accord-

ing to the Declaration of Helsinki. This was approved by the review board ('Medisch Ethische Toetsingscommissie, METc') of the VU University Medical Center, Amsterdam, The Netherlands. Classification of acute promyelocytic leukemia (APL) was based on standard genetic and molecular detection of t(15;17), as part of routine diagnostic procedures at our department. HLA-DR-negative AML patients contained high numbers of myeloid cells (>80% of the total WBC count), which were defined as CD45dim/SSClow-int by flow cytometry. Mononuclear cells were isolated using Ficoll-PaquePLUS (Amersham Biosciences, Freiburg, Germany) and directly used for protein analysis or cryopreserved in liquid nitrogen.

Cell lines and culturing

Human leukemic cell lines KG-1, THP-1 and Kasumi-1 were purchased from the American Type Culture Collection (ATTC). KG-1 cells were maintained in IMDM (Gibco, Paisley, UK) supplemented with 20% FBS (Greiner, Alphen a/d Rijn, The Netherlands), 25 mM Hepes (Sigma-Aldrich, St Louis, MO, USA), 1% L-glutamine and 50 µM 2-ME (both Gibco). THP-1 and Kasumi-1 were cultured in RPMI 1640 medium (Gibco) containing 10% and 15% FBS, respectively. For peptide elutions, Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (B-LCLs) were generated from peripheral blood mononuclear cells (PBMC) from healthy blood donors or patients. This was performed in approval with the Leiden University Medical Center review board. EBV-transformed B-LCL JY, PHEB and 5544 were cultured in IMDM containing 8% FBS, 100 IU/ml penicillin and 2 mM L-glutamine. For the competition-based peptide binding assays, B-LCLs expressing HLA-I alleles of interest were a generous gift from Dr. J. Kessler (Leiden University Medical Center; ref. 21). T2 cells were obtained from Dr. P. Cresswell (Yale University School of Medicine, New Haven, CT, USA).

Antibodies and immunofluorescence stainings

We used the following mouse anti-human monoclonal antibodies (MoAbs): PE-labeled anti-CLIP (clone cerCLIP.1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-HLA-ABC (clone W6/32; Dako, Glostrup, Denmark); FITC-labeled CD74, anti-HLA-DR (clone L243) and anti-HLA-DRPQ; PerCP-labeled CD45 (all BD Biosciences, San Jose, CA, USA). Anti-Ii MoAb (clone PIN1.1) was kindly provided by Dr. P. Cresswell. For stainings of murine cells, PE-labeled CD8 (clone 53-6.7; Biolegend, San Diego, CA, USA) and APC-labeled anti-IFN-γ (clone XMG1.2; BD Biosciences) antibodies were used.

For immunofluorescence, cells were incubated with 10% human γ-globulin (60 mg/ml; Sanquin, Amsterdam, The Netherlands) before the MoAb of interest was added. Moabs were added during 15 min at room temperature (RT). Intracellular staining for Ii was performed on cells fixed with PBS 1% paraformaldehyde and permeabilized with PBS 0.1% saponin (Sigma-Aldrich). After incubation with PIN1.1 for 30 min at RT, cells were stained for 20 min with a secondary, PE-labeled rabbit anti-mouse (RAM) IgG (Dako). Each incubation step was followed by two washing steps in PBS with 0.1% HSA and 0.05% sodium azide. We used a FACSCalibur flow cytometer and CellQuestPro software (BD Biosciences) or Flowjo software (Treestar Inc, Ashland, OR, USA) to analyze the percentage of positive cells. Leukemic cells of AML patients were defined by $CD45^{dim}/SSC^{low}$ expression.

Retrovirus production and transduction

Ii expression was silenced using the retroviral pSIREN-RetroQ vector (Clontech, Palo Alto, CA, USA) consisting of a puromycin resistance gene together with a cloned Ii siRNA insert (sequence no. 53; ref. 22) Retrovirus, a kind gift from Dr. S. Ostrand-Rosenberg (University of Maryland, Baltimore, USA), was produced as reported [23]. For retroviral transduction, 1 x 10^6 cells/ml were cultured until 40% confluency. Following washing with PBS, cells were resuspended in DMEM (Gibco) containing 10% FBS, 10 mM Hepes and polybrene, followed by the drop-wise addition of retroviral supernatant to attain a polybrene concentration of 4 µg/ml. Cells were incubated at 37°C for 6 h, washed three times with excess PBS and kept in culture medium for three days. Subsequently, 0.5 µg/ml puromycin was added and increased gradually during a period of two weeks to a final concentration of 1.0 µg/ml in order to select Ii siRNA-transduced cells. Ii expression in transduced cells was checked by flow cytometric analysis. The process of siRNA formation and retroviral transduction itself was validated not to affect Ii expression [6,22].

HLA class I peptide isolation, HPLC and mass spectrometry

HLA-I/peptide complexes were purified from >10^{10} EBV-transformed B-LCLs by affinity chromatography using protein A beads (GE Healthcare) covalently bound to MoAbs against HLA-I (clone W6/32; used for B-LCL JY) or HLA-A2 (clone BB7.2, ref. 24; used for B-LCL PHEB and 5544). Peptides were eluted from isolated HLA-I molecules and separated from class I heavy chains and β2m by passage through Centriprep filtration units with a 10 kD, and the complex peptide pool was fractionated on a 15 cm x 200 µm RP-C18 (Reprosil-Pur C18-AQ 3 µm) column, packed in house. The gradient was run from 0% to 50% solvent B (10/90/0.1 v/v/v water/acetonitrile/formic acid) in 45 min.

Peptide fractions from the first dimension separation

were reduced to near dryness and resuspended in 95/3/0.1 v/v/v water/acetonitrile/formic acid. These resuspended fractions were analyzed by on-line nano-HPLC mass spectrometry using a system earlier described [25]. Fractions were injected onto a precolumn (100 µm×15 mm; Reprosil-Pur C18-AQ 3 µm, 5 µm, Phenomenex) and eluted via an analytical nano-HPLC column (15 cm×50 µm; Reprosil-Pur C18-AQ 3 µm). The gradient was run from 0% to 50% solvent B (10/90/0.1 v/v/v water/acetonitrile/formic acid) in 90 min. The nano-HPLC column was drawn to a tip of approximately 5 µm and acted as the electrospray needle of the MS source. For mass spectrometry, we used a LTQ-FT Ultra mass spectrometer (Thermo, Bremen, Germany) that was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan mass spectra were acquired in the Fourier-transform ion cyclotron resonance (FT-ICR) with a resolution (m/Δm at full width half maximum) of 25,000 at a target value of 5,000,000. The two most intense ions were then isolated for accurate mass measurements by a selected ion monitoring scan in FT-ICR with a resolution of 50,000 at a target accumulation value of 50,000. The selected ions were then fragmented in the linear ion trap using collision-induced dissociation at a target value of 10,000. In a post analysis process, raw data were converted to peak lists using Bioworks Browser software, Version 3.1. For peptide identification, MS/MS data were submitted to the human IPI database using Mascot Version 2.2.04 (Matrix Science) with the following settings: 2 ppm and 0.8-Da deviation for precursor and fragment masses, respectively; no enzyme was specified. Mascot output files were loaded into Scaffold (http:// www.proteomesoftware.com) and exported to Excel.

HLA class I binding prediction and synthesis of peptides

The capability of peptides to bind to which HLA-I molecules was predicted via the netMHC server (http:// www.cbs.dtu.dk/services/NetMHC; refs 26,27), which makes use of approximation algorithms via artificial neural networks (ANNs) and is trained on 9- to 11-mer peptides to predict binding to HLA-I antigen binding pockets. Predicted peptides were synthesized by standard Fmoc chemistry and using a Syro II peptide synthesizer (MultiSyntech, Witten, Germany), as described previously [28]. The integrity of each peptide was routinely validated by HPLC and mass spectrometry.

Competition-based cellular peptide binding assay

To test binding affinity of eluted and predicted peptides to HLA-I, competition-based cellular peptide binding assays were performed as described earlier [21]. In short, B-LCLs were treated with a mild acid (1:1 mixture of 0.263M C6H8O7*H2O and 0.126M Na2HPO4*2H2O) for 1 min to remove the naturally HLA-I bound peptides. Cells were buffered with cold IMDM containing 2% FCS immediately thereafter and resuspended at a concentration of 4 x 105 cells/ml in 2% FCS and 1.5 mg/ml human β2m (Sigma-Aldrich). Then, 4 x 104 cells/well were incubated with 150 nM of fluorescently labeled reference peptide and a serial dilution of one of the eluted or predicted peptides. The following HLA-I allele-restricted reference peptides were used: FLPSDCFPSV (for HLA-A0201), KVFPCALINK (for HLA-A0301), APAPAP-CWPL (for HLA-B0702) and GEFGGCGSV (for HLA-

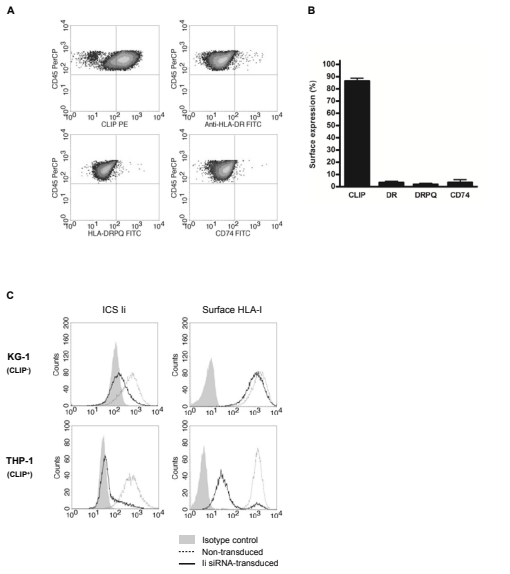

Figure 1. Surface display of the CLIP epitope on HLA-II-negative leukemic cells and influence of the invariant chain on HLA-I expression. (A) Surface expression of CLIP, HLA-DR, total HLA-II ('HLA-DRPQ') and invariant chain ('CD74') of myeloid cells from an acute promyelocytic leukemia (APL) patient, as determined by flow cytometry. Myeloid cells were defined as $CD45^{dim}/SSC^{low/int}$ and expression thresholds were set according to unstained myeloid cells. (B) Quantitative analysis on frequencies of myeloid cells from APL patients that express CLIP (n=9), HLA-DR (n=9), total HLA-II (n=6) and CD74 (n=6). Frequencies indicate percentage tumor cells that reach threshold expression based on unstained leukemic cells. (C) The effect of invariant chain Ii down-modulation in KG-1 ($CLIP^-$) and THP-1 ($CLIP^+$) leukemic cells on HLA-I expression at the cell surface. Intracellular staining (ICS) of Ii (PIN1.1) and surface staining of HLA-I (W6/32) were compared between Ii siRNA-transduced and non-transduced cells.

Table I. HLA-I binding affinity of eluted peptides derived from the invariant chain.

HLA-I allele		HLA-A0201		HLA-B0702	
Peptide no.*	Peptide sequence	IC50†	Affinity‡	IC50	Affinity
1	SRGALYTGFSIL	10.89	Int	1.39	High
2	LLAGQATT	>100	-	40.85	Low
3	RMATPLLMQALPM	13.66	Int	0.36	High
4	LPMGALPQGPM	>100	-	0.41	High
5	ETIDWKVFESW	>100	-	>100	-

* See Figure 2 for amino acid position in the invariant chain protein
† IC50 is the concentration used to obtain half maximal competition and represents the mean value of two independent experiments.
‡ Binding affinity is classified according to the following IC50 cut-off values: high affinity, ≤5 μM; intermediate (int) affinity, 5-15 μM; low affinity, 15-100 μM; no binding, >100 μM [21].

B4002), each containing a fluorescent label bound to the cysteine residue [21]. After overnight incubation at 4ºC, cells were washed twice in PBS supplemented with 1% BSA and fixed in 0.5% paraformaldehyde. Cells were analyzed with CellQuestPro or FlowJo software (Tree Star, Ashland, OR, USA). IC50 values were defined with GraphPad Prism 4.02 (GraphPad Software Inc., La Jolla, USA) using the following formula: Y=Bmax*X/(IC50+X), in which Bmax is the maximal binding capacity of the positive control peptide, X the concentration of peptide tested and IC50 the concentration of peptide needed to reach half-maximal binding.

Mouse immunizations

HLA-A2 transgenic mice (B6 background HLA-A2/H2-D; ref. 29) were immunized subcutaneously with 50 μg of invariant chain peptides and 150 mg HBV T helper peptide (TPPAYRPPNAPIL) in PBS. The injection side was covered with 60 mg of Aldara cream containing 5% imiquimod (3M Pharma Nederland BV). Immunization was repeated on day 7 combined with two intraperitoneal injections of 600.000 IU human recombinant IL-2 (Novartis) on day 7 and 8 [30]. The predicted invariant chain peptides were pooled to a total of 50 μg each. As positive control, mice were injected with 50 μg of MLIVYVRFWWL. At day 13, blood samples were taken and tested for CD8+ T cell reactivity by overnight incubation with the corresponding peptide and intracellular staining for IFN-γ.

Results

CLIP presented on HLA-DR-negative leukemic cells from patients is not explained by plasma membrane expression of HLA-II or CD74

We found that patients with acute promyelocytic leukemia (APL; ref. 31), a genetically determined subtype of HLA-DR-negative acute myeloid leukemia (AML), contained a high frequency of leukemic cells with surface display of the CLIP epitope. Since CLIP expression was not observed on normal promyelocytes from healthy individuals, this remarkable observation may suggest that CLIP presentation at the plasma membrane of these leukemic cells is leukemia-specific and occurs in the context of other types of HLA-II proteins or as unprocessed Ii. To examine the underlying mechanism of CLIP presentation, we analyzed protein expression of total HLA-II and CD74 (i.e. unprocessed Ii) at the plasma membrane. Flow cytometric analysis however showed a lack of both proteins on HLA-DR-CLIP+ leukemic cells (Figure 1A and B), which shows that the expression of CLIP can neither be attributed to its presentation by HLA-DP or HLA-DQ molecules, nor to the expression of unprocessed Ii.

Invariant chain silencing in myeloid leukemia cell lines differentially influences HLA-I surface expression

In the mutant TAP- and HLA-II-deficient T2 cell line, the precursor of CLIP, Ii, was shown to interact with H-2D^b alleles [32]. In addition, Ii can transport HLA-I molecules to endosomal compartments where exogenous peptides are present [33]. We next investigated

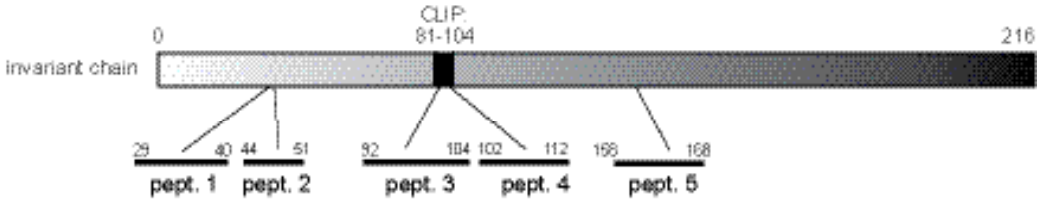

Figure 2. Invariant chain-derived peptides identified in isolated HLA-I molecules of B-LCLs. Peptide elutions of purified HLA-I molecules from EBV-transformed B-LCLs resulted in the identification of five peptides originating from the invariant chain. HLA-I purification and subsequent mass spectrometry analysis are described in *Materials and Methods*. Of note, peptide 3 and 4 are located in the CLIP region, known for universal binding to HLA-II molecules.

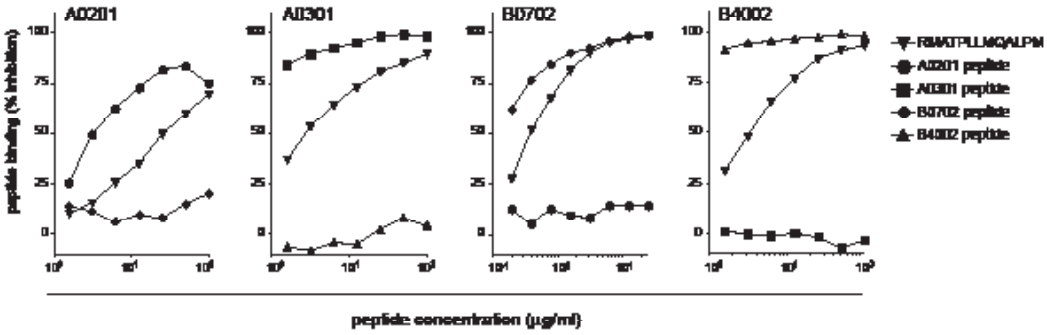

Figure 3. Promiscuous binding of CLIP-peptide to distinct HLA-I alleles, representing four supertypes. The CLIP peptide RMATPLLMQALPM (peptide 3) was tested for binding affinity in a competition-based cellular peptide binding assay. The four tested HLA-I alleles (HLA-A0201, -A0301, -B0702 and -B4002) harbor a completely distinct binding pocket and bind different peptide ligands. CLIP peptide shows intermediate to high binding affinity to all of these. Separate positive control peptides efficiently bind to their respective HLA allele: GILGFVFTL (A0201 peptide), QVPLRPMTYK (A0301 peptide), SPSVDKARAEL (B0702 peptide) and GEFGGFGSV (B4002 peptide) with IC50 values of 3.7, 0.2, 0.7 and 0.2, respectively [21]. The peptide concentration started at 100 µg/ml for HLA-A0201, -A0301 and -B4002 and 25 µg/ml for HLA-B0702, followed by a serial dilution of a factor two. Exact IC50 values of the CLIP peptides are depicted in Table II

whether there was a relation between Ii and HLA-I cell surface expression in CLIP-presenting and non-presenting leukemic cells. Retroviral introduction of Ii-specific siRNAs strongly downmodulates CLIP expression on several myeloid leukemia cell lines, as we recently reported [7]. When these cell lines were assessed for HLA-I protein expression by flow cytometry, KG-1 cells, which present low surface levels of CLIP [34], revealed hardly any effect of Ii silencing (Figure 1C). In THP-1 cells, which highly express CLIP [34], however, silencing of Ii induced a strong reduction in HLA-I surface expression (Figure 1C). In addition, we found indications that Ii was indeed able to associate with HLA-I-related products in leukemic cells, since Ii isoforms were present in HLA-I and Ii immunoprecipitates (Figure S1A), respectively. This was mostly seen in Ii-overexpressing Kasumi-1 cells (Figure S1A and S2), which also showed an increase in free β2m expression after Ii silencing (Figure S1B). The involvement of Ii in HLA-I plasma membrane expression in CLIP-presenting leukemic cells suggests a relation between surface expression of CLIP and HLA-I molecules. Also TAP expression was limited in $CLIP^+$ THP-1 cells, in contrast to $CLIP^-$ KG-1 cells (Figure S2), indicating that the involvement may rely on the ability to process endogenous antigens for loading in the ER.

Peptides derived from the invariant chain are presented at the cell surface by HLA-I molecules

We next assessed whether HLA-I molecules can indeed present Ii-derived peptides at the plasma membrane. The peptide repertoires of HLA-I molecules on EBV-transformed B cells, including CLIP-positive JY cells (Figure S3) were evaluated through biochemical purification. Out of the total characterized peptide repertoire, five peptides were derived from the Ii protein (Figure 2 and Table I), the sequences of which were validated by mass spectrometry according to their synthetic counterparts. Remarkably, two peptides were located within or near the CLIP region, known for its association with HLA-II peptide-binding grooves. Isolation of the 5 identified peptides was restricted to HLA-A0201 and HLA-B0702 alleles presented on EBV-transformed B cells. To examine the HLA-I allele specificity and binding affinity of these peptides, competition-based peptide binding assays were performed (Table I). Four out of five peptides had binding capacity to the B0702 allele and three of these were even categorized as high affinity binders. Notably, peptide 1 and peptide 3, the peptide located within the CLIP region, also bound to the A0201 allele, which might suggest a promiscuous binding capacity of these peptides to HLA-I. Altogether, these data demonstrate that surface HLA-I molecules are able to present a variety of peptides generated from Ii.

Invariant chain-derived peptides located at the CLIP region show promiscuous binding to various HLA-I alleles

As CLIP is known to bind promiscuously to the binding groove of a broad range of HLA-II molecules [35], we further explored the HLA-I binding specificity of the eluted peptide located at the CLIP region of Ii (peptide

Table II. HLA-I binding affinity of peptide length variants located in the CLIP region of the invariant chain.

HLA-I allele			HLA-A201		HLA-B0702		HLA-A0301		HLA-B4002	
Peptide sequence	Position†	Predicted allele‡	IC50§	Affinity¶	IC50	Affinity	IC50	Affinity	IC50	Affinity
RMATPLLMQALPM*	92-104	NA	13.81	Int	0.36	High	3.35	High	3.43	High
KMRMATPLL	90-98	A0201	10.90	Int	0.47	High	6.43	Int	0.44	High
RMATPLLMQA	92-101	A0201	8.08	Int	7.75	Int	2.45	High	2.05	High
RMATPLLMQAL	92-102	A0201+ B0702	4.49	High	1.85	High	4.28	High	0.48	High

* Peptide found with HLA-I elutions
† Amino acid (AA) position in the invariant chain protein
‡ Predicted HLA-I allele to which the peptide binds. Peptide binding prediction was done with netMHC (http://www.cbs.dtu.dk/services/NetMHC). Binding predictions can be made for peptide lengths between 8 and 11 for all alleles with a novel approximation algorithm using artificial neural networks trained on 9-mer peptides [26,27]. Only peptides are shown with a predicted binding affinity of 500 nM or stronger.
§ IC50 is the concentration used to obtain half maximal competition and represents the mean value of two independent experiments.
¶ Binding affinity is classified according to the following IC50 cut-off values: high affinity, ≤5 μM; intermediate (int) affinity, 5-15 μM; low affinity, 15-100 μM; no binding, >100 μM [21].

no. 3; Figure 2 and Table I). This naturally presented peptide (RMATPLLMQALPM) demonstrated a high affinity for both HLA-A0201 and -B0702 (Table I). To determine if peptide RMATPLLMQALPM could bind promiscuously to HLA-I molecules, we addressed its binding capacity to HLA-I molecules bearing structurally different binding grooves. HLA-I molecules are classified according to overlapping binding repertoires and consensus structures in the main peptide binding pockets, so-called supertypes [36]. HLA-A0201 and -B0702 are well known representatives of the A2 and B7 supertype, respectively. The same accounts for HLA-A0301 and -B4002, which represent the common supertypes A3 and B40 [36]. Interestingly, peptide RMATPLLMQALPM bound with a relatively high affinity to all four HLA-I alleles (Figure 3), which have completely different peptide-binding grooves, suggesting that the CLIP sequence involved in promiscuous HLA-II binding also underlies a promiscuous binding to HLA-I molecules.

The unexpected promiscuous binding of peptide RMATPLLMQALPM to distinct HLA-I molecules prompted us to systematically assess length variants of peptides located in the CLIP region of Ii for such binding feature. The CLIP amino acid sequence of Ii was subjected to standard HLA-I binding prediction algorithms for 9- to 11-mer peptides [26,27], which resulted in highly predictive values of three length variants (9-, 10- and 11-mer) of the naturally presented 13-mer RMATPLLMQALPM (Table II). The three peptides were predicted to selectively bind to HLA-A2 subtypes and, in case of the 11-mer, also to -B7 subtypes. Subsequently, we tested these predicted peptides for actual binding to our selected alleles representing four different supertypes, HLA-A0201, -B0702, -A0301 and -B4002. In contrast to their predictions, all the length variants bound to each of the four HLA-I alleles with reasonably high affinity (Table II), indicating that peptides located in the CLIP region of Ii have universal binding abilities to polymorphic HLA-I molecules. Finally, we tested whether the identified Ii-derived peptides were immunogenic in the context of HLA-I, as reported for self peptides termed T-cell epitopes associated with impaired peptide processing (TEIPPs; refs 37–39), but immunizations of HLA-A2 transgenic mice with these peptides showed no CD8+ CTL responses (Figure S4). From these data, we conclude that the CLIP region of Ii harbors a unique peptide sequence that can accommodate binding to structurally completely different grooves of both HLA-I and -II.

Discussion

Processing and presentation of antigens via HLA molecules by APCs is the key mechanism for generating a specific immune response against pathogens and TAAs. In normal APCs, Ii and CLIP have essential roles in the HLA-II antigen presentation pathway. We and others previously described that in tumor cells that are able to function as APC, expression of Ii and CLIP could serve as immune escape mechanism by interference with HLA-II-mediated TAA presentation [5,8,40]. Here, we reveal that Ii can also be associated with HLA-I antigen presentation in malignant cells. This study points at an alternative HLA-I antigen presentation pathway that may affect current concepts of antigen cross-presentation and tumor immune escape.

One of the early events during HLA-I processing is the binding of heavy chains to β2m in the ER. This results in HLA-I stabilization, appropriate folding and incorporation into the class I peptide-loading complex, consisting of TAP and other ER-resident chaperones important for efficient HLA-I peptide loading. Our obser-

vation that Ii is involved in HLA-I processing in leukemic cells (Figure 1C and Figure S1) indicates that it may take part of this process as well. This agrees with earlier studies using HLA-I-transfected T2 cells [32,41,42] and suggests that like newly synthesized HLA-II αβ complexes, also HLA-I/β2m heterodimers can interact with Ii in the ER lumen until peptides are loaded. As Ii inhibits premature peptide binding to HLA-II complexes in the ER [10], the question rises how the association of HLA-I with Ii is related to the function of the class I peptide-loading complex in this compartment. Ii-silenced THP-1 cells lacking the TAP molecule (Figure S2) revealed a strong reduction in HLA-I surface expression level, in contrast to TAP-expressing KG-1 cells (Figure 1C). Additionally, in transfected TAP-deficient T2 cells, HLA-I/Ii complexes relied on the absence of TAP-dependent HLA-I peptides for their stability [32,41] and were also part of the class I peptide-loading complex [43]. For this, we propose that two different types of competition mechanisms can be active in APCs: one involving the binding of Ii to either HLA-I or HLA-II and one involving the binding of either Ii or peptide loading complex peptides to HLA. In this model, the balance between TAP and Ii expression might be of major importance. The likelihood of Ii to interact with HLA-I molecules increases in the absence of TAP, as has been described for T2 cells [43], but likely also in the situation of Ii abundance, as we demonstrate for Ii-high Kasumi-1 cells (Figure S1 and S2). Our finding that peptides derived from Ii were predicted and able to bind to HLA-A0201 and -B0702 (Table I and Figure 2) further suggests that the peptide-binding groove is the HLA-I site with which Ii associates. Indeed, mutations in the binding groove of HLA-B7 molecules affected their interaction with Ii and expression at the plasma membrane in transfected T2 cells [42]. It remains to be determined which specific HLA-I alleles are prone to bind to Ii, but the promiscuous binding of CLIP-related Ii peptides to the four tested HLA-I superfamilies (Table II and Figure 3) indicates great similarity to the promiscuous binding capacity of the CLIP region in the context of HLA-II [44]. However, this contradicts with the large differences in key anchor motifs between each HLA-I superfamily. The shallow binding grooves of HLA-II binds peptides based on favorable interactions rather than need of specific amino acids at each position. Even one amino acid with high affinity to the HLA-II binding groove is sufficient for peptide binding, which could also be the case for HLA-I, as the methionine at position 99 of the CLIP region served as crucial anchor residue for H2-Kb molecules [43].

The observed involvement of Ii and CLIP in HLA-I processing in leukemic cells, as the result of a potentially imbalanced TAP/Ii ratio, can have different outcomes on tumor immune escape. In Ii overexpressing leukemias, such as AML (*e.g.* Kasumi-1 cells; Figure S2) and chronic lymphocytic leukemia (CLL; ref. 45), but also colorectal carcinoma [46], Ii instead of TAA-derived peptides may be bound to HLA-I molecules after synthesis in the ER, thereby preventing TAA presentation at the plasma membrane and recognition by TAA-specific CTLs. In TAP-deficient tumors, self peptides termed TEIPPs have been identified that are specifically detected by CTLs [37,39]. In addition, we observed a possible relation of TAP and CLIP expression in leukemia, with $CLIP^-$ leukemic cells positive and $CLIP^+$ leukemic cells negative for TAP (including APL cells in Figure 1A; Figure S2 and ref. 6). Therefore, the formation of HLA-I/Ii complexes may lead to processing of Ii into CLIP and surface presentation of this self-peptide as a TEIPP. In immunized HLA-A2 transgenic mice however, we could not detect CTL responses against CLIP-related Ii peptides (Figure S4). Another possibility is that HLA-I/Ii complexes in leukemic cells are transported from the ER into the endo-lysosomal pathway to be loaded with TAA-derived peptides obtained from authophagy and lysosomal processing. In normal APCs, Ii is classically known for its role in trafficking HLA-II molecules to the MIICs. It was recently demonstrated that in DCs Ii is also able to direct CD70 molecules to these compartments [20], indicating that the interaction of Ii with HLA-I molecules could account for such a transporting function as well and represent a novel cross-presentation pathway. Sugita and colleagues already demonstrated a role for Ii in the transport of HLA-I molecules to endosomes [33], which might explain the accumulation of newly synthesized HLA-I molecules in endosomal storage compartments [13]. Indeed very recently, in murine DCs, Ii was shown to be critical in MHC class I trafficking from the ER to late endosomes for antigen loading, serving as a mediator of cross-presentation [47]. Although further HLA-I/Ii processing for peptide loading in such endosomal compartments remains undefined, we detected CLIP-related Ii peptides in HLA-I-specific eluates of B-LCLs (Table I and Figure 2), indicating that Ii can be processed to CLIP in HLA-I. These peptides were not derived from HLA-II molecules, as validations for contamination with HLA-II during each step of peptide elution showed HLA-I heavy chains and β2m, but no HLA-II monomers. It is thus appealing to further examine HLA-I-mediated exchange of CLIP for antigenic peptides in the endo-lysosomal pathway as well as presentation of CLIP at the plasma membrane for the effect on CTL activation.

In this report, we present important data showing the promiscuous involvement of Ii and CLIP in the HLA-I antigen presentation pathway of leukemic APCs. To define the similarity with HLA-II processing, further

exploration of their role in intracellular transport and peptide loading of HLA-I molecules is necessary. Since Ii and CLIP are involved in both HLA-I and HLA-II antigen presentation, it will be attractive to design immunotherapeutic strategies that modulate their expression, thereby controlling antigen presentation with the purpose to target immune surveillance against leukemias and possibly prevent autoimmunity.

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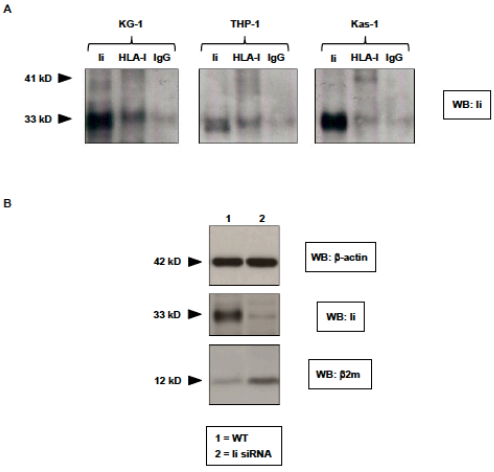
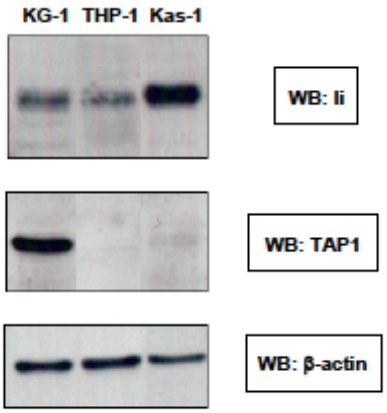
Supplementary methods

Western blotting

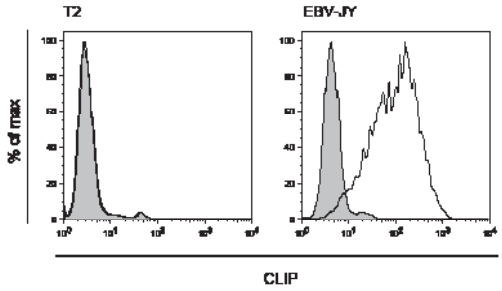
10-20 x 10^6 snap-frozen cells were dissolved in ice-cold lysis buffer, consisting of PBS with 1% Ipegal and 15% protease inhibitor cocktail [Complete, 1 tablet/7.5 ml H2O; Boehringer Mannheim, Mannheim, Germany]). Cell remnants and nuclei were removed by centrifugation (5 min, 10,000 g) and protein content was assessed by the Bio-Rad protein assay (Biorad Laboratories, Hercules, CA, USA). Western blotting was performed by electrophoresis of samples on 12.5% polyacrylamide gels with SDS and their subsequent transfer onto methanol-activated PVDF membranes. Different protein amounts from whole cell lysates were used for loading on gel: 20 µg for Ii and 70 µgfor TAP1. Membranes were pre-incubated for 1 h at 4°C in blocking buffer (5% blotting grade nonfat milk [Biorad]) in TBS-T; 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) and incubated overnight with MoAbs After washing with TBS-T (4x), membranes were incubated for 1 h with HRP-conjugated goat anti-mouse antibody (Dako). Protein complexes were visualized using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).



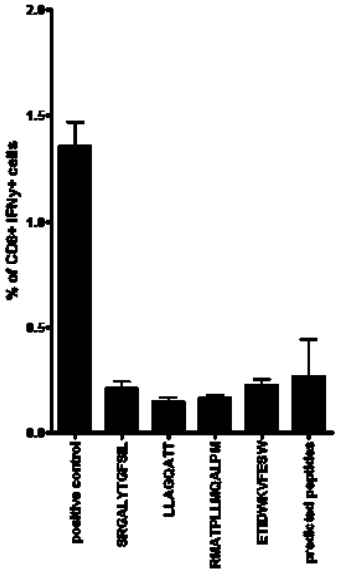
Supplementary Figure S1. (A) Immunoblotting of Ii in both HLA-I (W6/32) and Ii (PIN1.1) immunoprecipitates of KG- 1, THP-1 and Kasumi-1 cells. IgG immunoprecipiates were used as negative controls. (B) The Presence of free-form β2m (12 kD) in total lysates of leukemic cells derived from the Ii- overexpressing Kasumi-1 cell line. Immunoblots for Ii and β2m were performed under SDS conditions and antibodies used for staining were against Ii (PIN1.1) or β2m (rabbit polyclonal antibody, kindly provided by Dr. J.J. Neefjes, NKI, Amsterdam, The Netherlands).



Supplementary Figure S2. Immunoblot analysis of invariant chain (Ii) and TAP expression in total cell lysates of the KG-1 (CLIP-), THP-1 (CLIP+) and Kasumi-1 (CLIP+) leukemic cell line. Blots were stained with primary anti-Ii (clone PIN1.1) and anti-TAP1 (clone 148.3, a kind gift from Dr. E.J. Wiertz and Dr. M.E. Ressing, University Medical Center Utrecht, The Netherlands) MoAb, demonstrating specific bands of 33 kD and 74 kD, respectively.



Supplementary Figure S3. CLIP expression on the surface of T2 and EBV-transformed JY cells, as determined by flow cytometry using a PE-labeled cerCLIP.1 MoAb.



Supplementary Figure S4. HLA-A2 transgenic mice (n=3 per group) were immunized with Ii-derived peptides identified in peptide elution studies (see Table I for numbering), or with a pool of Ii-derived peptides identified on basis of an in silico prediction algorithm (see Table II). After 13 days, peptide-reactive CD8+ T cells from blood were stained with PE-labeled CD8 and APC-labeled anti-IFN-γ antibodies and analyzed by flow cytometry. IFN-γ-positive CD8+ T cell frequencies are expressed as the percentage within the total pool of CD8+ T cells.

CHAPTER 7

Discussion Part I: Identity of human TEIPP

Human TEIPP exist!

In this thesis I focus on the presence of the human TEIPP repertoire and its possible implications for immune therapy. I conclude that TEIPP exist within the human population and that cognate CD8 T cells are able to eradicate a broad diversity of processing-impaired cancer cells.

Previous studies showed the presence of TEIPP in a mouse model and they identified the Trh4 epitope as the first TEIPP (1, 2). This epitope is derived from the ER-membrane spanning Trh4 protein and belongs to the family of fatty-acid regulators (2). It is likely that the Endoplasmic Reticulum (ER) of humans harbors similar MHC-I peptides that are processed like the Trh4-epitope independently of TAP. In humans a set of TAP-independent peptides was presented in HLA-I molecules of TAP-deficient cells (3). This peptide repertoire was clearly different from its TAP+ counterpart, indicating that upon TAP-impairment neo-antigens including TEIPP emerge at the cell-surface (3).
To see whether the human population also harbors T cells with specificity for TAP-impaired targets we used an autologous system. Hereby we inhibited the TAP-function in professional antigen presenting cells and use these cells as target cells to stimulate T-cells. As described in **chapter 2** of this thesis, we observed strong TEIPP-reactivity at the polyclonal T-cell level of ten healthy donors. Remarkably, TEIPP-specificity was already observed four weeks after the initial stimulation and we were able to characterize three T-cell clones with different HLA-I restriction. Unfortunately, the exact peptides recognized by these T cells remain unknown. However, this chapter illustrate that like mice the human T-cell repertoire contains T cells specific for TAP-impaired targets and they can be rapidly induced. We conclude that T cells directed against TEIPP are present in humans.

The presence of TEIPP in humans is further illustrated by the identification of the CALCA-epitope. This epitope is derived from the carboxy-terminus region of the preprocalcitonin signal peptide (4, 5). The CTL was isolated from a lung carcinoma patient and recognized the autologous lung carcinoma cell-line (IGR-Heu), while the autologous B-LCL was not recognized. A cDNA library derived from the IGR-Heu tumor cell-line revealed the HLA-A2 presented minimal epitope VLLQAGSLHA (the CALCA-epitope). Recently it is shown that the expression of TAP is low in the IGR-Heu lung carcinoma cell-line and once the levels of TAP are increased by IFNg the recognition of the CALCA specific CTL is decreased (5). We, therefore, conclude that the CALCA-epitope represents the first molecularly characterized human TEIPP.

To identify other TEIPP, we used a reverse immunology approach in **chapter 3**. Hereto a candidate list of self-peptides, containing potential TEIPP, was created using in silico prediction programs based upon the biological knowledge of the two known TEIPP antigens Trh4 and CALCA. Reactivity against five of these peptides was detected in HLA-A2 transgenic mice. Minicultures of human CD8 T-cell libraries from healthy donors confirmed the immunogenicity of these peptides. Three of these peptides triggered immune responses in naïve T-cell libraries from three healthy donors. We conclude that this confirms the existence of human TEIPP-specific T cells. To show that these peptides are indeed bona fide TEIPP-antigens, we need to isolate specific T-cell clones and characterize their capacity to recognize and kill TAP-impaired tumors.

Characteristics of TEIPP

TEIPP is processed via TAP-independent antigen presentation pathways

TEIPPs are presented on cells that have an impaired MHC-I antigen-processing pathway. T-cells to TEIPP exhibit higher reactivity to processing-defective target cells than to the processing-proficient counterparts. This is illustrated by both known TEIPPs: Trh4-specific CTL in mice fails to recognize the TAP-positive counterpart of RMA-S cells (RMA) (2) and restoration of TAP levels in the IGR-Heu lung carcinoma decreased the recognition by the CALCA specific CTL (5).
A major target for disruption of the antigen-processing pathway in cancer is the transporter molecule TAP. The majority of peptides presented at the cell-surface are transported into the ER through this TAP molecule, and therefore upon inhibition these peptides cannot be presented anymore. It is frequently observed that the expression of TAP is down regulated in tumors, and resulting in low MHC-I surface expression as a result (6, 7). However, TEIPP profit from the absence of TAP-dependent peptides as they are processed in a TAP-independent fashion. Their chances to be presented in MHC-I molecules are increased due to the absence of TAP-dependent peptides.

The CALCA-epitope is processed through the signal sequence pathway, which involves the enzymes Signal Peptidase (SPase) and Signal Peptide Peptidase (SPPase) (4). The signal sequence of the preprocalcitonin is directed towards the ER membrane where cleavage of SPase, and SPPase occurs. After cleavage the CALCA-epitope remains inside the ER and in the absence of TAP it is loaded in HLA-A*0201 molecules (4, 5). Inhibition of the enzymes SPPase and SPase confirmed that processing of the CALCA-epit-

ope was dependent on the activity of these enzymes (4). Moreover, this process is independent of the proteasome, as inhibition with epoxomicin had no effect on the recognition (4).

The Trh4-epitope in mice is derived from the ultimate C-terminus of the membrane spanning protein Trh4. Possible candidates involved in the antigen processing route of this epitope are furin, and enzymes belonging to the family of carboxypeptidases or endoproteases (8-15). Furin cleavage sites can be predicted by ProP 1.0 server (http://www.cbs/dtu.dk/services/ProP/ (16)) and analysis of amino-acid sequence of the Trh4-protein reveals that there is no furin cleavage site near the Trh-4 epitope (data not shown). This is confirmed by an experiment in which the furin enzyme is blocked by 50µM of decanoyl-RVKR-chloro-methylketone (10), in which no decrease in recognition by the Trh4-specific CTL (data not shown). It is, therefore, unlikely that furin is involved in the processing of the Trh4 epitope. Enzymes from the family of carboxypeptidases or endoproteases are more likely candidates to be involved in the TAP-independent processing of the Trh4-epitope (11, 12). Further research needs to reveal the exact proteolytic enzyme responsible for the liberation of this C-terminal epitope.

It is interesting to see that the Trh4-epitope in mouse is derived from the C-terminus of the protein whereas the CALCA-epitope is derived from the N-terminus, indicating that numerous TAP-independent pathways can lead to presentation of TEIPP.

TEIPP benefits from the absence of TAP-dependent peptides

Even though both preprocalcitonin and Trh4 are expressed in a variety of tissues, their corresponding epitopes are not presented on the cell-surface if these cells have no disruption in their TAP-dependent antigen presentation pathway (4, 5, 17). This is most likely due to two factors: 1: the presence of large quantities of TAP-dependent peptides, 2: the expression levels of the protein.

Transfecting the IGR-Heu cell-line with TAP, or inducing TAP expression via IFNg results in decreased recognition by the CALCA-specific CTL, showing that upon restoration of TAP levels, the availability of TAP-dependent peptides decreases the chances of the CALCA-epitope to be presented in HLA-A2 molecules (5). Cell-lines that were recognized by the CALCA-specific CTL, highly overexpressed preprocalcitonin in comparison to PBMCs (5). Also the CALCA-specific T-cell recognized the Medullary Thyroid Carcinoma (MTC) cell-line TT, once TAP was down regulated. This means that the CALCA-epitope is also an interesting target for MTCs.

At this moment, it is unclear if these high expression levels are needed for surface display of CALCA peptides. T-cell recognition is observed in TAP-impaired cells in which they introduce low concentrations of the CALCA-epitope. However, higher gene copies were needed if the TAP transporter was active (5). For the application of the CALCA-epitope in immunotherapy, it has to be investigated how much expression of preprocalcitonin is needed for presentation of the CALCA-epitope at the cell-surface of TAP-deficient cells. Testing whether the CALCA-specific CTL recognizes the TAP-deficient T2 cell-line is an attractive experiment as the T2 cells do express CALCA but in much lower levels than the lung-carcinoma cell-lines or the Medullary Thyroid Carcinoma cell-line TT.

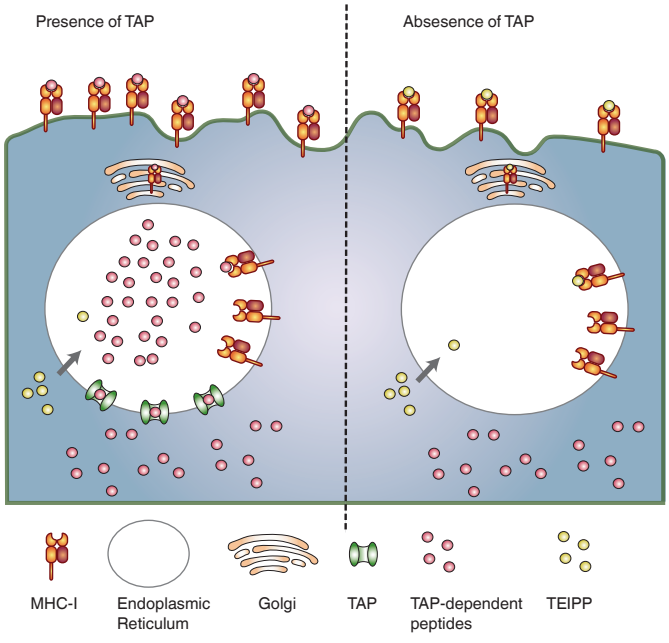


Figure 1: Availability of TEIPP. TEIPPs are not presented at the cell-surface in the presence of TAP-function. The majority of peptides are transported into the ER via TAP, and we hypothesize that in the presence of TAP-dependent peptides TEIPP peptides are not loaded in MHC-I molecules due to their low frequency (left part of figure). Once TAP is impaired, the majority of peptides do not enter the ER anymore. In this case, TEIPPs have a much higher chance to be loaded in MHC-I molecules (right part of figure).

In mice, the limited availability of TEIPP, in TAP proficient cells is illustrated by overexpression of the Trh4-epitope in TAP+ RMA cells. Overexpression results in recognition by the Trh4-specific CTL (17), showing that the availability of the epitope is limited in TAP-proficient cells. It can be concluded that under normal circumstances TEIPPs are not presented at the cell-surface due to low quantities of the epitope in comparison to the TAP-dependent peptides (figure 1).

Presentation of TEIPP in HLA-E

Apart from presentation of TEIPP in classical MHC-Ia molecules there are indications that TEIPP can also be presented in non-classical MHC-Ib molecules, like HLA-E (18). This MHC-Ib molecule mainly present one set of peptides, derived from the leader sequence of classical MHC-Ia molecules (19-21). These leader sequences are conserved throughout mammalian species pointing at a conserved function of this HLA-E/peptide complex (20). HLA-E displaying the leader sequence can be recognized by Natural Killer cells through the CD94/NKG2 complex, serving either as an inhibitory or activating signal.

Studies in mice reveal that the peptide repertoire of Qa-1b (the mouse homolog of HLA-E) is altered upon the inhibition of the TAP-molecule (18). The TAP-dependent classical leader sequences are hereby replaced by a broad repertoire of peptides. T-cells recognizing peptides presented in Qa-1b were identified, but the epitopes remain unknown (18).
We show that like Qa-1b the peptide repertoire of HLA-E is altered upon the inhibition of TAP (**chapter 4**). Like Qa-1b it is observed that upon inhibition of the TAP-molecule the peptide repertoire presented in HLA-E is altered compared to the TAP+ counterpart. Only eight peptides were found in both TAP+ and TAP- cells, indicating the impact in peptide presentation upon disruption of the antigen-processing pathway. T cells responses against HLA-E presented peptides derived from Mycobacterium-, and Epstein Bar Virus (EBV), have been identified, indicating that the T cell receptor fits with this HLA molecule (22-24).

The conserved nature of HLA-E makes it an interesting target for immunotherapy. In humans only two HLA-E variant are known, which differ by only one amino acid. To explore the possible application of immunotherapy, we examined in **chapter 5** the HLA-E expression levels in large panels of ovarium- and cervical carcinomas. It appeared that high levels HLA-E positively correlates with the levels of components of the antigen presentation pathway. One could, therefore, argue that HLA-E up-regulation in tumors results in presentation of more leader peptides instead of tumor specific peptides to which T cells can react. Moreover we show in chapter 5 that the T-cell infiltrate in tumors express the inhibiting CD94/NKG2A receptor suggesting that up-regulation of HLA-E has a more regulatory than pro-inflammatory effect in tumors. This is further illustrated in cells infected with Mycobacterium tuberculosis, were both HLA-E restricted CD8+ T-cells effector cells and regulatory CD8+ T-cells were found (24).
Another interesting feature of HLA-E is its binding-motif. In **chapter 4** we defined a peptide-binding motif based on 407 9mer peptides eluted from TAP-impaired K562.HLA-E cells. This motif was remarkably similar to the HLA-A0201 binding-motif and numerous HLA-A0201 described peptides were presented in HLA-E upon TAP-impairment. Furthermore the peptides have affinity for both HLA-E and HLA-A0201 as shown in **chapter 4**. The HLA-A0201 molecule is present in 25% of the Caucasian population. Peptide vaccination induced T cells could be generated on either the HLA-A0201 molecule or the HLA-E molecule. The effects of this possibility should be explored in great detail to see whether this effect is beneficial or not. Hereby, it is important to investigate under which conditions HLA-E restricted T-cells shows either an effector or regulatory function.

Exploitation of the human TEIPP CALCA in immunotherapy

The CALCA-epitope is so far the only identified human TEIPP epitope and the application of CALCA-specific immunotherapy remains to be investigated. Expression of preprocalciton is only detected in 27 out of the 209 tested lung carcinoma samples (4). This indicates that the application of a CALCA based immunotherapeutic approach is limited for treatment of lung-carcinoma patients. In medullary thyroid carcinoma, however, preprocalcitonin is frequently over expressed and serves as one of the biomarkers to detect MTC (25). Moreover, the CALCA-specific CTL also recognizes the MTC cell-line TT once TAP is down-regulated (5). Therefore a CALCA based immunotherapy approach might be applicable for MTC patients.
To successfully apply the CALCA-based immunotherapy I highly recommend that the expression levels of preprocalcitonin will be determined in combination with the TAP-expression levels and correlated to the recognition by the CALCA CTL. It might also be interesting to study the expression levels of SPase, and SPPase in order to find out whether this forms a factor could influencing the availability of the CALCA-epitope at the cell-surface.

Apart from the absence of TAP, there might be more antigen processing mechanisms which could influence the peptide repertoire presented at the cell-surface. For example it is shown that the furin-mediated pathway can contributes to roughly one third of the peptides presented at the cell-surface of cells (10). In the absence of TAP, contribution of this furin-mediated pathway is increased (10).

Binding of CLIP in HLA-I molecules

In our search for human TEIPP we came across leukemic cells showing surface expression of CLIP, a region derived from the invariant chain (Ii). It is known that CLIP is expressed at the cell-surface in HLA-II molecules. However these leukemic patients were negative for HLA-II, meaning that CLIP was presented in another molecule.
Inhibition of the Ii in leukemic cells resulted in reduced levels of HLA-I surface expression, indicating that the Ii is somehow involved in the antigen processing of HLA-I molecules. This involvement was illustrated by immune-precipitation were we observed a unique feature of a complex of Ii and HLA-I molecules. Previous studies showed that HLA-I/Ii complex formation is more frequently observed in cells with a down-regulated TAP expression (26, 27). In **chapter 6** of this thesis we noticed a CLIP region derived 13mer peptide, eluted from HLA-I molecules, which showed promiscuous binding to a variety of HLA-A and HLA-B alleles. This 13mer could play a role in the complex formation of the Ii and HLA-I molecules. To examine this further, one could remove this 13mer within the Ii and then test whether the complex formation between Ii and HLA-I molecules is absent.

The presentation of CLIP at the cell-surface could also indicate that this CLIP peptide might represent a TEIPP antigen in humans, since it is normally not presented in HLA-I. To confirm this, T cells have to be isolated recognizing the CLIP peptide in HLA-I. However, injection in HLA-A2 transgenic mice showed no T-cell responses against the CLIP peptide and further studies need to confirm whether T-cell responses against the CLIP peptide can be observed.

Regardless whether the CLIP-peptide represents a human TEIPP, the complex formation of the Ii and HLA-I in leukemic patients is an interestingly finding. Previous reports observed Ii and HLA-I complexes in the T2 cell-line and EBV-JY. In line with our study, a role for the CLIP peptide in the complex forming of the Ii and HLA-I is seen (28). Moreover the Ii/HLA-I complex is directed towards the endosomal compartment (26). This indicates that Ii/HLA-I complex formation could play a role in cross-presentation by delivering "empty" HLA-I molecules to endolysosomal compartments where it then can be loaded with exogenously derived peptides.
So far, two cross-presentation routes have been proposed and we hypothesize that the Ii/HLA-I complex plays a role in the vacuolar route in which the exogenous derived peptides remain in the endocytic/endosomal compartment. I envisage that due to Ii/HLA-I complex formation the HLA-I is transported to the endosomal compartment where it engages with exogenously derived peptides, and subsequently transported to the cell surface.
Vaccination studies in mice in which antigens are directly coupled to Ii in adenoviral vectors also points to the existence of such a pathway (29, 30). Furthermore this vaccination resulted in increased CD8+ T-cell responses and better memory formation compared to vaccination expressing only the CD8+ epitope in adenoviral vector (29, 30). The addition of the invariant chain in peptide-vaccination might therefore enhance CD8+ T-cell responses and it is interesting to study the antigen processing upon vaccination with a CD8+ T-cell epitope in combination with the Ii.
We believe that vaccination with TEIPP-antigens combined with adjuvants is an attractive strategy to counteract MHC-I deficiencies in tumors. However there are other therapies that focus on restoration of the MHC-I antigen-processing pathway. These therapies will be discussed in part 7.2 of this discussion.

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Discussion Part II: Strategies to counteract MHC-I defects in tumors

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Summary

Defects in MHC-I antigen presentation represent a common feature of cancer and allow evasion from T cell recognition. Recent findings from immunotherapy in melanoma suggested that irreversible MHC-I defects enable escape from immune pressure. Although loss of antigen presentation is known for many years, strategies to counteract these defects are scarce and largely unexamined. Now that the first forms of T-cell based immunotherapy show clinical efficacy and reach FDA approval, this issue deserves urgent awareness. Here we describe possible roads leading to corrections of MHC-I defects in tumors and describe a salvage pathway for CTL by targeting novel tumor antigens that we recently uncovered.

Introduction

$CD8^+$ T cell immunity depends on MHC-I mediated antigen presentation. The polymorphic MHC-I surface molecules heavily impact on T cell repertoires and mediate surveillance of all nucleated cells of the body sensing malfunctioning. The classical processing pathway engages multiple components of the Antigen Processing Machinery (APM), including proteasomes and the peptide pump TAP (see Box I for a summary). Impediments in this pathway are frequently found in chronic viral infections and are a common feature of cancer. Consequently it mediates evasion from surveillance by cytotoxic T cells. The first reports on loss of MHC-I expression on tumors encountered some doubts, due to confusion on reagents and mouse strains. However, by now these defects have overwhelmingly been reported in most tumor types and, more importantly, the underlying molecular mechanisms have largely been unraveled. Interestingly, the frequent finding of loss of heterogeneity (LOH) at the MHC-I and β2m loci at chromosome 6 and 15, respectively, resembles inactivation mechanisms of tumor suppressor genes [1], suggesting that MHC-I mediates resistance to tumor progression.

The apparent selection and outgrowth of tumor variants with low MHC-I expression might represent a sign of immunoediting by which an active immune system shapes tumor phenotypes [2;3]. Several mouse models have shown that processing defects indeed represent a real escape mechanism [4-6]. On the contrary, loss of MHC-I expression might merely correlate with genetic instability of cancer cells. Nonetheless, two recent case reports showed that the difference between regressing and progressing melanoma metastases during immunotherapy was related to the failure to restore MHC-I expression in the progressing lesions [7;8]. This implies the urgent need for strategies that can counteract antigen presentation defects, especially now the first forms of T-cell based immunotherapy show clinical efficacy and reach FDA approval [9-12]. Defects in MHC-I can not be neglected anymore, despite the seeming lack of possibilities that deal with this problem.

Mechanisms of antigen processing defects in cancer: epigenetics into the picture.

We will not elaborate here on all described MHC-I defects in tumors, since excellent reviews on this subjects are already available [1;6;13;14]. We just like to stress that impediments in MHC-I antigen presentation are present in most cancer types, ranging from approximately 30–80%; are already present in early malignant lesions, but are regularly associated with tumor progression; and can be divided in reversible and irreversible defects [1] Irreversible (or 'hard') defects usually result from structural defects of MHC-I, b2m and APM genes, for example. LOH or point mutations. Lack of one MHC-I allele or haplotype will only mildly affect total MHC-I surface levels, but still might result in functional escape from a focused CTL response. Lack of the peptide transporter TAP, as frequently found in cancers [12], results in a more generalized MHC-I decrease. Although genetic alterations are frequently found in some cancer types [1;15], the dominant underlying molecular mechanism seems to reside at the transcriptional level [1]. Repression of gene transcription (or 'soft' defects) can be operated by activated oncogenes, like Her2/Neu, HPV E7 and p53 [16-18], and is often exerted via histone modifications. Indeed, lack of MHC-I and APM gene expression in tumor cells can be restored by DNA de-methylating agents (e.g. 5-azacytidine and decitabine) and inhibitors of histone de-acethylases (HDAC) (e.g. trichostatin A (TSA)) [1;13;19;20]. These agents modulate the histone code, induce relaxation, and enhance accessibility of the chromatin. Alternatively, immunostimulatory cytokines, like interferons, can restore MHC-I expression in tumors. Most involved genes encode interferon-stimulated responsive elements (ISRE) in their promoter regions [14]. The fact that MHC-I antigen presentation in tumors is frequently controlled at the transcriptional level, and not by structural alterations of the genes, suggests that they are often reversible and can be corrected. However, the situation is somewhat more complex, since resistance to epigenetic modification or interferon signal transduction pathways have been reported [1;13;14]. This indicates that not all transcriptional defects can be re-

stored, and turn out to belong to the so-called 'hard', irreversible aberrations [1]. Moreover, upregulation of interfering miRNAs has been described as a novel mechanism for silencing MHC-I molecules in tumors [21], suggesting that regulation at even additional levels needs to be taken into account.

Strategies to correct antigen presentation defects.

Although the problem of MHC-I defects in tumors has extensively been portrayed, solutions remain challenging. We will mention three approaches that have been described in literature and will close with a novel strategy that we recently unraveled.

Gene therapy

A straight forward approach is the replacement of the defective genes. This solves structural alterations in MHC-I and APM genes, but also transcriptional repression, since strong heterologous promoters in the vectors take over the endogenous gene regulation. Introduction of viral vectors encoding the peptide transporter TAP or β2m were shown to reconstitute surface expression of MHC-I molecules in APM deficient tumor cells [22-25]. Recognition by antigen-specific CTL *in vitro* was also restored, indicating that these single defects were the key factors. Interestingly, application of recombinant viruses in established tumors *in vivo* resulted in expression at the tumor site, restoration of surface MHC-I in the tumor area and, furthermore, augmented immune cell infiltration. Moreover, gene therapy resulted in delayed tumor outgrowth, compared with empty viruses [22-24]. The major limitation of this approach, however, is the local delivery of the gene, especially when the tumor is situated in internal organs.

Epigenetic modulation

Decreased expression levels of antigen presentation genes due to low recruitment of RNA polymerase II complex and hyper-methylation has been described for MHC-I, APM components, but also for the proinflammatory signal transducers STAT-1 and IRF-1, illustrating the wide de-regulation of antigen processing genes in cancer [13;26;27]. It was found that treatment with TSA and Valproic acid could change the histone code and restore the promoter activity of TAP-1 to similar levels as observed in TAP-expressing cells [19;27]. Administration of TSA in an *in vivo* mouse model demonstrated improved survival due to increased immunogenicity of the tumor. Importantly, the effects were absent in T-cell deficient nude mice, demonstrating that the TSA-induced tumor control was immune mediated [27]. HDAC inhibitors and de-methylating agents are clinically applied for a variety of cancers, but thus far no studies were reported on their impact on MHC-I reconstitution. HDAC inhibitors are in general well tolerated, but accumulation of acetylated histones in normal tissues may induce toxicity and lead to unwanted gene expression profiles. For instance, long term application of TSA in animal models has shown an enhanced frequency of regulatory T cells, which dampen immune responses [28;29]. In our opinion, the clinical application of epigenetic modifiers with the aim to increase immune recognition of tumors first requires better pre-clinical analysis.

Interferons

Interferons are at the heart of immune-mediated control of tumors as demonstrated by the sensitivity to tumor formation of mice deficient for one of the associated genes (cytokines, receptors or signal transducers) [3]. Both types of interferon (type I: IFN-α and IFN-β; and type II: IFN-γ) are known to potently stimulate MHC-I mediated antigen presentation via transcriptional activation. Indeed, interferon treatment regularly restores MHC-I defects in human cancer cell lines and improves CTL-mediated lysis [14]. In high risk cutaneous melanoma, IFN-α treatment does lead to improved survival [30] and has been shown to shift the balance in tumors from STAT-3 towards a favorable STAT-1 signaling, resulting in increased expression of TAP in the tumor cells [31]. Interestingly, a case study reported that metastatic melanoma lesions with irreversible MHC-I defects progressed during IFN-α treatment, whereas all reversible lesions regressed [8]. Together, these findings suggest that interferons are potential modifiers to restore 'soft' defects in antigen presentation, in particular because IFN-α is already a FDA approved therapeutic agent for several types of cancer [1;14]. By contrast, administration of IFN-γ is not very successful for treatment of cancers due to its severe adverse effects. Local delivery of IFN-γ, however, can indeed be very effective, as recently shown in a mouse model [32]. Unfortunately, cancers can develop permanent resistance to interferons, via downmodulation of the receptor, activation of the cytokine suppressor SOCS or decreased STAT1 expression [20;33;34].

We conclude that gene delivery, epigenetic regulation and interferons might be exploited to counteract MHC-I defects in tumors, but that all three have significant limitations, in particular for the irreversible alterations such as genetic loss and mutations.

Box I: The manifold roads to load MHC-I molecules

The presentation of peptide antigens to $CD8^+$ T-cells is largely mediated via the classical MHC-I processing pathway in which the proteasome and the peptide loading complex (PLC) in the ER are involved. Components of the PLC include: peptide transporter TAP, tapasin, calreticulin, and the thiol oxidoreductase ERp57. This pathway is further facilitated by the proteases TPPII, TOP, and ERAP-1 for fine tuning of the peptide repertoire [43-45]. In addition to this main TAP-dependent pathway, other pathways are clearly independent from TAP. One of these TAP-independent routes is the processing of peptides located in N'-terminal signal sequences, which are released in the ER by Signal Peptidase (SPase), and Signal Peptide Peptidases (SPPase) [46]. Only a few substrates for SPPase have been indentified, and the exact specificity of SPPase remains largely unknown [46]. The Golgi-route represents yet another pathway. The protease furin is located in the *trans*-Golgi network where it mediates maturation of many proproteins by cleaving at precise stretches of three of four basic residues. If MHC-I epitopes are located downstream of the furin cleavage sites they can be loaded in MHC-I molecules through a TAP-independent secretory pathway [47]. A couple of furin-processed peptides-epitopes have been described so far [47;48], but the exact loading compartments are elusive. MHC-I molecules normally travel to the cell-surface in their peptide-filled conformation, therefore peptides generated in the Golgi network have to be either transported back to the ER, or bind to empty MHC-I molecules en route to the cell-surface [47]. Sufficient evidence points at yet more processing pathways.

TAP-independent peptide-epitopes play an important role in viral infections and it is striking that TAP-deficient individuals do not succumb of viral infections, but are prone for bacterial problems [49;50]. Some of the involved TAP-independent antigens have been characterized in EBV [51], melanoma [52], and a calcitonin signal peptide in lung carcinoma [41].

A novel CTL-based strategy for tumors with processing-defects.

Some years ago we discovered a unique population of CTL that was capable of recognizing MHC-I^{low} tumors [35]. Surprisingly, these CTLs displayed preferential killing for tumors with defects in the antigen processing pathway and targeted a novel category of tumor antigens [35;36]. This novel peptide-repertoire emerges in MHC class I at the cell-surface upon impediments in the classical processing route, for example proteasome, TAP or tapasin, implying that processing defects block the conventional tumor antigens, but stimulate alternative processing routes (see Box I). We designated this novel category of tumor antigens as TEIPP (T-cell Epitopes associated with Impaired Peptide Processing) as they are selectively presented by processing deficient cells (see Table I). TEIPP peptides are derived from normal housekeeping proteins and represent widely shared tumor antigens [35]. Tumor cells with complete absence of MHC-I at the cell surface, due to for instance β2m loss, are not recognized by TEIPP-CTL and we demonstrated that the cognate T cell receptors engage peptide-epitopes in the residual MHC-I molecules of tumor immune escape variants. In mouse models, we showed the exploitability of TEIPP antigens in several ways: vaccination with the molecular identified TEIPP peptides, cellular vaccination with dendritic cells and adoptive transfer of *in vitro* expanded CTL [35;37]. In all three cases, TEIPP targeted immunotherapy mediated clearance of tumors with irreversible MHC-I defects, in particular TAP-deficiencies. Although the reactivity of TEIPP CTL resembles that of natural killer cells, TEIPP

Table I: 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*

antigens behave like conventional tumor antigens for CTL. In addition to irreversible defects in APM components, a subpopulation of TEIPP CTL was capable to eradicate cells with total loss of MHC-I. This subset appeared to target peptide-epitopes when presented by the non-classical MHC-I Qa-1, the homolog of HLA-E [36;38]. This non-classical MHC-I is very conserved in the human population, suggesting that the same TEIPP peptides are presented in all tumor escape variants [39]. Moreover, HLA-E expression in tumors is frequently preserved or even overexpressed (our own observations). Next to tumors with irreversible defects in MHC-I, we envisage a role for TEIPP CTL in the context of reversible MHC-I defects, since tumors with partial deficiencies also present TEIPP at their cell surface [35;36;38], albeit to lower degree. TEIPP CTL might provide the first local immune activation and cytokine release upon recognition of these reversible defects, leading to correction of transcriptional silencing. Recently we demonstrated TEIPP-reactive CTL in the human population and found that this CTL specificity was readily detectable, pointing at a relative high precursor frequency [40]. We have indications that the previously reported signal peptide from the CALCA gene might represent a human TEIPP [41]. Molecular identification of additional human TEIPP antigens will enable the development of TEIPP targeted immunotherapy in the future.

Several basic questions on the TEIPP concept remain, especially concerning the TAP-independent processing pathway that leads to presentation of these novel antigens (see Box I) and the reason why they are not presented by normal, processing-proficient cells. We are also examining the most optimal protocol to activate TEIPP CTL *in vivo*, as this might be different from conventional CTL. TEIPP CTL seems to be poorly cross-primed from cellular vaccines and need a combined T cell co-stimulation with TAP-inhibition on the same cell [24;42]. Preliminary data also indicate that peptide formulations, including the powerful long synthetic peptides [10], do elicit TEIPP CTL *in vivo*.

Conclusion

The commonly observed MHC-I defects in tumors constitute a potential problem for the upcoming forms of T-cell based immunotherapy. Convincing objective tumor regressions have been reported for prostate cancer, melanoma and HPV-induced neoplasia for a fraction of the patients, however the impact of MHC-I defects on the non-responding tumors is largely unknown and corrections of antigen presentation in these tumor types might result in much higher success rates. Two recent case reports nicely illustrated that the progressing metastases in a melanoma patient were the ones that failed to restore MHC-I expression during immunotherapy, whereas all regressing metastatic lesions expressed residual MHC-I [7;8]. In this review we speculate that reversible (so-called 'soft') defects might be corrected via epigenetic modulation or interferon treatment, however this remains to be confirmed in clinical tests. Histone methylase- or de-acetylase inhibitors or interferons are safe and well tolerated as monotherapy and might simply be tested in combination with different forms of immunotherapy. Resistance to these modulators via unresponsiveness or because of 'hard' genetic MHC-I defects, necessitates other options. In theory, one could correct the defects by gene therapy, however, we propose to pursue a strategy in which the novel TEIPP antigens are exploited. Although this discovery is still in an early phase, the presentation of these antigens on tumors with reversible and irreversible MHC-I defects makes them attractive immune targets. We are currently unraveling the human TEIPP antigens and investigate the efficacy of different immunotherapeutic protocols. In the future, T cell based immunotherapy might be complemented with TEIPP-directed immune responses in order to prevent immune escape via MHC-I defects.

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• = of great interest
•• = of outstanding interest

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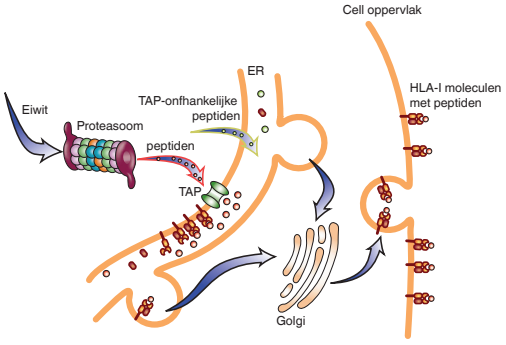
Nederlandse Samenvatting

In het jaar 2010 overleden ongeveer 44 duizend mensen in Nederland aan de gevolgen van kanker. Dit is een stijging van 2% ten opzichte van 2009. Toch is er hoop. Door vele onderzoeken is de diagnostiek en behandeling van kanker al sterk verbeterd. Naast de traditionele bestralings- en chemotherapie wordt er steeds vaker gebruik gemaakt van immuuntherapie. Deze therapie is erop gericht om het immuunsysteem van het lichaam beter te activeren en het te helpen om de kankercellen op te ruimen.

De immuuntherapieën die nu in ontwikkeling zijn, en waarvan sommige al zijn goedgekeurd als behandelingsmethode, zijn erop gericht om het immuun systeem tumorantigenen te laten herkennen. Dit wordt ook wel therapeutische vaccinatie genoemd. Deze tumorantigenen zijn stukjes eiwit die specifiek door kankercellen worden gepresenteerd in HLA-I of HLA-II moleculen. HLA moleculen kunnen herkend worden door T cellen. Een T cel komt in actie wanneer deze een HLA molecuul met daarin een tumorantigeen herkent. De immuunreactie die hierdoor op gang is erop gericht om de kankercel te vernietigingen. Bij patiënten met kanker is de immuunreactie niet krachtig genoeg waardoor de kanker kan uitgroeien. Met de komst van immuuntherapieën is dat hopelijk voorbij.

Tumorantigenen kunnen in verschillende klassen onderverdeeld worden. Zo heb je virus afkomstige tumorantigenen die worden gepresenteerd door kankercellen die zijn ontstaan als gevolg van een virale infectie. Een goed voorbeeld hiervan is baarmoederhalskanker wat ontstaat als gevolg van een infectie met het humaan papiloma virus (HPV). Een andere groep tumorantigenen kenmerkt zich door mutaties. Door die mutaties komt een eiwit er iets anders uit te zien waardoor het als lichaamsvreemd herkend kan worden door de T cel. Daarnaast zijn er eiwitten die specifiek tot expressie worden gebracht in een aantal organen en hierdoor ook in eventuele kankercellen die ontstaan uit deze organen. De expressie en herkenning van deze zogenoemde differentiatie antigenen zijn met name onderzocht bij melanoompatiënten. Andere groepen van tumorantigenen zijn de zogenoemde kanker/testis-antigenen en een kleine groep genaamd cryptische antigenen.

Recentelijk is er een nieuwe groep tumorantigenen ontdekt. Deze antigenen die wij TEIPP noemen worden alleen op kankercellen gepresenteerd die het immuunsysteem proberen te ontwijken door de presentatie van tumorantigenen in HLA-I moleculen tegen te houden. Dit kan een kankercel op een aantal manieren doen: 1. De kankercel zorgt ervoor dat er minder of geen HLA-I moleculen meer gemaakt worden; 2. De kankercel blokkeert de route die tumorantigenen moeten afleggen om in het HLA-I molecuul gepresenteerd te worden.
Voordat eiwitten in een HLA-I molecuul gepresenteerd kunnen worden gaat het door de antigeen presenterende route (figuur 1). Om beladen te worden in een HLA-I molecuul wordt een eiwit eerst door het proteasoom in kleine stukjes eiwitten gehakt. Daarna vervolgen de kleine stukjes eiwitten binnen de cel hun route richting het endoplasmatisch reticulum (ER). Om het ER binnen te komen worden ze in het algemeen getransporteerd door het TAP molecuul, wat een kanaal in het ER-membraan is dat veel kleine stukjes eiwit gemakkelijk naar binnen pompt. Als de stukjes eiwit eenmaal in het ER zijn kunnen ze geladen worden in een HLA-I molecuul waarna het gehele complex naar de buitenkant van de cel wordt getransporteerd om eventueel herkend te worden door T cellen.
Kankercellen blokkeren soms het TAP molecuul waardoor de tumorantigenen niet meer het ER kunnen binnenkomen. Deze tumorantigenen worden dan niet meer door HLA-I aan T cellen gepresenteerd waardoor het moeilijker wordt om de cel als eventuele kankercel te herkennen. Gelukkig zijn er ook stukjes eiwit die het ER weten binnen te komen zonder gebruik te maken van het TAP molecuul. Deze TAP-onafhankelijk eiwitten zijn echter sterk in de minderheid en hebben normaliter geen kans om in voldoende mate gepresenteerd te worden door een HLA-I molecuul (figuur 1). De kankercel die TAP blokkeert geeft deze peptiden de kans om in het HLA-I molecuul te komen.



Figuur 1: De klasse I antigeen presenterende route. Eiwitten in de cel worden in kleine stukjes peptiden gebroken door het proteasoom alvorens zij het ER binnengaan via het TAP-molecuul. In het ER worden de stukjes peptiden beladen op klasse I moleculen. Het klasse I molecuul met peptiden wordt vervolgens naar het cel-oppervlak getransporteerd waar CD8 T-cellen het kunnen herkennen. Een aantal peptiden kan ook het ER binnenkomen zonder gebruik te maken van het TAP-molecuul.

Doordat de T cellen deze TAP-onafhankelijke eiwitten normaal niet zien worden beschouwen ze deze peptiden als vreemd en komt een immuunreactie op gang.

De blokkering van het TAP molecuul komt in kanker best vaak voor. Hoe vaak verschilt per soort kanker, maar gemiddeld genomen komt het in 50% van alle kankergevallen voor. Als de kankercellen ook nog uitzaaien gebeurt het zelfs nog vaker dat het TAP molecuul geblokkeerd wordt. De toevoeging van TEIPP-antigenen aan therapeutische vaccins wat gebruikt wordt bij de immuuntherapie van kanker kan dus van groot belang zijn.

In **hoofdstuk 2** van dit proefschrift wordt beschreven dat mensen T cellen hebben die specifiek reageren op cellen waarvan het TAP-molecuul geblokkeerd is. Dit betekent dus dat de ontdekking van humane TEIPP-antigenen mogelijk is. In 2011 werd ook het eerste humane TEIPP-antigeen beschreven. Dit antigeen is gevonden in een longtumor, maar kan misschien ook gebruikt worden voor medullair schildkliercarcinoma. Op dit moment wordt onderzocht in hoeverre dit antigeen toegepast kan worden voor de immuuntherapie van kanker, en welke patiënten voor deze therapie geschikt zijn. Om nog meer TEIPP-antigenen te identificeren hebben we in dit proefschrift in **hoofdstuk 3** een methode beschreven waarbij met behulp van bio-informatica mogelijke TEIPP-antigenen ontdekt kunnen worden.

Een uitdaging voor de ontwikkeling van de immuuntherapie van kanker is de grote hoeveelheid verschillende HLA moleculen. Zo zijn er 6 groepen HLA-I moleculen: HLA-A, -B, -C, -E, -F, -G. Deze 6 groepen vertegenwoordigen vervolgens ook weer allemaal varianten. Zo zijn er 124 varianten HLA-A moleculen, 258 HLA-B moleculen en 74 HLA-C moleculen. Elk persoon heeft maar twee varianten van elke HLA-A, -B, -C, -E, -F en –G, in zijn immuunsysteem wat dus voor een grote diversiteit in de bevolking zorgt. Door deze grote verscheidenheid is het dus lastig om een therapie te ontwikkelen die voor iedereen geschikt is.

Recent is echter ontdekt dat elk persoon bijna dezelfde HLA-E moleculen heeft. Tot nu toe zijn er maar twee varianten van HLA-E ontdekt. Dit betekent dat elk persoon bijna hetzelfde stukje eiwit in HLA-E toont aan het immuunsysteem. Het is dus interessant om te ontdekken welke eiwitten door HLA-E gepresenteerd worden en of er T cellen zijn die deze eiwitten herkennen.

In **hoofdstuk 5** van dit proefschrift is er gekeken naar de HLA-E expressie op ovarium en baarmoederhals tumoren. In 80% van alle tumoren werd een hogere HLA-E expressie aangetroffen dan op de gezonde cellen. Deze HLA-E expressie was gecorreleerd aan de expressie van andere HLA-I moleculen, maar ook aan moleculen die betrokken zijn bij de antigeen presentatie route. Vervolgens werd er ook onderzocht of een hoge HLA-E expressie zorgde voor een betere overleving. Bij mensen met een ovarium tumor was dit helaas niet het geval. De T cellen die aanwezig waren binnen deze tumoren werden niet goed geactiveerd omdat er op de oppervlakte van de T cel een molecuul aanwezig was dat voor een remmend signaal zorgde als HLA-E herkent werd. Dit CD94/NKG2A molecuul zorgde er dus voor dat de T cellen niet actief werden, wat met name in ovarium kanker voor een probleem zorgde omdat in deze tumoren al weinig T cellen te vinden waren.

Ook is er onderzocht wat voor soort eiwitten een HLA-E molecuul presenteert. Een gezonde cel laat maar een paar eiwitten in het HLA-E molecuul aan T cellen zien. Deze eiwitten zijn afkomstig van stukjes HLA-A, -B en –C moleculen. Het lijkt er dus op dat HLA-E moleculen aan T cellen kunnen laten zien dat alles in orde is. Als de antigeen presenterende route echter verstoord wordt door het blokkeren van TAP laat het HLA-E molecuul hele andere stukjes eiwit zien. In **hoofdstuk 4** van dit proefschrift laten we zien dat deze stukjes eiwit lijken op de stukjes die een HLA-A2 molecuul presenteert aan een T cel. Het zou interessant zijn om een vaccin te ontwikkelen die verschillende T cellen via beide moleculen kan stimuleren omdat dan blokkade van TAP niet automatisch leidt tot ontsnapping van de kankercel aan het immuunsysteem.

Nog een belangrijke stap die zou kunnen helpen bij het ontwikkelen van immuuntherapieën is de optimalisering van antigenpresentatie in HLA-I en HLA-II moleculen. Behalve de hierboven beschreven route zijn er namelijk nog vele andere routes die kunnen leiden tot het presenteren van eiwitten in HLA-I en HLA-II moleculen. Een molecuul dat voor deze andere routes een grote rol kan spelen is de invariante keten. In **hoofdstuk 6** van dit proefschrift is onderzocht hoe de invariante keten een rol speelt in het presenteren van eiwitten in HLA-I moleculen. Tot nu toe wist men dat de invariante keten helpt om HLA-II moleculen te beladen met eiwitten. In dit proefschrift wordt aangetoond dat de invariante keten ook bij de belading van HLA-I moleculen kan helpen. Met name kan dit een rol kunnen spelen bij leukemiepatiënten omdat hier de binding

van de invariante keten met het HLA-I molecuul wordt waargenomen. De toevoeging van de invariante keten aan immuuntherapie zou dus voor een betere T cel reactie kunnen zorgen, maar dit moet nog verder onderzocht worden.

De hoofdstukken beschreven in dit proefschrift tonen aan dat de immuuntherapie nog volop in ontwikkeling is en dat er nog veel winst behaald kan worden door het bestuderen van de antigeen presenterende routes en de eventuele uitschakeling hiervan in tumoren.

Curriculum Vitae

Margit Heilkelien Lampen werd geboren op 26 april 1982 te Utrecht. Zij groeide in Utrecht op en in het jaar 2000 behaalde zij haar VWO diploma op het Montessori Lyceum Herman Jordan te Zeist. Hierna vertrok zij voor een jaar richting Engeland om daar aan het Chichester College of Art, Science, and Technology te gaan studeren alvorens in het jaar 2001 aan haar studie Biotechnologie te beginnen. Deze studie volgden zij aan de universiteit van Wageningen.

Gedurende haar studie liep zij stage bij de afdeling Virologie aan de Wageningen Universiteit onder de leiding van Dr. M. van Oers. Hier deed zij onderzoek naar de functionaliteit van photolyase genen gevonden in Chrysodeixis Chalcites nucleopolyhydrovirus.
Vervolgens ging zij naar het Nederlands Vaccin Instituut (tegenwoordig behorende bij het Rijksinstituut voor Volksgezondheid en Milieu) om daar op de afdeling Onderzoek en Ontwikkeling de synergie tussen Toll-like receptoren, en NOD receptoren, in combinatie met het “outer membrane complex” van Meningitidis te onderzoeken. Dit deed zij onder de leiding van Dr. F Fransen en Dr. P van der Ley. Haar laatste stage volgde zij op de afdeling Zoölogie van de University of British Colombia in Vancouver, Canada. Onder de leiding van Dr. N Pante deed zij onderzoek naar de nuclear import van baculovirusen.

In juni 2007 behaalde Margit haar diploma aan de Wageningen Universiteit en in september 2007 begon zij aan haar promotieonderzoek zoals beschreven in dit proefschrift. Dit deed zij op de afdeling Klinische Oncologie onder de leiding van Dr. T van Hall.

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a,b= these authors equally contributed to this paper
in press
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