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**ALTERNATIVE ANTIGEN PROCESSING
AND PRESENTATION PATHWAYS BY TUMORS**

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ALTERNATIVE ANTIGEN PROCESSING AND PRESENTATION PATHWAYS BY TUMORS

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1

INTRODUCTION

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A novel category of antigens enabling CTL immunity to
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| ANTIGEN PRESENTATION FOR CD8 T-CELL IMMUNITY

CD8⁺ T-cells

The immune system plays a crucial role in the surveillance of the host, detecting and eradicating threats such as bacterial and viral infections, as well as transformed tumor cells. The triggering of this effective defense mechanism starts with the activation of antigen presenting cells (APC), such as dendritic cells (DCs), that are able to detect the presence of pathogens in the body, for instance via surface innate receptors called “Toll-like receptors” (TLR). Through other types of receptors they can detect dying cells, for instance derived from a cancer. To initiate a pathogen-specific immune response, the DCs ingest the extracellular pathogens, infected cells or transformed cells and process them into single antigens (normally peptides). Then, they present these disease-related antigens to other cells of the immune system. This process enables the priming and activation of T-cells, such as the CD8⁺ T-cells, that are critical effector cells in the adaptive immune response. Activation of CD8⁺ T-cells occurs when their T-cell receptor (TCR) binds specifically to Major Histocompatibility Complex class I (MHC-I) molecules and small peptides displayed into these MHC-I molecules at the surface of APCs. Once activated, the CD8⁺ T-cells are potent effector cells that release effector molecules like interferon- γ (IFN γ), perforin and granzyme B to induce apoptosis (cell death) in their targets. CD8⁺ T-cells are well known for their function to kill their target cells via the release of effector molecules and because of this they are often called as Cytotoxic T-lymphocytes (CTL).

CD8⁺ T-cells develop in the thymus from precursors called thymocytes. During their development thymocytes are subjected to two subsequent processes called positive and negative selection. According to the affinity model of thymocyte selection, the affinity of the TCR-peptide-MHC interaction is the key determinant of T-cell selection¹. During positive selection, thymocytes with intermediate affinity for self-peptide-MHC complexes receive a survival signal and commit to the CD4 or CD8 T-cell lineage. T cells that express MHC class II-restricted receptors are positively selected to the CD4 lineage, while T cells expressing MHC class I-restricted TCRs are selected to the CD8 lineage². High-affinity binding of the TCR to self-peptide-MHC complexes induces cell death by apoptosis, a process that is known as negative selection (or clonal deletion). Negative selection is necessary for the maintenance of self-tolerance as it induces the deletion or inactivation of potentially autoreactive thymocytes. In the thymic medulla, the medullary thymic epithelial cells (mTECs) have a key function in this process as they express a large number of tissue-specific self-antigens that are presented to developing T cells^{1,3}. After positive and negative selection, thymocytes migrate out of the thymus and become part of the peripheral T cell pool.

MHC-I processing and presentation pathway

MHC-I molecules are present on the surface of virtually all cells of the body where they present peptides to ensure that rogue cells can be recognized by CTL. These peptides are generated inside the cell by proteolysis of endogenous or pathogen derived proteins.

Potentially, a multitude of proteolytic systems may generate antigenic peptides, but the proteasome is responsible for the liberation of the majority. Other endo- and exoproteases complement proteasome activity by further degrading proteasomal products or, sometimes, by directly generating the defined peptide sequences that fit into MHC-I molecules. For instance, tripeptidyl peptidase II (TPPII), thimet oligopeptidase (TOP) and nardilysin have been implicated in the generation of some CTL epitopes⁴. Peptides generated in the cytosol need to be translocated into the ER by the TAP peptide transporters, to have access to the peptide loading complex (PLC) which is located in the ER. Once in contact with the PLC, the suitable peptides for binding are associated with the different nascent MHC-I heavy chains and β 2-microglobulin (β 2m) with help from chaperones such as calreticulin, tapasin and ERp57 of the PLC⁵⁻⁷. Figure 1 summarizes the MHC-I presentation pathway.

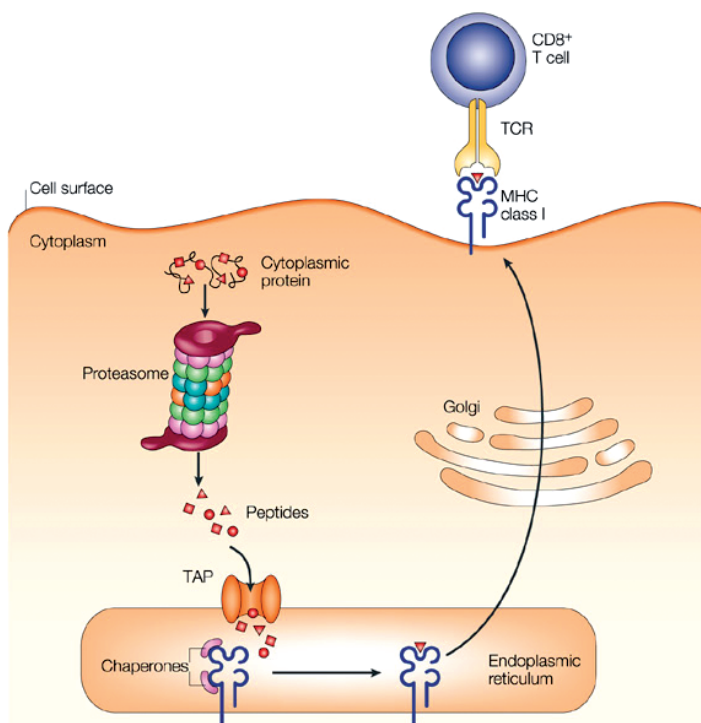


Figure 1. MHC-I antigen presentation: the basics. Small peptide sequences derived from proteolysis in the proteasome are generated in the cytosol and loaded into the ER by TAP. These peptides bind to MHC class I molecules which are routed to the cell surface. Adapted from: Yewdell J.W. *et al*, Making sense of mass destruction 2003, Nature Reviews Immunology, 3(12):952-61.

| CD8⁺ T-CELL IMMUNITY AGAINST TAP-DEFICIENT CELLS

Importance of TAP-independent processing pathways

The proteasome-TAP pathway is considered as the conventional processing route⁸⁻¹⁰. However, cells are equipped with alternative routes leading to liberation and loading of peptides into MHC-I molecules. These routes are independent of the molecules proteasome, tapasin or TAP from the conventional pathway. This has become apparent from studies on cells with deficiencies in the conventional processing pathway. The levels of surface MHC-I display are much decreased at the surface of cells from TAP-deficient mice¹¹⁻¹³. However, the generation of TAP-independent peptides enables the residual expression of surface class I molecules. In human beings, TAP-deficiency syndrome is rare but can be observed in independent families and results from mutations in both subunits of the peptide transporter, TAP1 and TAP2¹⁴. These patients suffer from chronic necrotising lesions in the lungs and skin associated with recurrent bacterial infections. Surprisingly, they are not unusually susceptible to infections by viruses despite the fact that the conventional MHC-I antigen processing pathway is considered very important for the presentation of viral peptides in infected cells. Peripheral TCRαβCD8⁺ T cells were present in TAP-deficient patients and their TCR repertoire is polyclonal. It was possible to isolate CTL recognizing a peptide from the EBV protein LMP2 presented by a HLA-B allele on TAP-deficient cells¹⁵. These observations argue that the TAP-independent processing pathway sufficiently compensate for the loss of the conventional pathway in order to select a functional CD8⁺ T-cell repertoire and, moreover, to control virus infections¹⁴. In fact, the phenotype of families with genetic TAP-defects resembles that of TAP-knockout mice, with lower surface expression levels of MHC-I but a remaining broad polyclonal repertoire of CD8⁺ T-cells¹¹⁻¹³. In an attempt to identify the nature of TAP-independent peptides, pioneering studies by Peter Cresswell and Victor Engelhard in 1992 revealed that TAP-deficient T2 cells predominantly present peptides derived from signal sequences^{16, 17}. Some of these peptides were also observed within HLA-A2 of normal, TAP-proficient cells, indicating that in normal cells alternative processing pathways operates side-by-side with the conventional route.

TAP-independent processing routes: targeting to the ER

The best characterized alternative, TAP-independent processing pathway comprises peptides generated in the secretory compartments. Initial studies with TAP-deficient T2 cells showed that signal sequences can efficiently function as TAP-independent class I binding peptides. Signal sequences are typically composed of three domains: a hydrophobic core (h region) of 6-15 amino-acids, a polar C-terminal end (c region) with small uncharged amino acids and a polar N-terminal region (n region) with a positive net charge¹⁸. Signal sequences are targeted to the ER membrane and cleaved at their carboxyterminus by signal peptidase (SP). The remaining transmembrane trunks are then further processed by membrane-associated signal peptide peptidase (SPP) which cuts the signal sequences within their transmembrane region into several

fragments^{16, 19, 20}. Peptide fragments in the vicinity of the ER lumen can serve as TAP-independent class I ligands. The other fragments get access to the cytosol again and are further processed. The precise loading mechanism of TAP-independent signal peptides into MHC-I molecules is not known, since processing by SP and SPP is thought to take place outside of the PLC. Thus the question arises how TAP-independent signal peptides find their way to peptide-receptive MHC-I molecules in the ER. Normally, the transport by TAP, trimming by ERAAP and loading into MHC all occurs in the PLCs, which are specialized multi-unit machines. The PLC greatly facilitates peptide loading by physical bridging transporters to chaperones for loading and also ‘edits’ the repertoire of bound peptides to maximize their affinity⁵. In the absence of TAP however, the PLC misses one of its important components and thereby detaches the peptide influx from the loading machinery. Therefore loading of TAP-independent peptides, such as signal peptides, might take place outside of the PLC. The hydrophobicity of signal peptides might enable them to quickly associate with nearby proteins of the PLC, however, signal peptides are efficiently loaded in the ER, suggesting a strong degree of regulation and organization. These considerations prompt us to speculate the existence of specialized chaperones or loading complexes for TAP-independent peptides.

Snyder *et al.* (1997) showed that peptides located at the C-terminus of ER-targeted proteins can be generated and presented very efficiently. In this case the very end of the C-terminus of the protein should represent the epitope, not requiring C-terminal trimming, in line with the fact that there is poor carboxypeptidase activity in the ER²¹. They described the presentation of TAP-independent peptides from one ER-resident protein, Jaw1, and proteins in the secretory pathway, like ovalbumin and CD23²²⁻²⁵. In each case the peptides were efficiently liberated from the very C-terminus by the activity of yet unidentified endoproteases to be generated as class I ligands. Based on this “pathway” of peptide liberation, the authors gave the term “C-end rule” to highlight the capacity of ER-resident proteases to liberate class I ligands from the C-terminal ends of ER-targeted proteins.

TAP-independent processing routes: the secretory route

A second characterized processing pathway that bypasses TAP is active in the secretory route. The work of Margarita del Val demonstrated that presentation of TAP-independent peptides can also start in the trans-Golgi network^{26, 27}. This pathway was studied with the use of a model peptide at the C-terminus of the secreted Hepatitis HBe protein. The responsible proteolytic enzyme was shown to be furin, a known protease of the trans-Golgi network normally required for the maturation of secreted proteins (e.g. growth factors and neurotransmitters) by cleaving at precise stretches of three to four basic residues²⁸. Furin processes a wide variety of precursor proteins after the C-terminal arginine residue in the preferred consensus motif -Arg-X-Arg/Lys-Arg↓-X- (X is any amino acid and “↓” indicates the cleavage position)²⁹. Furin-processed peptide-epitopes have been described but the exact MHC-I loading compartments are still unknown. One theoretical option is that recirculating surface MHC-I molecules pick up these peptides

in endosomal vesicles. Some MHC-I molecules are actually observed in endolysosomal compartments and seem to be chaperoned by the invariant chain, which is known to guide MHC-II molecules to their loading vesicles. A recent paper confirmed this role for the invariant chain in cross-presentation of exogenous antigens for MHC-I, leaving the option that the MHC-I antigen presentation system is equipped with such a vesicular route, at least in dendritic cells³⁰. Strikingly, complexes of MHC-I and invariant chain seem more pronounced in cells with TAP deficiency, suggesting that the importance of alternative processing pathways increases in the absence of a functional conventional pathway^{31,32}. The invariant chain-derived peptide CLIP was detected on HLA-I molecules at the surface of leukemic cells. Interestingly, one eluted CLIP peptide efficiently bound a wide variety of HLA-I molecules (-A2, -B7, -A3, -B40) suggesting that this reflects a general non-allele specific mechanism³³.

Other alternative processing pathways have also been described but are poorly characterized. Viral peptides from the LMP2 protein of the Epstein-Barr virus (EBV) can be produced by a TAP-independent and proteasome-dependent pathway and presented by MHC-I³⁴. The TAP-independent peptides were highly hydrophobic, so it was speculated that due to their highly hydrophobicity these cytosolically produced peptides traversed to the ER membrane to reach MHC-I loading compartments. Furthermore, a recent study by Tey *et al.* showed that the presentation of a peptide antigen from the human cytomegalovirus (HCMV) latency associated protein, pUL138, occurs via a TAP-independent and proteasome-independent mechanism³⁵. Interestingly, the MHC-I presentation of this antigen occurred entirely in the vesicular pathway and was mediated by autophagy. The autophagy-mediated pathway generated the same epitope as that generated through the conventional pathway and the MHC-I loading of the peptides occurred within the autophagolysosomal compartment. This shows that the autophagy-mediated pathway can contribute to circumvent viral immune evasion strategies that are common to target the MHC class I machinery, such as viral TAP-inhibiting molecules.

| THE CASE OF IMMUNE ESCAPE OF TUMORS

Tumors frequently display processing defects

One hallmark of tumors is their ability to evade immune recognition³⁶. Among others, a common way to escape is through loss of antigen presentation by MHC-I molecules³⁷⁻⁴¹. Defects in the intracellular processing pathway are often the underlying mechanism of the MHC-I downregulation. For instance, TAP impairment is observed from 10% to 74%, varying with tumor types³⁹⁻⁴². Interestingly, MHC-I downregulation has been associated with progressive disease and is very frequent in metastases of cervical carcinoma, breast cancer, melanoma and Ewing sarcoma^{39, 43, 44}. Moreover, several studies found a clinical correlation between MHC-I expression and enhanced survival in different malignancies as cervical cancer and head and neck squamous cell carcinoma (HNSCC)^{41, 45-47}. Two recent case reports show a strong relation between MHC-I expression on metastatic

melanoma lesions and progression of these individual lesions during immunotherapy with IFN- α ^{48, 49}. All regressing lesions maintained MHC-I surface expression, whereas progressing metastases were characterized by low levels of MHC-I. The underlying mechanisms of MHC-I downregulation include loss of genes encoding MHC class I heavy chain and β 2m as in loss-of-heterozygosity (LOH), decreased transcription of MHC-I locus products and defects in components of the antigen-processing machinery (APM) comprising peptide transporter TAP, tapasin and proteasome subunits³⁷.

Conventional anti-tumor CTL recognize tumor-specific (TSA) or tumor-associated antigens (TAA) presented by MHC-I molecules on the surface of tumor cells. Upon loss of antigen presentation, tumor cells become invisible for recognition by these CTL and therefore turn resistant to CTL-mediated killing⁵⁰. Since impairment of TAP leads to a limited availability of all peptide precursors in the ER, this dysfunction impacts on the total MHC-I surface levels and may lead to general impairment of recognition and elimination of tumor cells by tumor-reactive CTL⁵¹.

Discovery of a novel CTL specificity combating immune escaped tumors

During the search for approaches to counteract immune escape via this route, the group of Dr. Klas Kärre originally discovered a CD8⁺ T-cell subset that selectively recognizes TAP-deficient cells⁵². This T-cell population was raised in mice after immunization with B7.1-expressing TAP-deficient RMA-S lymphoma cells. We performed an in-depth investigation of this phenomenon and characterized a unique category of CTL that exclusively recognizes tumor cells with defects in their APM, but not cells with proficient APM (Figure 2)⁵²⁻⁶⁰. Recognition depended on the β 2m light chain and residual MHC-I molecules on the tumor cells⁶⁰. Restoration of TAP function by gene transfer of TAP subunits or by IFN- γ treatment significantly decreased recognition of target cells by these CTL. Conversely, inhibition of TAP in dendritic cells by the varicella virus-encoded evasion protein UL49.5, which mediates degradation of mouse and human TAP proteins⁶¹, induced recognition of these cells by the novel CTL category^{53, 56}. Furthermore, reactivity of these CTL was clearly T-cell receptor dependent and their phenotype was indistinguishable from that of conventional CD8⁺ CTL⁶⁰. These findings implied that the specificity of the novel CTL category is based on MHC-I/peptide complexes which are exclusively presented by processing deficient cells. This alternative peptide repertoire emerges due to their APM defects and therefore we named the target structures “*T cell epitopes associated with impaired peptide processing*” (TEIPP)⁶⁰.

Characterization of TEIPP antigens

TEIPP-specific CTL do not recognize processing proficient cells, which makes possible that these alternative peptides are immunogenic. Peptide elution studies combined with mass spectrometry and synthetic peptide libraries enabled the molecular identification of the first mouse TEIPP⁶⁰. The peptide recognized by this first CTL clone was not derived from a tumor antigen as such, but from the housekeeping protein TRAM-protein homolog 4 (Trh4 or CerS5)⁶⁰. The epitope was located at the very C-terminus

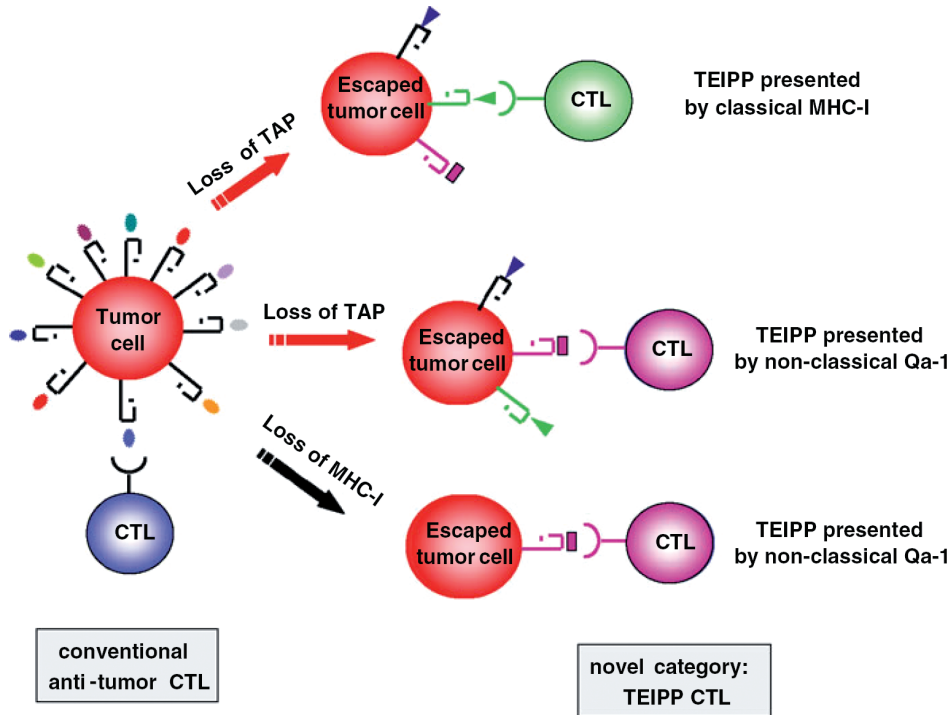


Figure 2. Schematic diagram of TEIPP-specific CTL. Tumor cells with defects in the antigen processing machinery are recognized by TEIPP-specific CTL, but not tumors with intact processing. TEIPP antigens in mouse models are presented by classical MHC-I (upper part) as well as the non-classical MHC-I Qa-1 molecule (lower part). Qa-1-restricted TEIPP CTL recognize TEIPP peptides on cells with TAP-defects and on cells that lost classical MHC-I heavy chains.

of Trh4, which is an ER membrane spanning fatty acid regulator⁶². Since this protein is ubiquitously expressed, it is logical that every tumor type harboring an APM defect was recognized by this CTL⁶⁰. This implies that TEIPP antigens constitute a novel category of CTL epitopes presented by a broad range of tumors with APM defects. Furthermore, these studies showed that the identified TEIPP-specific CTL clones had distinct MHC restriction patterns including not only the classical MHC-I molecules K^b and D^b, but also the non-classical MHC-I molecule Qa-1^{b 57,60}.

TEIPP antigens in the human population

Alternative peptide repertoires presented by TAP-deficient human cells have been described and provided first indications for the presence of TEIPP candidates^{17,63,64}. Further evidence of human TEIPPs was provided by experiments with TAP inhibitors derived from herpes viruses. Many viruses, in particular of the herpes family, deploy mechanisms to target APM components in order to circumvent immunosurveillance by CTL⁶⁵. Examples of viral proteins targeting TAP are ICP47 (HSV), US6 (HCMV), BNLF2a (EBV), and

UL49.5 (varicella viruses). By introducing UL49.5 via gene transfer in human DCs, TAP activity was successfully inhibited⁵⁵. These human TAP-impaired DCs were used as stimulators for autologous T-cell cultures and the resulting CTL displayed selective killing of TAP-impaired target cells. These findings formally demonstrate the existence of TEIPP-specific CTL which are likely to come with a relative high frequency among the precursor population in the human CD8⁺ T-cell pool as they are readily detectable at the bulk T-cell culture level⁵⁵. Furthermore, *in silico* predictions for immunogenic TAP-independent classical HLA-I-binding peptides and HLA-E peptide elution studies with TAP-deficient cells provided a great variety of potential TEIPP candidates. The most immunogenic peptides among them will be defined in current investigations.

Interestingly, a CTL epitope derived from the precursor signal peptide of preprocalcitonin (ppCT) was recently shown to be processed in a TAP-independent manner⁶⁶. The described HLA-A2 restricted CTL clone was isolated from a lung cancer patient and recognizes autologous lung carcinoma cells in which overexpression of ppCT transcripts was detected. Recent data of the group of Dr. Mami-Chouaib show that downregulation of TAP is required to allow presentation of this ppCT peptide⁶⁷. Therefore, we consider this CTL epitope, derived from a primary human tumor, as the first molecular defined human TEIPP.

| SCOPE OF THIS THESIS

The studies of TEIPP antigens thus far have revealed that these antigens are promising candidates for the combat of immune escaped tumors. However, several aspects about TEIPPs are poorly understood: what are the processing pathways that lead to generation and presentation of TEIPP antigens; what is the mechanism behind the immunogenicity of TEIPP; what are characteristics of TEIPP peptides presented by non-classical MHC-I. The studies presented in this thesis are focused on these topics. In **chapter 2** the mechanism underlying the immunogenicity of TEIPP antigens was explored. The mouse TEIPP antigen from the Trh4 protein was used as a model. We induced the overexpression of the Trh4 protein and MHC-I heavy chains in TAP-intact cells, to identify the limiting step of Trh4 presentation. The increased expression of Trh4 was the only mechanism that achieved Trh4-peptide presentation in these TAP⁺ cells. On the other hand, decreased TAP-activity gradually induced the presentation of Trh4-peptide and inhibited the presentation of the TAP-dependent repertoire. Therefore, we proposed a model of competing peptide pools that are governed by TAP-activity, which can be seen as a control lever in shifting the presented peptide repertoire gradually towards TAP-independent or TAP-dependent peptides. In **chapter 3** we studied the processing pathway that lead to the liberation of the Trh4-derived peptide. To identify the involved proteolytic enzymes, a panel of chemical protease inhibitors was used in cellular assays and the re-appearance of the MHC-I/Trh4 complexes in the presence of the inhibitors was measured. These experiments revealed that the SPP enzyme, which belongs to the family of intramembrane cleaving aspartyl

proteases (I-CLiPs), liberated the 9-mer Trh4-antigen in the ER. The participation of SPP in this process was independent from its known role in the release of leader peptides in the ER membrane and therefore revealed a new role for SPP in the liberation of immunogenic C-terminal peptides. In **chapter 4** additional processing pathways participating in the liberation of TEIPP antigens were studied. Here, we studied a TEIPP antigen presented by the classical MHC-I molecule K^b to CTL. Protease inhibitor experiments were performed to identify the proteolytic enzymes responsible for the liberation of this antigen, similarly to what was done in the study of the Trh4 peptide. Surprisingly, we found that the presentation of this antigen was strictly dependent on proteasome function. Furthermore, our data suggested a role of autophagy in the presentation of this TAP-independent and proteasome-dependent antigen.

In **chapter 5**, the topic of TEIPP-specific T-cells restricted by conserved non-classical MHC-I molecules was studied. The non-classical MHC molecule Qa-1 normally presents monomorphic leader-derived peptides and binds CD94/NKG2 receptors on natural killer (NK) cells and CD8⁺ T-cells. The presentation of these Qa-1 determinant modifier (Qdm) peptides is strictly dependent on TAP activity. However, primary studies on TEIPP-specific CTL revealed that Qa-1 molecules activated the Qa-1-restricted TEIPP-CTL in a TCR-dependent manner under conditions of TAP-deficiency but not TAP-proficiency. This prompted us to analyze the repertoire of ligands presented by Qa-1 in TAP-deficient cells. We set out to determine this via biochemical purification and tandem mass spectrometry (MS). A list with more than 80 sequences was elaborated. Several of these peptides were highly immunogenic *in vivo* in mice, showing that they are promising targets in the combat of TAP-deficient cells, such as tumor cells. These data revealed a new role for Qa-1 in adaptive immunity by displacing the monomorphic leader peptides and by presenting a novel repertoire of immunogenic peptides to CD8⁺ T-cells in processing-deficient cells. In **chapter 6** we reviewed the known roles of Qa-1 and the human variant, HLA-E, in immunity in the context of the new findings concerning Qa-1 as described in chapter 5. In **Chapter 7**, an overview of the findings presented in this thesis is discussed.

I REFERENCES

1. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* **9**, 833-44 (2009).
2. Sebзда, E. et al. Selection of the T cell repertoire. *Annu Rev Immunol* **17**, 829-74 (1999).
3. Koble, C. & Kyewski, B. The thymic medulla: a unique microenvironment for intercellular self-antigen transfer. *J Exp Med* **206**, 1505-13 (2009).
4. Kessler, J.H. et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* **12**, 45-53 (2011).
5. Cresswell, P., Ackerman, A.L., Giodini, A., Peaper, D.R. & Wearsch, P.A. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol. Rev* **207**, 145-157 (2005).
6. Jensen, P.E. Recent advances in antigen processing and presentation. *Nat Immunol* **8**, 1041-8 (2007).
7. Kloetzel, P.M. & Ossendorp, F. Proteasome and peptidase function in MHC-class-I-mediated antigen presentation. *Curr Opin Immunol* **16**, 76-81 (2004).
8. Neefjes, J., Jongsma, M.L., Paul, P. & Bakke, O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* **11**, 823-36 (2011).

9. Rock, K.L., Farfan-Arribas, D.J. & Shen, L. Proteases in MHC class I presentation and cross-presentation. *J Immunol* **184**, 9-15 (2010).
10. Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* **8**, 607-18 (2008).
11. Aldrich, C.J. et al. Positive selection of self- and alloreactive CD8+ T cells in TAP-1 mutant mice. *Proc. Natl. Acad. Sci. USA* **91**, 6525-6528 (1994).
12. Sandberg, J.K., Chambers, B.J., Van Kaer, L., Karre, K. & Ljunggren, H.G. TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. *Eur. J. Immunol.* **26**, 288-293 (1996).
13. Van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L. & Tonegawa, S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell* **71**, 1205-1214 (1992).
14. Cerundolo, V. & de la Salle, H. Description of HLA class I- and CD8-deficient patients: Insights into the function of cytotoxic T lymphocytes and NK cells in host defense. *Semin Immunol* **18**, 330-6 (2006).
15. de la Salle, H. et al. Human peptide transporter deficiency: importance of HLA-B in the presentation of TAP-independent EBV antigens. *J Immunol* **158**, 4555-63 (1997).
16. Henderson, R.A. et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* **255**, 1264-1266 (1992).
17. Wei, M.L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443-6 (1992).
18. Martoglio, B. & Dobbertin, B. Signal sequences: more than just greasy peptides. *trends in Cell Biol.* **8**, 410-415 (1998).
19. Martoglio, B. & Dobbertin, B. Signal sequences: more than just greasy peptides. *Trends Cell Biol* **8**, 410-5 (1998).
20. Wei, M.L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443-446 (1992).
21. Reits, E. et al. Peptide diffusion, protection, and degradation in nuclear and cytoplasmic compartments before antigen presentation by MHC class I. *Immunity* **18**, 97-108 (2003).
22. Snyder, H.L. et al. Two novel routes of transporter associated with antigen processing (TAP)-independent major histocompatibility complex class I antigen processing. *J. Exp. Med.* **186**, 1087-98 (1997).
23. Snyder, H.L., Bacik, I., Yewdell, J.W., Behrens, T.W. & Bennink, J.R. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur. J. Immunol.* **28**, 1339-46 (1998).
24. Snyder, H.L., Yewdell, J.W. & Bennink, J.R. Trimming of antigenic peptides in an early secretory compartment. *J. Exp. Med.* **180**, 2389-94 (1994).
25. Yewdell, J.W. et al. TAP-independent delivery of antigenic peptides to the endoplasmic reticulum: therapeutic potential and insights into TAP-dependent antigen processing. *J. Immunother.* **21**, 127-131 (1998).
26. Gil-Torregrosa, B.C., Raul Castano, A. & Del Val, M. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-Golgi network protease furin. *J Exp Med* **188**, 1105-16 (1998).
27. Medina, F. et al. Furin-processed antigens targeted to the secretory route elicit functional TAP1-/-CD8+ T lymphocytes in vivo. *J Immunol* **183**, 4639-47 (2009).
28. Thomas, G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat Rev Mol Cell Biol* **3**, 753-66 (2002).
29. Yamada, Y. & Liu, D.X. Proteolytic activation of the spike protein at a novel RRRR/S motif is implicated in furin-dependent entry, syncytium formation, and infectivity of coronavirus infectious bronchitis virus in cultured cells. *J Virol* **83**, 8744-58 (2009).
30. Basha, G. et al. A CD74-dependent MHC class I endolysosomal cross-presentation pathway. *Nat Immunol* **13**, 237-45 (2012).
31. Sugita, M. & Brenner, M.B. Association of the invariant chain with major histocompatibility complex class I molecules directs trafficking to endocytic compartments. *J Biol Chem* **270**, 1443-8 (1995).
32. Vigna, J.L., Smith, K.D. & Lutz, C.T. Invariant chain association with MHC class I: preference for HLA class I/beta 2-microglobulin heterodimers, specificity, and influence of the MHC peptide-binding groove. *J Immunol* **157**, 4503-10 (1996).
33. van Luijn, M.M. et al. Promiscuous binding of invariant chain-derived CLIP peptide to distinct HLA-I molecules revealed in leukemic cells. *PLoS One* **7**, e34649 (2012).
34. Lautscham, G. et al. Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell recognition reveals a proteasome-dependent, transporter associated

- with antigen processing-independent pathway. *J Exp Med* **194**, 1053-68 (2001).
35. Tey, S.K. & Khanna, R. Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway. *Blood* **120**, 994-1004 (2012).
 36. Cavallo, F., De Giovanni, C., Nanni, P., Forni, G. & Lollini, P.L. 2011: the immune hallmarks of cancer. *Cancer Immunol Immunother* **60**, 319-26 (2011).
 37. Garrido, F. & Algarra, I. MHC antigens and tumor escape from immune surveillance. *Adv Cancer Res* **83**, 117-58 (2001).
 38. Garrido, F., Cabrera, T. & Aptsiauri, N. "Hard" and "soft" lesions underlying the HLA class I alterations in cancer cells: implications for immunotherapy. *Int J Cancer* **127**, 249-56 (2010).
 39. Hicklin, D.J., Marincola, F.M. & Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol Med Today* **5**, 178-86 (1999).
 40. Khong, H.T. & Restifo, N.P. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat Immunol* **3**, 999-1005 (2002).
 41. Seliger, B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol Immunother* **57**, 1719-26 (2008).
 42. Chang, C.C., Campoli, M. & Ferrone, S. HLA class I defects in malignant lesions: what have we learned? *Keio J Med* **52**, 220-9 (2003).
 43. Berghuis, D. et al. Reduced human leukocyte antigen expression in advanced-stage Ewing sarcoma: implications for immune recognition. *J Pathol* **218**, 222-31 (2009).
 44. Ryu, K.S. et al. Alterations of HLA class I and II antigen expression in preinvasive, invasive and metastatic cervical cancers. *Exp Mol Med* **33**, 136-44 (2001).
 45. Jordanova, E.S. et al. Human leukocyte antigen class I, MHC class I chain-related molecule A, and CD8+/regulatory T-cell ratio: which variable determines survival of cervical cancer patients? *Clin Cancer Res* **14**, 2028-35 (2008).
 46. Mehta, A.M., Jordanova, E.S., Kenter, G.G., Ferrone, S. & Fleuren, G.J. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol Immunother* **57**, 197-206 (2008).
 47. Meissner, M. et al. Defects in the human leukocyte antigen class I antigen processing machinery in head and neck squamous cell carcinoma: association with clinical outcome. *Clin Cancer Res* **11**, 2552-60 (2005).
 48. Cabrera, T. et al. HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. *Cancer Immunol Immunother* **56**, 709-17 (2007).
 49. Carretero, R. et al. Analysis of HLA class I expression in progressing and regressing metastatic melanoma lesions after immunotherapy. *Immunogenetics* **60**, 439-47 (2008).
 50. Maeurer, M.J. et al. Tumor escape from immune recognition: lethal recurrent melanoma in a patient associated with downregulation of the peptide transporter protein TAP-1 and loss of expression of the immunodominant MART-1/Melan-A antigen. *J Clin Invest* **98**, 1633-41 (1996).
 51. van Endert, P.M. Genes regulating MHC class I processing of antigen. *Curr Opin Immunol* **11**, 82-8 (1999).
 52. Wolpert, E.Z. et al. Generation of CD8+ T cells specific for transporter associated with antigen processing deficient cells. *Proc Natl Acad Sci U S A* **94**, 11496-501 (1997).
 53. Chambers, B. et al. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer Res* **67**, 8450-5 (2007).
 54. Lampen, M.H. & van Hall, T. Strategies to counteract MHC-I defects in tumors. *Curr Opin Immunol* **23**, 293-8 (2011).
 55. Lampen, M.H. et al. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. *J Immunol* **185**, 6508-17 (2010).
 56. Oliveira, C.C. et al. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. *Eur J Immunol* **41**, 3114-24 (2011).
 57. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-21 (2010).
 58. van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b1. *J Immunol* **178**, 657-62 (2007).
 59. van Hall, T., Oliveira, C.C., Joosten, S.A. & Ottenhoff, T.H. The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress. *Microbes Infect* **12**, 910-8 (2010).
 60. van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat Med* **12**, 417-24 (2006).
 61. Verweij, M.C. et al. Inhibition of mouse TAP by immune evasion molecules encoded by non-murine herpesviruses. *Mol Immunol* **48**, 835-45 (2011).

62. Riebeling, C., Allegood, J.C., Wang, E., Merrill, A.H., Jr. & Futerman, A.H. Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J Biol Chem* **278**, 43452-9 (2003).
63. Henderson, R.A. et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* **255**, 1264-6 (1992).
64. Weinzierl, A.O. et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur J Immunol* **38**, 1503-10 (2008).
65. Horst, D., Verweij, M.C., Davison, A.J., Rensing, M.E. & Wiertz, E.J. Viral evasion of T cell immunity: ancient mechanisms offering new applications. *Curr Opin Immunol* **23**, 96-103 (2011).
66. El Hage, F. et al. Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway. *Proc Natl Acad Sci U S A* **105**, 10119-24 (2008).
67. Durgeau, A. et al. Different Expression Levels of the TAP Peptide Transporter Lead to Recognition of Different Antigenic Peptides by Tumor-Specific CTL. *J Immunol* (2011).

2

PEPTIDE TRANSPORTER TAP MEDIATES BETWEEN COMPETING ANTIGEN SOURCES GENERATING DISTINCT SURFACE MHC CLASS I PEPTIDE REPERTOIRES

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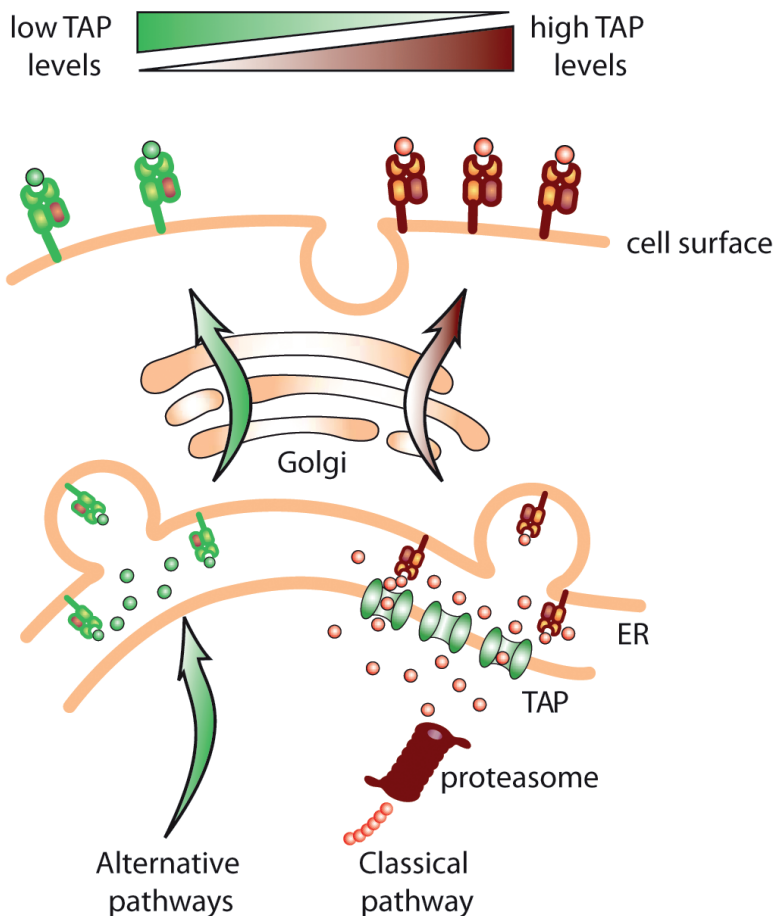
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PEPTIDE TRANSPORTER TAP PREVENTS PRESENTATION OF ALTERNATIVE PEPTIDES

The peptide transporter TAP mediates the entry of peptide precursors from the cytosol into the ER where they are loaded into MHC-I molecules. Part of the MHC-I presented peptides do not require the action of TAP or the proteasome and derive from alternative processing pathways. Interestingly, some of these alternative peptides are only presented when there are impairments in the classical processing pathway and do not reach the cell surface in normal cells. In this issue, Oliveira *et al* now describe that the peptide transporter TAP actually prevents the presentation of this alternative repertoire due to the overwhelming influx of competitor peptides in the ER. Strong over-expression of the antigen-encoding gene was needed to push the alternative peptide towards MHC-I surface display. Thus, TAP behaves like a lever of control to shift the presented peptide repertoire gradually towards TAP-independent or TAP-dependent peptides.



| ABSTRACT

We recently described a category of TAP-independent peptide-epitopes that are selectively presented by cells with processing defects in the classical MHC class I (MHC-I) pathway. Here, we studied the ER-resident ceramide synthase Trh4 as a prototypic example of these neo-antigens and found that moderate inhibition of TAP permits cell surface presentation of the Trh4 peptide. The absence of this peptide from WT cells was not related to the binding or stability of the Trh4/D^b complexes, or to the availability of MHC-I heavy chains, but rather to the limited expression of the antigen. Strongly elevated antigen levels were needed to reach comparable peptide display on WT as on TAP-deficient cells. Our data suggest that the normal influx of TAP-transported peptides in the ER during routine processing creates an efficient barrier for peptides from alternative processing routes. Impairment of TAP function, as commonly found in cancers and virus-infected cells, lowers this resistance allowing for MHC-I presentation of other peptide sources.

| INTRODUCTION

Cytotoxic T lymphocytes (CTLs) are key effector cells of the adaptive immune system and circulate throughout the body in search for their cognate peptides that are presented by MHC class I (MHC-I) molecules. T-cell receptors determine the antigen specificity of CTLs and engagement with peptide/MHC-I complexes leads to their activation and elimination of target cells. Therefore, the process of MHC-I antigen processing and presentation, which operates in all nucleated cells of our body, is crucial for CTL immune surveillance¹⁻³. The highly complex repertoire of MHC-I presented peptides reflects the total proteome of cells and derives from physiological turnover of proteins, a process that is largely operated by the multicatalytic enzyme proteasome^{4,5}. In addition to the proteasome, other proteolytic enzymes in the cytosol have been implicated in the liberation of peptides for MHC-I presentation, some of which can compensate for the lack of proteasome activity^{1,6,7}. For instance, tripeptidyl peptidase II (TPPII), insulin-degrading enzyme (IDE), thimet oligopeptidase (TOP) and nardilysin have been implicated in the generation of some CTL epitopes⁸⁻¹⁰. However, the relative contributions of these novel peptidases and their cooperation with the proteasome have not been fully characterized.

The intermediate peptide products are rescued from total breakdown by these cytosolic proteases through translocation into the ER. Subsequently, peptides are trimmed and loaded into the grooves of MHC-I molecules, a dynamic process that is mediated by the peptide loading complex (PLC) consisting of MHC-I, β_2m , ERp57, TAP, tapasin and chaperones¹¹⁻¹³. The TAP peptide transport is operated by the heterodimer pump TAP1/TAP2, members of the ABC transporter family. The importance of the TAP transporter is highlighted by the strong decrease in cell surface MHC-I molecules in the absence of TAP¹⁴. Furthermore, this bottleneck of the antigen processing pathway is frequently targeted by viruses, especially from the herpes group, which successfully evade complete eradication by CTL immunity^{15,16}. Moreover, loss of TAP expression

is often found in cancers and results in resistance to CTL attack¹⁷⁻¹⁹. All these findings together convincingly demonstrate that the conventional proteasome-TAP pathway plays a dominant role in the surface display of peptide/MHC-I.

In addition to the conventional MHC-I processing pathway described above, cells are equipped with alternative routes that lead to the liberation and loading of peptides in MHC-I molecules. The exact contribution of these alternative routes to the overall peptide repertoire is difficult to assess, but it is intriguing that TAP-knockout mice still harbor a rather diverse CD8⁺ T-cell repertoire that can functionally respond to viral antigens^{14, 20}. Moreover, TAP-deficient patients seem to cope quite well with viral infections, in contrast to infections with Gram-negative bacteria²¹⁻²⁴. Apparently, TAP-independent processing pathways can partly compensate for the loading of peptides in MHC-I molecules. Biochemical analysis of the peptide repertoire of TAP-deficient T2 cells revealed abundant presentation of signal sequence derived peptides²⁵⁻²⁷. Indeed, signal sequences are liberated by the combined action of signal peptidase (SP) and signal peptide peptidase (SPP) and are directly available for loading into MHC-I^{28, 29}. A second characterized processing pathway that bypasses TAP is active in the secretory route and is mediated by members of the proprotein convertase (PC) family, like furin³⁰⁻³². This enzyme is located in the trans-Golgi network and mediates the proteolytic maturation of many proproteins, e.g. growth factors and matrix metalloproteinases³³. Peptides located at the C-terminus of secreted proteins can be liberated by furin and subsequently gain access to MHC-I in a TAP-independent way^{30, 32}.

Previously we reported the TAP-independent presentation of a C-terminal peptide from the ceramide synthase Trh4, which is a multiple membrane-spanning protein in the ER³⁴⁻³⁶. This peptide was the first natural example of a C-terminal processing pathway of ER resident proteins, while previous studies suggested the existence of this route³⁷⁻³⁹. The Trh4 protein has a housekeeping function and is ubiquitously expressed. Intriguingly, the C-terminal peptide-epitope was not presented by processing-intact cells, but only emerged in MHC-I of cells with processing defects, like proteasome, tapasin or TAP³⁶. In our current study, we show that peptide repertoires from the conventional processing route and the TAP-independent route are shifting arrays controlled by TAP function. The TAP-mediated peptide influx in the ER seems to constitute a resistance barrier for the presentation of peptides from alternative processing routes.

| RESULTS

Selective presentation of self-peptide Trh4 by processing-deficient cells

The surface presentation of the Trh4 peptide was determined for two panels of tumor cells using previously established CTL clones. RMA-S lymphoma cells are TAP-deficient due to an incomplete TAP2 chain and the Trh4 peptide, but not a proteasome- and TAP-dependent control peptide, was detected at the surface of these cells (Fig 1A). Vice versa, TAP-intact RMA cells efficiently presented the control epitope, but the Trh4 peptide

was not detected by the CTL clone (Fig 1A). In the fibrosarcoma tumor model MCA, a chemically induced tumor isolated from a TAP1-knockout mouse, we observed the same dichotomy in presented peptide repertoires between TAP-deficient and TAP-proficient cells (Fig 1B). MCA cells only presented the Trh4 peptide, but not the TAP-dependent control. Importantly, restoration of the mouse TAP1 gene in this tumor resulted in decreased stimulation of the Trh4-specific CTLs and simultaneously in an increased stimulation of the control CTLs (Fig 1B). This indicated that MCA.TAP1 cells display a mixed peptide repertoire in their MHC-I molecules comprised of both TAP-dependent and -independent peptides. The introduction of the TAP1 subunit did not fully restore TAP function in these cells but showed an inhibitory effect of TAP on Trh4 presentation. This effect was potentiated by IFN- γ treatment of MCA.TAP1 cells, a strong inducer of the conventional antigen processing and presentation machinery. IFN- γ -treated MCA.TAP1 cells displayed the other extreme of the peptide profile, in that the Trh4 peptide was virtually absent from the cell surface (Fig 1B). IFN- γ treatment of parental TAP-knockout MCA cells did not abrogate Trh4 presentation, suggesting that deficiency of TAP is sufficient for the surface display of Trh4/MHC-I complexes.

Next, we studied the presentation of the Trh4 peptide by dendritic cells, which are highly efficient antigen presenting cells (APCs). Introduction of the viral evasion protein UL49.5 that targets TAP function⁴⁰ resulted in modest inhibition of peptide transport and MHC-I presentation of these cells as we previously showed⁴¹. Control CTLs specific for a TAP-dependent epitope were still capable of recognizing these UL49.5-expressing dendritic cells, albeit to lower extent, attesting to the partial inhibition of TAP (Fig 1C). Nevertheless, this partial TAP impediment led to the emergence of the Trh4 peptide on these dendritic cells (Fig 1C).

These data corroborated our previous work and showed that the endogenous Trh4 protein is ubiquitously present in cells, but its C-terminal epitope is only presented by MHC-I on cells harboring a partially impaired TAP function. Furthermore, these results demonstrated that the peptide repertoire from the conventional proteasome-TAP pathway can co-exist with that of alternative routes and that TAP function mediates the shift between these repertoires.

Trh4 peptide presentation by primary tissue cells

Trh4 presentation was thus far studied with *in vitro* cultured cell lines. To obtain a broader profile of Trh4 peptide presentation by primary tissues we examined a panel of *ex vivo* isolated tissue cells from organs of wild type mice and TAP1-knockout mice. Parenchymal cell populations were purified by mechanical and enzymatic disruption of organs that were perfused with heparin. *Ex vivo* depletion of CD45⁺ hematopoietic cells, including antigen presenting immune cells resulted in purified epithelial with stromal cell populations. Tissue cells from TAP1-knockout mice were recognized by the Trh4-specific CTLs, except from spleen and liver (Fig 2A). Interestingly, the degree of recognition varied between the organs and was particularly high for bone marrow, lymph

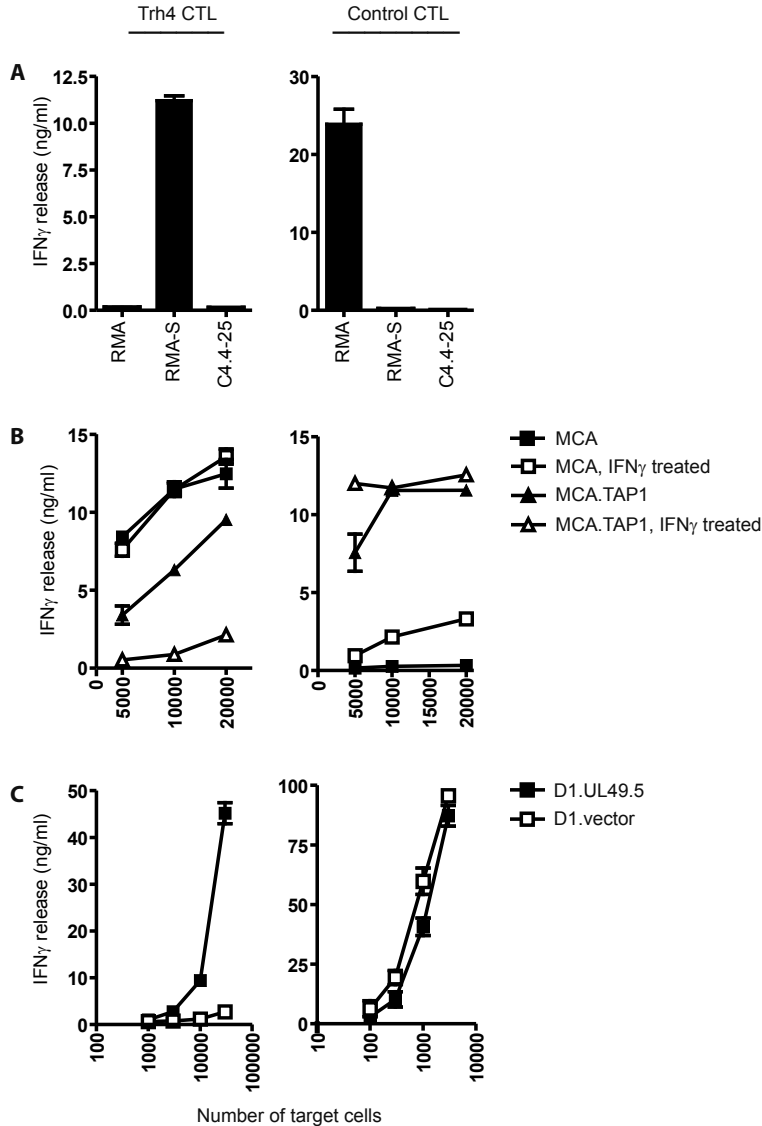


Figure 1. The Trh4 peptide is only presented on cells with impaired TAP function. Peptide presentation was evaluated by incubating CTLs (Trh4-specific or controls) with target cells and measuring IFN- γ levels in the supernatant by ELISA after culture for 18 hours. The Trh4-specific CTL clone recognizes the MCLRLMTAVM peptide from the Trh4 protein; the control T cells recognize the TAP- and proteasome-dependent peptides (A) CCLCLTVFL, an MuLV-derived peptide, and (B, C) the H-2D^b-leader derived peptide AMAPRTLTL. The target cells are (A) RMA, a TAP-proficient lymphoma cell line; RMA-S, a TAP2-mutant variant of RMA; and C4.4-25, a β 2m-deficient lymphoma cell line; (B) MCA fibrosarcoma cells derived from TAP1^{-/-} mice either untreated or reconstituted with the TAP1 gene (MCA.TAP1) and, where indicated, pre-treated with IFN- γ to boost the antigen processing and presentation machinery before culture with the CTLs; (C) D1 dendritic cells transduced with the viral TAP-inhibitor UL49.5 (D1.UL49.5) or an empty vector (D1.vector). Means and standard deviations of triplicates are shown from one out of three independent experiments.

nodes and thymus, suggesting that the epithelial and connective tissue cells of these organs efficiently support MHC-I antigen presentation. Parenchymal cell populations of liver and spleen appeared to lack Trh4/MHC-I complexes and we speculate that these organs do not express the processing enzymes for Trh4 peptide liberation. None of the tissue populations of wild type mice was recognized by the Trh4-specific CTLs (Fig 2A). Analysis of Trh4 gene expression by the tissues revealed no difference between wild type and TAP1-knockout cell populations, indicating that the Trh4 gene was expressed by all tissue cells of the wild type mouse as well (Fig 2B). To determine Trh4 gene expression in thymus in more detail, we performed expression analysis on separated thymus subpopulations that are known to mediate negative selection⁴². Trh4 transcripts were detected in medullary thymic epithelial cells (mTEC), dendritic cells and macrophages (Fig 2C). In addition, Trh4 was also expressed by thymocytes. Notably, thymus stromal cells from wild type mice did not present the Trh4 epitope in contrast to their TAP1-knockout counterparts, suggesting that negative selection of this CTL specificity in wild type mice is negligible.

These results implied that Trh4 is ubiquitously expressed *in situ* in the body, including thymic cells that mediate negative selection, but that the conventional TAP-facilitated peptide repertoire prevents its MHC-I presentation. As a consequence, T cell receptors with Trh4/H-2D^b specificity will not be deleted from the CTL repertoire in normal mice.

Trh4 is a stable and high affinity peptide for binding to H-2D^b

In order to study the underlying mechanisms leading to the absence of the Trh4 peptide on cells with normal antigen processing functions we assessed the capacity of the Trh4 peptide to compete for MHC-I loading as weak interactions with the presenting molecule H-2D^b might explain its failure to stimulate T cells. It is known that the ER-resident peptide loading complex actively selects high quality peptides for MHC-I binding^{11, 43} and, potentially, weak binding affinity might thus preclude Trh4 loading. We determined the binding affinity of Trh4 to H-2D^b as well as the dissociation rate of these complexes and compared these values with those for a viral CTL epitope and five other tumor-associated CTL epitopes (Fig 3). The measured peptide binding affinities showed that Trh4 was positioned in the group of high affinity binders, comparable to the viral gp33 epitope from LCMV (Fig 3A). Trh4 was clearly distinguishable from low affinity peptides like the MDM2- and gp100-derived epitopes. Moreover, the stability of Trh4/H-2D^b complexes, a parameter that strongly associates with immunogenicity⁴⁴, was superior to all the other tested peptides (Fig 3B). After 6 hours, virtually all Trh4/H-2D^b complexes were still detected, whereas the tumor-associated peptides showed a fast decay.

Considering these results, we concluded that the Trh4 peptide binds to its presenting MHC-I molecule with high affinity and stability. These features would rather facilitate than avoid its presentation by normal cells.

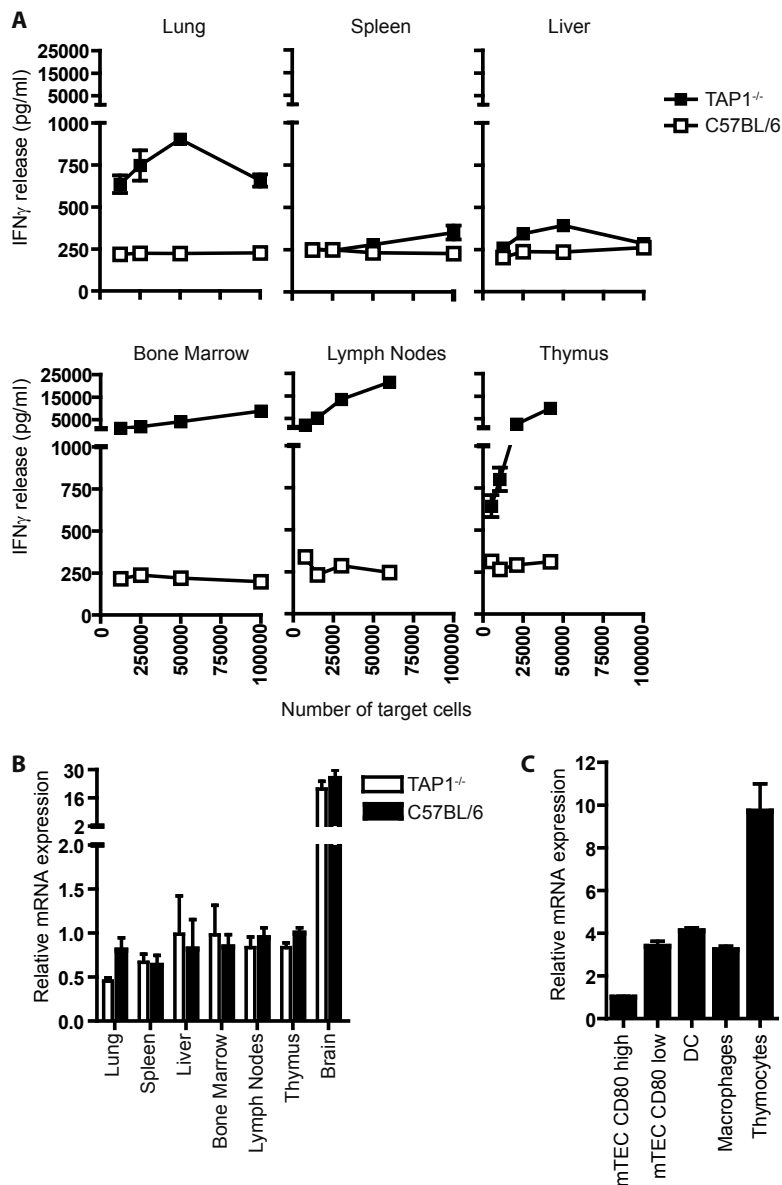


Figure 2. *Trh4* is widely expressed and presented on TAP-deficient primary tissue cells. (A) The indicated organs/tissues were collected from C57BL/6 and TAP1 $^{-/-}$ mice and processed *in vitro* by mechanical and enzymatic disruption. CD45 $^{+}$ cells were depleted from the isolated tissue cells by use of magnetic CD45 MicroBeads and the remaining cells were incubated with the *Trh4*-specific CD8 $^{+}$ T-cell clone and IFN- γ production determined. (B-C) Total RNA was extracted from the isolated primary cells derived from (B) the indicated tissues/organs of C57BL/6 and TAP1 $^{-/-}$ mice or (C) the specific thymic subpopulations of C57BL/6 mice. cDNA was synthesized from the RNA samples and *Trh4* mRNA expression determined by quantitative PCR with normalization to the (B) *GAPDH* or (C) β -*actin* housekeeping genes and expressed relative to (B) thymus TAP1 $^{-/-}$ and (C) mTEC CD80 $^{\text{high}}$ *Trh4* mRNA levels. Means and standard deviations of triplicates are shown from one out of three independent experiments.

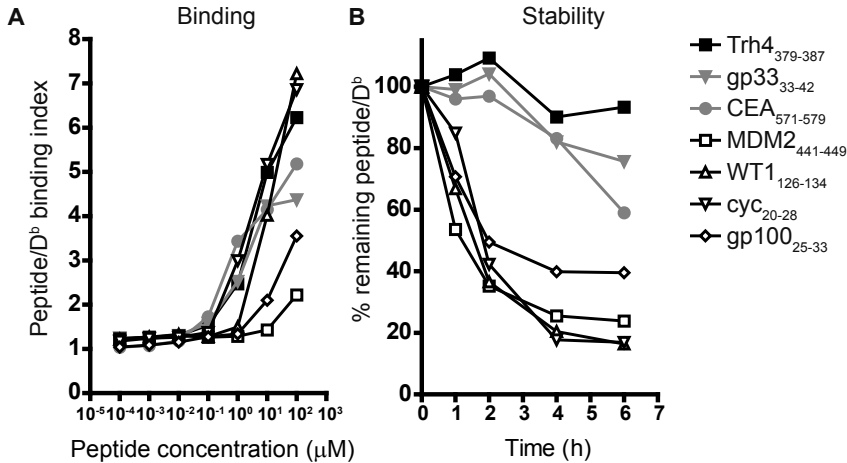


Figure 3. Binding and stability of Trh4 peptide/H-2D^b complexes. (A) The binding affinity of the Trh4 peptide to H-2D^b was evaluated. RMA-S cells were incubated with varying concentrations of the indicated H-2D^b binding peptides and H-2D^b cell-surface levels after 4h incubation were determined by flow cytometry. The binding index of peptides to MHC was calculated as the ratio of the mean fluorescence intensity (MFI) between loaded and unloaded cells. The index of a control H-2K^b binding peptide was one in all concentrations (not depicted). (B) The decay of the Trh4/H-2D^b complexes was tested by incubation of RMA-S cells with the indicated peptides and evaluation of surface H-2D^b levels by flow cytometry over time. The data are the percentages of remaining peptide/H-2D^b complexes on the cell surface as compared to initial levels. Results shown are representative of three independent experiments.

MHC availability is not the limiting step for Trh4 loading and presentation

Next, we tested whether the availability of MHC-I molecules was limiting for the presentation of Trh4. Higher expression levels for the heavy chain might enable the Trh4 peptide to be loaded into H-2D^b molecules even in the presence of the peptide repertoire of the conventional processing pathway. A retroviral expression vector containing the H-2D^b gene was introduced into TAP-positive RMA cells and MHC-I protein levels were analysed by flow cytometry. The surface display of H-2D^b molecules increased nearly two-fold, indicating that extra availability of heavy chain proteins indeed enhances peptide loading and surface presentation of peptide/MHC-I complexes (Fig 4A). Nevertheless, analysis of Trh4 presentation showed that additional heavy chains did not bring this peptide to the cell surface (Fig 4B). These data suggested that the supplementary MHC-I molecules predominantly accommodated peptides from the classical repertoire. Furthermore, a three-fold increase of H-2D^b molecules at cell surface of TAP-deficient RMA-S cells neither enhanced the surface display of Trh4 peptides (Fig 4A and 4B). This result suggested that TAP deficiency, which blocks the presentation of the classical peptide repertoire, creates the opportunity for all available Trh4 peptides to be loaded, irrespective of MHC-I levels. Together, these results showed that the availability of MHC-I molecules is not the rate-limiting step for the restricted presentation of Trh4 by processing-deficient cells.

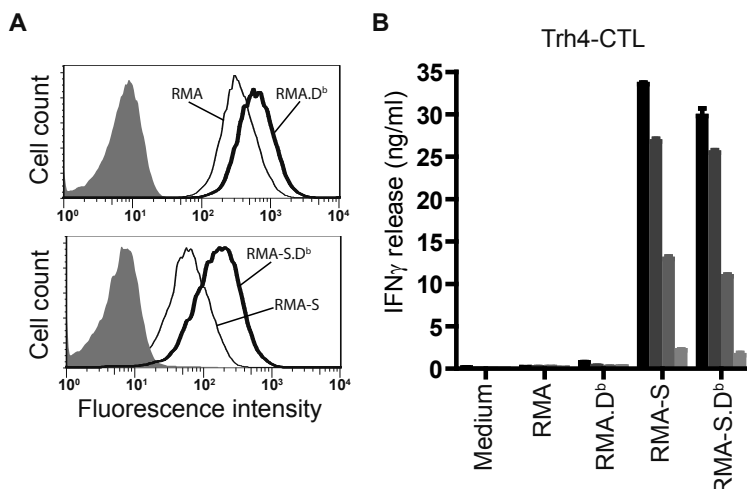


Figure 4. Over-expression of MHC-I does not facilitate presentation of the Trh4 peptide. (A) H-2D^b was over-expressed in RMA and RMA-S cells (RMA.D^b and RMA-S.D^b) by retroviral transduction and cell surface expression of H-2D^b was determined in transduced and control (RMA and RMA-S) cells. The filled grey line represents the background staining with the secondary antibody alone. The results are representative of three independent experiments. (B) H-2D^b over-expressing RMA and RMA-S target cells (RMA.D^b and RMA-S.D^b), non-transduced RMA and RMA-S cells or medium alone were incubated with Trh4-specific CTLs and IFN- γ production determined. The black bar represents culture with 20,000 target cells and the grey bars represent culture with five fold serial dilutions of the target cells. Means and standard deviations of triplicates are shown from one out of three independent experiments.

Higher antigen levels in TAP-intact cells allow Trh4 presentation

Then we assessed whether the restricted presentation of our Trh4 peptide was based on quantity limitations. Processing-intact cells encounter large influxes of peptides into the ER via the TAP transporter. This peptide pool from the conventional proteasome-TAP processing pathway might easily overwhelm the peptides arriving in the ER via alternative routes and compete with the Trh4 peptides for MHC-I binding. We over-expressed Trh4 by gene transfer using vectors with a strong heterologous promoter. RMA and RMA-S cells were stably transfected with cDNA encoding the long transcript variant of Trh4, which contains the CTL epitope at the very C-terminus. The short Trh4 transcript lacks one exon and does not encode for the epitope due to a reading frame shift³⁶. Analysis of processing-proficient RMA cells with higher Trh4 gene expression revealed that the Trh4 peptide was efficiently presented by these cells to an extent that was comparable with RMA-S cells (Fig 5A). On the other hand, over-expression of Trh4 in the TAP-negative RMA-S hardly enhanced the Trh4 presentation. This suggested that rate limiting steps other than peptide quantity might be in place, for example related to processing enzymes. These results indicated that the processing pathway for the generation of Trh4 is active in normal TAP-positive cells and, moreover, that physiological quantities of the Trh4 peptide precluded the loading and surface presentation by these cells. By feeding higher levels

of the antigen, a successful competition with the peptide repertoire of the conventional pathway was achieved. To assess the expression level of Trh4, quantitative PCR analysis was performed, since antibodies against the protein were not available. The Trh4 gene in our RMA.Trh4 cells was expressed 120 times higher than the endogenous levels in RMA cells (Fig S1). The expression of the short Trh4 transcript did not vary between the cell lines, because this cDNA was not involved in gene transfer (Fig S1). We anticipated that over-expressed Trh4 protein was still ER localized like the endogenous protein, since a previous study confirmed this using HA-tagged Trh4³⁵. Moreover, the over-expressed Trh4 is not shuttled to the cytosol for proteasome-mediated degradation, because epoxomicin did not inhibit the presentation of the Trh4 peptide by these RMA.Trh4 cells (Fig S2). Interestingly, the proteasome inhibitor rather increased the presentation of the TAP-independent epitope, again confirming that the classical processing route of proteasome-TAP hampers the presentation of peptides from alternative pathways.

We then examined the quantity range that was needed for the presentation of Trh4 on TAP-positive cells. RMA.Trh4 cells were sorted in three populations on basis of fluorescent GFP proteins levels, which corresponded to Trh4 levels due to a coupled translation initiation by an internal ribosome entry site in our gene construct. Gene expression analysis showed that the three RMA.Trh4 populations harbored approximately 800, 400 and 20 times the amount of endogenous Trh4, respectively (Fig 5B). Again, over-expression of the long Trh4 transcript did not influence the expression of the shorter isoform. RMA.Trh4 cells with 20 times over-expression were recognized by the CTL clone, but just above the quantity threshold (Fig. 5C). RMA.Trh4 cells with 400 times over-expression presented the epitope to convincing levels. However, this high expression level was still not saturating, as RMA.Trh4 cells with 800 times over-expression were clearly better recognized by the Trh4-specific CTL clone (Fig 5C). The fact that these extreme levels of Trh4 expression were needed to reach optimal peptide presentation in TAP-positive cells implied that the conventional peptide repertoire entering the ER via TAP mediates strong resistance to the presentation of the alternatively processed Trh4 peptide. Importantly, vigorous over-expression of this one transcript did not alter or inhibit the presentation of the TAP-mediated control peptide (Fig 5C). We wondered if the over-expressed *Trh4* gene in RMA.Trh4 cells would reach transcript levels comparable to those of classical antigens. The endogenously expressed retroviral *gag* gene encodes the immunodominant H-2D^b-presented epitope CCLCLTVFL, which is TAP- and proteasome-dependent⁴⁵. Whereas endogenous Trh4 expression in the tumor cells was very low, the over-expressed gene approached levels comparable to the *gag* gene (Fig. S3). This indicated that the Trh4 epitope is not presented by TAP-positive cells because its expression level is too low.

Increased Trh4 expression in professional antigen-presenting cells (dendritic D1 cells) also induced recognition by Trh4-CTLs (Fig S4). Stimulation of the conventional processing route through LPS-mediated maturation of these cells decreased Trh4 peptide presentation, supporting our notion on competing peptide repertoires (Fig. S4).

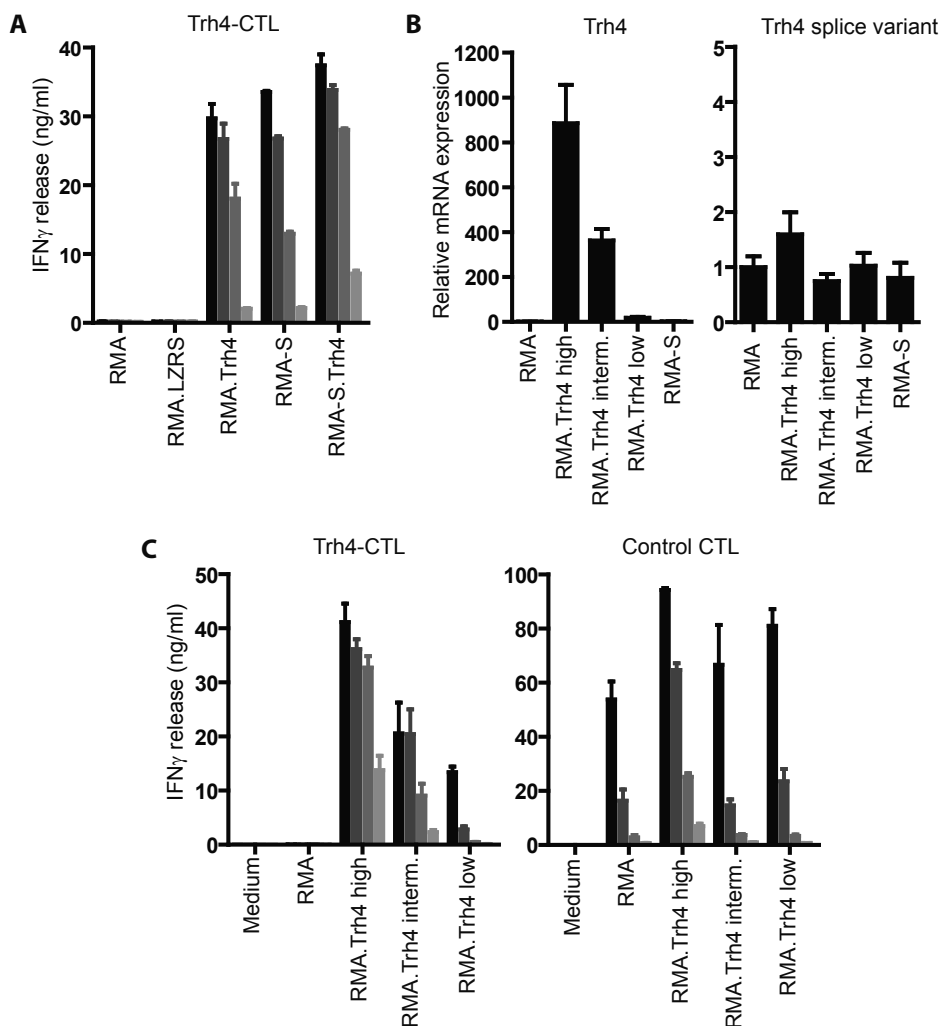


Figure 5. Trh4 peptide is presented on TAP-proficient cells upon over-expression of Trh4. (A) Reactivity of Trh4-specific T-cell clone against TAP-proficient RMA cells and TAP2-deficient RMA-S cells transduced or not with the *Trh4* gene (RMA.Trh4, RMA-S.Trh4) or an empty vector (RMA.LZRS) as determined by IFN- γ production. Four fold serial dilutions of targets were performed starting at 20,000 target cells as indicated by the grey scale bars. (B) mRNA expression, in the indicated cells, of *Trh4* and a natural *Trh4* splice variant as determined by quantitative PCR using specific primers to distinguish both transcripts. The splice variant, used as a control, is a shorter transcript that does not contain the sequence of the Trh4 peptide MCLRMTAVM. *Trh4* expression levels were normalized with the $\beta 2m$ housekeeping gene and expressed relative to the RMA sample. (C) RMA cells over-expressing Trh4 protein (RMA.Trh4) either weakly or at medium or high levels (low, interm. or high respectively) were incubated with Trh4-specific or MuLV control T cell clones and IFN- γ production measured. Two fold (control CTLs) or four fold (Trh4-CTLs) serial dilutions of targets were performed starting at 20,000 target cells and are indicated by the grey scale bars. Means and standard deviations of triplicates are shown from one out of three independent experiments.

We concluded that the TAP-independent peptide repertoire needs to compete with the conventional repertoire for loading and presentation by MHC-I. This resistance to the alternative repertoire is swiftly alleviated by decreasing levels of the TAP transporter. The newly emerging peptides on TAP-impaired cells therefore represent immunogenic neo-antigens and constitute unique CTL targets for TAP-deficient tumors and cells infected with immune evading herpes viruses.

| DISCUSSION

MHC-I presented peptides constitute protein breakdown intermediates that are rescued from complete destruction in the catabolic milieu of the cytosol. The total pool of cytosolic peptides formed by proteolysis is very diverse and estimated to be composed by millions of peptides^{2, 3, 46-48}. From this pool only a small repertoire is selected for presentation by MHC-I molecules at the surface of cells. In our current study we demonstrate that this repertoire selection is largely governed by the peptide transporter TAP, which pumps peptides from the cytosol into the ER. The TAP complex behaved like a lever of control to shift the presented repertoire gradually towards TAP-independent or TAP-dependent peptides. Cells with normal antigen processing function did not present the TAP-independent Trh4-derived peptide at the cell surface, despite the fact that this peptide is generated in TAP-positive cells and is capable of forming stable complexes with H-2D^b (Fig 3). Modest inhibition of TAP function, however, resulted in surface display of this Trh4/D^b complex. In addition, partial deficiencies still allowed the presentation of TAP-dependent peptide species, yielding a mixed peptide repertoire of both pools. The highest presentation of Trh4 peptide occurred when TAP function was completely blocked. These data revealed that TAP supports a highly competitive environment for class I loading in the ER most likely due to the overwhelming flow of peptide species that it pumps from the cytosol. Partial inhibition of TAP function alleviates this competition and induces the presentation of novel peptides and gradually inhibits the presentation of the classical TAP-dependent pool. This hypothesis is corroborated by the finding that over-expression of the Trh4 protein results in higher amount of peptide epitopes that are able to successfully compete for presentation in processing-intact cells (Fig 5). Alternatively, the selective Trh4 presentation could be explained by difference in organization of the peptide-loading complexes in the absence of TAP. A change in stoichiometry of the peptide-loading complex might favor an alternative peptide repertoire. This alternative hypothesis is, however, less likely because over-expression of the Trh4 protein is not expected to change the loading complex, while it still leads to surface presentation of the CTL epitope by H-2D^b. This forced presentation of a TAP-independent peptide was operable in leukemia cells as well as in primary dendritic cells (Fig 5 and Fig S4), illustrating the general character of this mechanism. We therefore conclude that TAP activity normally contributes to the retention of this TAP-independent peptide inside the cell. Apparently, not all peptides that can be presented on basis of accessibility to the ER and of binding affinity are actually presented. A consequence of this reasoning is that peptide availability is not the

rate limiting step in antigen presentation. The fact that MHC-I surface expression can be enhanced by gene transfer of heavy chains, further supports this notion (Fig. 4).

The studied Trh4 peptide represents a much broader repertoire of TAP-independent peptides, as we have shown that TAP-deficient cells are recognized by a diverse pool of CTL clones with distinct MHC restriction patterns^{36, 41}. Interestingly, the non-classical MHC molecule Qa-1^b seems to play a dominant role in the presentation of these TAP-independent antigens⁴⁹⁻⁵¹. Recently, we showed the existence of the human equivalent of this novel CTL category^{52, 53}. These peptide sequences, called TEIPP (T-cell epitopes associated with impaired peptide processing) are not presented by normal cells and we can speculate, based on the results of the present study, that one mechanism governing the absence from normal cells is related to the low expression level of the cognate proteins. In a parallel study, a proteasome- and TAP-independent tumor antigen from the signal sequence of the preprocalcitonin protein (ppCT₁₆₋₂₅) was found to represent a human TEIPP, in that this HLA-A2 presented peptide was selectively presented by tumor cells with TAP-deficiency⁵⁴. This peptide is liberated in the ER lumen by sequential cleavage with SP and SPP⁵⁶ and is a clear example of the alternative TAP-independent peptide repertoire.

We like to emphasize that not all TAP-independent peptides fail to be presented by processing intact cells. Mass-spectrometry analyses have revealed that part of this repertoire can be detected on the surface of TAP-proficient cells²⁵⁻²⁷. We anticipate that the expression levels of these proteins or the rate of peptide formation is higher than that of TEIPPs. Consequently, the immunogenicity of these peptides is expected to be much lower, due to central and peripheral T cell tolerance, as these peptides are derived from ubiquitously expressed housekeeping proteins.

The emerging picture of MHC-I antigen processing incorporates novel proteolytic enzymes next to the multicatalytic proteasome complex as the central player⁶⁻⁹. Most of these novel breakdown systems liberate peptides in the cytosol and produce substrates that still need TAP transport for MHC-I loading. However, processing by yet other proteolytic systems deliver peptides that are presented in a TAP-independent way. The above mentioned SP and SPP proteases produce such TAP-independent peptides within the ER and proprotein convertases like PC7 and furin have been shown to facilitate TAP-independent presentation in the secretory route²⁹⁻³². Interestingly, our preliminary data show that presentation of the Trh4 peptide is independent of these known enzyme systems, indicating that yet other pathways exist.

In this study we show that TAP influences the balance of peptide repertoires and that potential MHC-I ligands compete for loading and surface display. TAP down-modulation facilitates the presentation of the suppressed repertoire. Importantly, inhibition of this processing bottleneck is a common feature in cancer^{55, 57} and TAP is also a frequently targeted molecule by most viruses that cause lifelong infections¹⁵. The direct consequences are a generalized decrease in MHC-I presentation and the emergence of alternative peptide repertoire. These TAP-independent peptides might constitute an important line of host defense that might be exploited in therapeutic intervention strategies.

| MATERIAL AND METHODS

Cell lines and mice

The tumor cell lines RMA, RMA-S (TAP2 deficient), C4.4-2S ($\beta 2m$ deficient), MCA (TAP1 deficient) and the D1 dendritic cell line have been described previously^{36, 41, 49}. Cell variants overexpressing H-2D^b or Trh4 (accession number UniProtKB/Swiss-Prot Q9D6K9 LASS5_MOUSE) were generated by retroviral gene transfer using the LZRS vector containing GFP behind an internal ribosome entry site⁵⁰. RMA.Trh4 cells were differentially sorted according to GFP levels to generate variants with low, intermediate and high expression of Trh4. MCA.TAP1 is a variant of MCA fibrosarcoma where the mouse TAP1 gene was introduced. UL49.5 is a gene from the Bovine Herpes Virus-1 and blocks mouse TAP activity^{40, 50}. Generation and culture of T cell clones was described previously^{36, 45, 50}. CD8⁺ T cell clones used in this study: Qa-1-restricted CTL B12i is specific for the TAP-dependent peptide Qdm (AMAPTRLRL); H-2D^b-restricted CTL clone 1 is specific for the TAP-dependent MuLV gag-leader peptide (CCLCLTVFL); H-2D^b-restricted CTL clone B5 is specific for the TAP-independent Trh4 peptide (MCLRMTAVM). All cells were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS, 100 U/mL penicillin, 100 μ g/mL streptomycin (Life Technologies, Rockville, MD), 2mM L-glutamine (Invitrogen) and 30 μ mol/L of 2-mercaptoethanol (Merck, NJ, USA) at 37°C in humidified air with 5% CO₂.

C57BL/6 mice were purchased from Charles River Laboratories (France and Germany) or were bred at the German Cancer Research Center (DKFZ) and housed in the animal facility of the Leiden University Medical Center and DKFZ under specified pathogen-free conditions. Mice were used between 8 and 12 weeks of age. TAP1-knockout mice were purchased from Jackson laboratories. Experiments were performed in accordance with national legislations and institutional guidelines and were approved by the local ethical committees.

T-cell activation assays and flow cytometry

T cell activity was measured by IFN- γ secretion. Supernatants were harvested after overnight incubation of T cells with target cells and concentrations of IFN- γ were measured by ELISA as previously described⁵⁸. Surface expression of H-2D^b molecules was determined using purified mouse anti-H-2D^b mAb (clone 28.14.8S; BD Biosciences, NJ, USA) followed by allophycocyanin labeled goat anti-mouse Ig (Southern Biotech, Birmingham, USA). Cells were analyzed using a FACS Calibur with Cellquest software (BD Biosciences) or Flowjo software (Tree Star, Ashland, OR).

Quantitative PCR analysis

Total RNA isolation and cDNA preparation was performed using RNeasy Mini Kit (Qiagen, Maryland, USA). 500 ng of purified total RNA was used to synthesize cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, USA). Quantitative PCR on short and long transcripts of Trh4 was done as described before³⁶.

SensiMix™ SYBR No-ROX kit from GC Biotech Bioline (Alphen aan den Rijn, NL) was used in a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) and results were analysed using Bio-Rad CFX manager software.

Quantitative PCR on thymic stromal cells purified by cell sorting was performed as published⁵⁹. Long Trh4 transcripts were amplified with Power SYBR Green Master Mix (Applied Biosystems) on a GeneAmp 7300 System (Applied Biosystems).

Peptide binding and stability assays

Peptides for which MHC-I binding and stability were analyzed were Trh4₃₇₉₋₃₈₇ (MCLRMTAVM), LCMV gp33₃₃₋₄₂ (KAVYNFATC), CEA₅₇₁₋₅₇₉ (CGIQNSVSA), MDM₄₄₁₋₄₄₉ (GRPKNGCIV), WT1₁₂₆₋₁₃₄ (RMFPNAPYL), cyc₂₀₋₂₈ (TNLLNDRVL), gp100₂₅₋₃₃ (EGSRNQDWL). RMA-S cells were cultured for 2 days at 26°C to accumulate peptide receptive MHC-I molecules on the cell surface⁶⁰. Cells were washed in serum free medium and incubated at 2x10⁵ cells/well with varying concentrations of peptides for 4h at 37°C. Cells were washed and stained for H-2D^b as described above. For peptide/MHC-I stability determination, the RMA-S cells were plated (3x10⁶ cells/mL) with peptides (10 or 100 µg/mL) and 10 µg/mL Brefeldin A for 1h at 26°C. After incubation, cells were washed 3 times, plated at 3x10⁵ cells/well with Brefeldin A and incubated at 37°C for 1h, 2h, 4h and 6h to study the decay of peptide/MHC complexes. Cells were washed, fixed with 1% paraformaldehyde and stained for H-2D^b.

Proteasome inhibition assay

RMA.Trh4 cells were incubated at 2x10⁶ cells/mL with 2 uM epoxomicin (Sigma) before (1h) and after (6h) peptide-stripping with acetic acid as previously described⁴⁵. Control cells were treated with peptide-stripping, but not with epoxomicin. Target cells were then incubated with CTLs in a 2:1 ratio with Brefeldin A to retain produced IFN-γ intracellular. Incubation was performed overnight at 37°C and percentage of cytokine-producing CTLs was analysed by flow cytometry as described⁶⁰.

Isolation of primary cells from mice

Anaesthetized mice were perfused with heparin solution to remove blood cells from the tissues. To obtain single cell suspensions, spleens were mechanically disrupted with cell strainer and bone marrow cells were obtained from femurs by flushing with RPMI medium. Other tissues were mechanically disrupted or sliced in small pieces and incubated with enzyme mixes. Thymuses were incubated with 0.5 mL of collagenase IV solution (0.5 mg/mL, Sigma-Aldrich, MO, USA) per thymus at 30 °C and slowly agitated for 15 minutes. Two incubations were performed. The remaining fragments were digested with a mixture of collagenase and dispase I (0.1 mg/mL, Sigma-Aldrich) during three incubations of 30 minutes at 37 °C (0.5 mL of enzyme mix per thymus). Enzyme mix was replaced and fractions were collected and “pooled” at the end. Lymph nodes were incubated with 0.2 mg/mL collagenase IV, 0.1 mg/mL DNase I (Sigma-Aldrich) and 0.8 mg/mL dispase I at 37 °C under agitation. After 15 minutes the enzyme mix was replaced and the fragments were incubated again. This procedure was repeated 5 times⁶¹.

Lung tissue fragments were collected to gentleMACS C tubes (Miltenyi Biotec, Bergisch Gladbach, Germany) and mixed with 0.05mg/mL DNase I and 250 U/mL collagenase IV diluted in serum-free medium (5 mL enzyme mix/2 lungs). Tubes were transferred to gentleMACS Dissociator (Miltenyi Biotec) and the protocol was run according to manufacturer's recommendations. Cell suspension was passed through a cell strainer to remove cell clumps. Livers were collected to gentleMACS C tubes and mixed with 0.05 mg/mL DNase I and 500 U/mL collagenase IV diluted in serum-free medium (5 mL enzyme mix/2 livers). The fragments were incubated at 37 °C for 30 minutes under slow mixing. After incubation, tubes were transferred to gentleMACS Dissociator and the protocol was run according to the manufacturer's recommendations. Cell clumps were removed with a cell strainer.

Blood-derived CD45⁺ cells were depleted from the tissue cell suspensions by negative selection with magnetic CD45 MicroBeads (Miltenyi Biotec) according to manufacturer's protocol. Contamination with CD45 cells was estimated by staining with antibodies against the epithelial cell marker CD326 (Ep-CAM) and CD45.

For the isolation of thymic subpopulations, thymic lobes were cleaned of fat and connective tissue, cut in little pieces and stirred for 10 min in 15mL RPMI medium at room temperature to release thymocytes. Tissue fragments were then resuspended in 1 mL medium per thymus containing 0.2 mg/mL Collagenase IV (Worthington), 10mM HEPES and 2% FCS. The mixture was slowly stirred for 15 min at 30°C, released cells removed and fresh enzyme mixture added for a total of 3 incubations. The remaining fragments were digested with a mixture of Collagenase IV and Neutral Protease (0.2 mg/mL each; Worthington), 25 mg/mL DNaseI (Roche), 10 mM HEPES and 2% FCS in RPMI1640. Five incubations for 25 min at 37°C were performed. For DC and macrophage isolation all cells from the collagenase incubations and the first round of collagenase/neutral protease digestion were pooled, washed and rosettes dissociated by 5 min incubation at 37°C in PBS containing 25mM EDTA. Cells were stained with anti-CD11c microbeads (Miltenyi Biotec), run on an AutoMACS (Miltenyi Biotec) using the "Possel_S" program, blocked with anti-FcR mAb 2.4G2 supernatant including 5 % rat serum and stained with anti-CD11c-PE (clone HL3; BD), anti-F4/80-FITC (clone CI:A3-1; Serotec) and PI. Thymic epithelial cells were enriched by pooling cells from collagenase/neutral protease digestion rounds two to five, followed by staining with anti-CD45 microbeads (Miltenyi Biotec), run on an AutoMACS using the "Deplete" program, blocked with anti-FcR mAb 2.4G2 supernatant and stained with anti-Ly51-FITC (clone 6C3; BD), anti-CD80-PE (clone 16-10A1; BD), anti-EpCAM-Alexa 647 (clone G8.8; BD), anti-CD45-PECy5 (clone 30-F11; BD) and PI. Dendritic cells were identified as CD11c⁺F4/80⁻ and macrophages as CD11c^{-/lo} and F4/80⁺. mTECs were identified as CD45⁺Ly5.1⁺EpCAM⁺ and sorted according to their CD80 expression, as CD80^{hi} or CD80^{lo}, representing the top and bottom 30% of the population. Cell sorting was performed with a FACSAriaI cell sorter (Becton Dickinson).

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| REFERENCES

1. Rock, K.L., Farfan-Arribas, D.J. & Shen, L. Proteases in MHC class I presentation and cross-presentation. *J.Immunol.* **184**, 9-15 (2010).
2. Wearsch, P.A. & Cresswell, P. The quality control of MHC class I peptide loading. *Current Opinion in Cell Biology* **20**, 624-631 (2008).
3. Yewdell, J.W. in Current Opinion in Immunology Innate immunity/Antigen processing and recognition 79-86 (2007).
4. Cresswell, P., Ackerman, A.L., Giodini, A., Peaper, D.R. & Wearsch, P.A. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol.Rev.* **207**, 145-157 (2005).
5. Jensen, P.E. Recent advances in antigen processing and presentation. *Nat.Immunol.* **8**, 1041-1048 (2007).
6. Geier, E. et al. A giant protease with potential to substitute for some functions of the proteasome. *Science* **283**, 978-981 (1999).
7. Glas, R., Bogoy, M., McMaster, J.S., Gaczynska, M. & Ploegh, H.L. A proteolytic system that compensates for loss of proteasome function. *Nature* **392**, 618-622 (1998).
8. Kessler, J.H. et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat.Immunol.* **12**, 45-53 (2011).
9. Parmentier, N. et al. Production of an antigenic peptide by insulin-degrading enzyme. *Nat. Immunol.* **11**, 449-454 (2010).
10. Seifert, U. et al. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat.Immunol.* **4**, 375-379 (2003).
11. Hammer, G.E., Kanaseki, T. & Shastri, N. The final touches make perfect the peptide-MHC class I repertoire. *Immunity* **26**, 397-406 (2007).
12. Peaper, D.R. & Cresswell, P. Regulation of MHC class I assembly and peptide binding. *Annu.Rev.Cell Dev.Biol.* **24**, 343-368 (2008).
13. Scholz, C. & Tampe, R. The peptide-loading complex--antigen translocation and MHC class I loading. *Biol.Chem.* **390**, 783-794 (2009).
14. Van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L. & Tonegawa, S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell* **71**, 1205-1214 (1992).
15. Griffin, B.D., Verweij, M.C. & Wiertz, E.J. Herpesviruses and immunity: the art of evasion. *Vet.Microbiol.* **143**, 89-100 (2010).
16. Hansen, T.H. & Bouvier, M. MHC class I antigen presentation: learning from viral evasion strategies. *Nat.Rev.Immunol.* **9**, 503-513 (2009).
17. Campoli, M. & Ferrone, S. HLA antigen changes in malignant cells: epigenetic mechanisms and biologic significance. *Oncogene* **27**, 5869-5885 (2008).
18. Carretero, R. et al. BCG immunotherapy of bladder cancer induces selection of HLA class I-deficient tumor cells. *Int.J.Cancer* (2010).
19. Seliger, B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol.Immunother.* **57**, 1719-1726 (2008).
20. Sandberg, J.K., Chambers, B.J., Van Kaer, L., Karre, K. & Ljunggren, H.G. TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. *Eur.J.Immunol.* **26**, 288-293 (1996).
21. Cerundolo, V. & de la Salle, H. Description of HLA class I- and CD8-deficient patients: Insights into the function of cytotoxic T lymphocytes and NK cells in host defense. *Semin.Immunol.* **18**, 330-336 (2006).
22. de la Salle, H. et al. Human peptide transporter deficiency: importance of HLA-B in the presentation of TAP-independent EBV antigens. *J.Immunol.* **158**, 4555-4563 (1997).
23. Gadola, S.D., Moins-Teisserenc, H.T., Trowsdale, J., Gross, W.L. & Cerundolo, V. TAP deficiency syndrome. *Clin.Exp.Immunol.* **121**, 173-178 (2000).
24. Moins-Teisserenc, H.T. et al. Association of a syndrome resembling Wegener's granulomatosis with low surface expression of HLA class-I molecules. *Lancet* **354**, 1598-1603 (1999).
25. Henderson, R.A. et al. HLA-A2.1-associated peptides from a mutant cell line: a second

- pathway of antigen presentation. *Science* **255**, 1264-1266 (1992).
26. Wei, M.L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443-446 (1992).
 27. Weinzierl, A.O. et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur.J.Immunol.* **38**, 1503-1510 (2008).
 28. Lemberg, M.K. & Martoglio, B. Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. *Mol.Cell* **10**, 735-744 (2002).
 29. Martoglio, B. & Dobberstein, B. Signal sequences: more than just greasy peptides. *Trends Cell Biol.* **8**, 410-415 (1998).
 30. Gil-Torregrosa, B.C., Raul, C.A. & Del Val, M. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-Golgi network protease furin. *J.Exp.Med.* **188**, 1105-1116 (1998).
 31. Leonhardt, R.M. et al. Post-endoplasmic reticulum rescue of unstable MHC class I requires proprotein convertase PC7. *J.Immunol.* **184**, 2985-2998 (2010).
 32. Medina, F. et al. Furin-processed antigens targeted to the secretory route elicit functional TAP1-/-CD8+ T lymphocytes in vivo. *J.Immunol.* **183**, 4639-4647 (2009).
 33. Thomas, G. Furin at the cutting edge: from protein traffic to embryogenesis and disease. *Nat.Rev.Mol.Cell Biol.* **3**, 753-766 (2002).
 34. Levy, M. & Futerman, A.H. Mammalian ceramide synthases. *IUBMB.Life* **62**, 347-356 (2010).
 35. Riebeling, C., Allegood, J.C., Wang, E., Merrill, A.H., Jr. & Futerman, A.H. Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J.Biol.Chem.* **278**, 43452-43459 (2003).
 36. van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat.Med.* **12**, 417-424 (2006).
 37. Aladin, F., Lautscham, G., Humphries, E., Coulson, J. & Blake, N. Targeting tumour cells with defects in the MHC Class I antigen processing pathway with CD8+ T cells specific for hydrophobic TAP- and Tapasin-independent peptides: the requirement for directed access into the ER. *Cancer Immunol. Immunother.* **56**, 1143-1152 (2007).
 38. Snyder, H.L. et al. Two novel routes of transporter associated with antigen processing (TAP)-independent major histocompatibility complex class I antigen processing. *J.Exp.Med.* **186**, 1087-1098 (1997).
 39. Snyder, H.L., Bacik, I., Yewdell, J.W., Behrens, T.W. & Bennink, J.R. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur.J.Immunol.* **28**, 1339-1346 (1998).
 40. Verweij, M.C. et al. Inhibition of mouse TAP by immune evasion molecules encoded by non-murine herpesviruses. *Mol.Immunol.* **48**, 835-845 (2011).
 41. Chambers, B. et al. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer Res.* **67**, 8450-8455 (2007).
 42. Klein, L., Hinterberger, M., Wirnsberger, G. & Kyewski, B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat.Rev.Immunol.* **9**, 833-844 (2009).
 43. Momburg, F. & Tan, P. Tapasin-the keystone of the loading complex optimizing peptide binding by MHC class I molecules in the endoplasmic reticulum. *Mol.Immunol.* **39**, 217-233 (2002).
 44. van der Burg, S.H., Visseren, M.J., Brandt, R.M., Kast, W.M. & Melief, C.J. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. *J.Immunol.* **156**, 3308-3314 (1996).
 45. van Hall, T. et al. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J.Exp.Med.* **192**, 483-494 (2000).
 46. Shastri, N., Cardinaud, S., Schwab, S.R., Serwold, T. & Kunisawa, J. All the peptides that fit: the beginning, the middle, and the end of the MHC class I antigen-processing pathway. *Immunol.Rev.* **207**, 31-41 (2005).
 47. Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. The known unknowns of antigen processing and presentation. *Nat.Rev.Immunol.* **8**, 607-618 (2008).
 48. Yewdell, J.W., Reits, E. & Neefjes, J. Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat.Rev.Immunol.* **3**, 952-961 (2003).
 49. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J.Exp.Med.* **207**, 207-221 (2010).
 50. van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b1. *J.Immunol.* **178**, 657-662 (2007).

51. van Hall, T., Oliveira, C.C., Joosten, S.A. & Ottenhoff, T.H. The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress. *Microbes Infect.* **12**, 910-918 (2010).
52. Lampen, M.H. & van Hall, T. Strategies to counteract MHC-I defects in tumors. *Curr. Opin.Immunol.* **23**, 293-298 (2011).
53. Lampen, M.H. et al. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. *J.Immunol.* **185**, 6508-6517 (2010).
54. Durgeau, A. et al. Different expression levels of the TAP peptide transporter lead to recognition of different antigenic peptides by tumor-specific CTL. *J Immunol* **187**, 5532-9 (2011).
55. Chang, C.C., Campoli, M. & Ferrone, S. HLA class I defects in malignant lesions: what have we learned? *Keio J.Med.* **52**, 220-229 (2003).
56. El Hage, F. et al. Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway. *Proc.Natl.Acad.Sci.U.S.A* **105**, 10119-10124 (2008).
57. Hicklin, D.J., Marincola, F.M. & Ferrone, S. HLA class I antigen downregulation in human cancers: T-cell immunotherapy revives an old story. *Mol.Med.Today* **5**, 178-186 (1999).
58. van Hall, T. et al. Identification of a novel tumor-specific CTL epitope presented by RMA, EL-4, and MBL-2 lymphomas reveals their common origin. *J.Immunol.* **165**, 869-877 (2000).
59. Derbinski, J. et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J.Exp.Med.* **202**, 33-45 (2005).
60. van Stipdonk, M.J. et al. Design of agonistic altered peptides for the robust induction of CTL directed towards H-2Db in complex with the melanoma-associated epitope gp100. *Cancer Res.* **69**, 7784-7792 (2009).
61. Fletcher, A.L. et al. Lymph node fibroblastic reticular cells directly present peripheral tissue antigen under steady-state and inflammatory conditions. *J.Exp.Med.* **207**, 689-697 (2010).

| SUPPLEMENTARY FIGURES

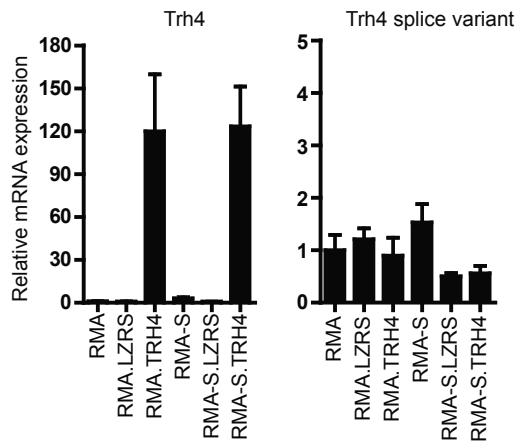


Figure S1. Expression levels of *Trh4* on RMA and RMA-S cell targets. RMA and RMA-S targets were transduced with *Trh4* (RMA.Trh4, RMA-S.Trh4) or an empty vector (RMA.LZRS, RMA-S.LZRS) to over-express the *Trh4* protein. *Trh4* expression levels were determined by quantitative PCR with specific primers. The expression of a natural splice variant which does not encode the C-terminal peptide MCLRMTAVM was included as control. *Trh4* expression levels were normalized with $\beta 2m$ housekeeping gene and expressed relative to RMA sample. Means and standard deviations of triplicates are shown from one out of three independent experiments.

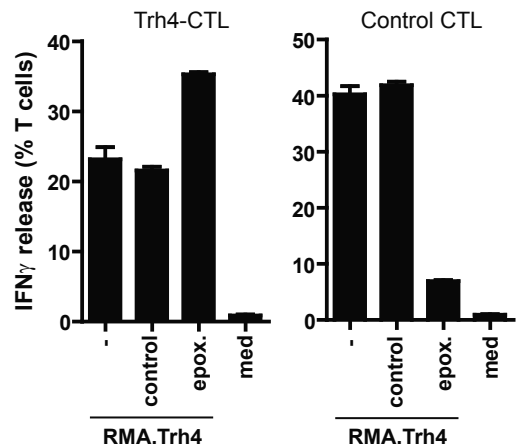


Figure S2. The presentation of *Trh4* peptide in cells over-expressing the *Trh4* protein is independent of proteasome activity. RMA.Trh4 cells were submitted to mild acid treatment to strip surface MHC-I molecules and then recovered in the presence of the proteasome-specific inhibitor epoxomicin. Target cells were tested for CTL recognition overnight and percentage CTL producing IFN γ was determined by flow cytometry. Control CTL (right panel) recognize the *gag* leader peptide CCLCLTVFL, which is TAP- and proteasome-dependent⁴⁵. Means and standard deviations of triplicates are shown from one out of two independent experiments.

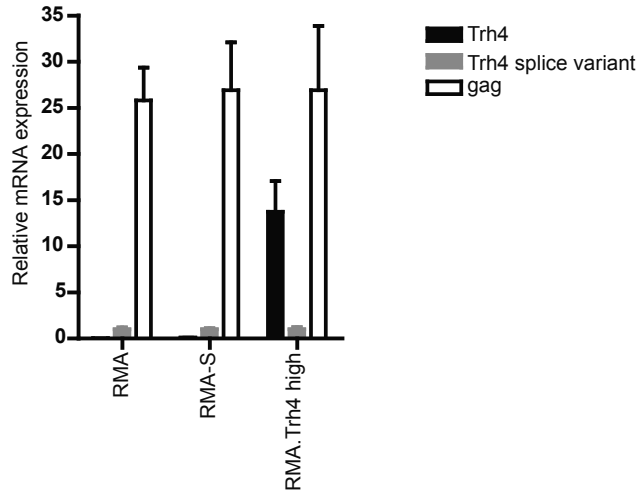


Figure S3. Relative mRNA expression of *Trh4* and *gag* on TAP-positive and -negative cell targets. The expression levels of *Trh4* and the MuLV-derived *gag* polypeptide were determined by quantitative PCR with specific primers. RMA and RMA-S are MuLV-induced T cell lymphomas. We compared the expression of *gag*, which produces a CTL epitope, with the expression of *Trh4* on these targets and on an RMA-derived cell line that overexpressed *Trh4*. *Trh4* and *gag* expression levels were normalized with *GAPDH* housekeeping gene and expressed relative to *Trh4* splice variant for each sample. Means and standard deviations of triplicates are shown from one out of two independent experiments.

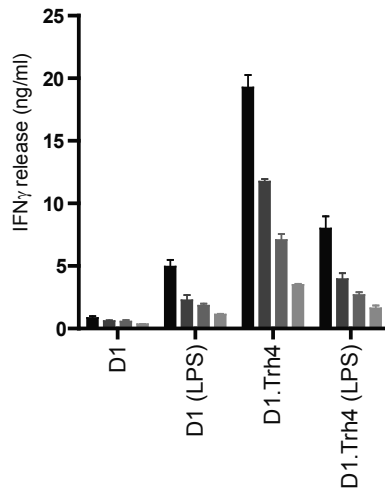


Figure S4. *Trh4* peptide is presented on dendritic cells with increased *Trh4* expression. D1 dendritic cells were transduced with a retroviral construct containing *Trh4* long transcript (D1.Trh4) or an empty vector (D1). Immature dendritic cells or matured with LPS for 24 hours were incubated with *Trh4*-specific T cell clone. Activation of T cells was measured by IFN γ production on supernatants after overnight incubation with targets. Two fold serial dilutions of targets were performed starting at 5,000 and represented by grey scale bars. Means and standard deviations of triplicates are shown from one out of two independent experiments.

3

NEW ROLE OF SIGNAL PEPTIDE PEPTIDASE TO LIBERATE C-TERMINAL PEPTIDES FOR MHC CLASS-I PRESENTATION

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| ABSTRACT

The signal peptide peptidase (SPP) is an intra-membrane cleaving aspartyl protease involved in release of leader peptide remnants from the ER membrane, hence its name. We now found a new activity of SPP that mediates liberation of C-terminal peptides. In our search for novel proteolytic enzymes involved in major histocompatibility complex class I (MHC-I) presentation, we found that SPP generates the C-terminal peptide-epitope of a ceramide synthase. The display of this immunogenic peptide/MHC-I complex at the cell surface was independent of conventional processing components like proteasome and peptide transporter TAP. Absence of TAP activity even increased the MHC-I presentation of this antigen. Mutagenesis studies revealed the crucial role of the C-terminal location of the epitope and 'helix-breaking' residues in the transmembrane region just upstream of the peptide, indicating that SPP directly liberated the minimal 9-mer peptide. Moreover, silencing of SPP and its family member SPPL2a led to a general reduction of surface peptide/MHC-I complexes, underlining the involvement of these enzymes in antigen processing and presentation.

I INTRODUCTION

All nucleated cells of our body display a representative selection of the cellular proteome in Major Histocompatibility class I (MHC-I) molecules at their surfaces. This wide array of peptides serves as ligands for CD8⁺ T-cells for cellular immunity. The process of peptide presentation starts with the proteolysis of aged or misfolded proteins, a process mainly executed by the multi-catalytic action of proteasomes. The generated peptide sequences are translocated from the cytosol into the ER by the TAP peptide transporters, where they are assembled with different MHC-I molecules with help from members of the peptide loading complex, including calreticulin, tapasin and ERp57. Stable peptide/MHC-I complexes are routed to the cell surface. This proteasome-TAP pathway is considered as the conventional processing route, used to generate and shuttle the majority of peptides¹⁻³. Additional players, like tripeptidyl peptidase II (TPPII) and nardilysin are responsible to further shorten proteasomal intermediates or, sometimes, to directly generate minimal peptide sequences that fit in MHC-I molecules^{4,5}.

Although these central elements of the conventional antigen processing pathway are important for the mainstream of peptides, substantial MHC-I antigen presentation is still detectable on cells in which the function of key components is compromised. For example, deficiency in the peptide transporter TAP, which can be regarded as a bottleneck in the pathway, results in a decreased surface MHC-I, but the residual peptide repertoire is sufficient to generate a diverse CD8⁺ T cell subset in mice⁶⁻⁸ and man^{9,10}. Importantly, this T cell repertoire is capable to control common viral infections and from some TAP-deficient patients TCRαβ⁺ CD8⁺ T-cell clones were isolated that recognized TAP-independent viral antigens^{11,12}. Therefore, TAP-independent processing pathways may sufficiently compensate for the loss of the conventional route in order to select a functional CD8⁺ T-cell repertoire in these patients.

This reasoning is in line with our recent findings on a novel category of T cell epitopes presented by cancer cells with TAP-defects. We showed that deficiency in the expression of the peptide transporter TAP, a feature frequently found in human cancers, leads to the presentation of a broad repertoire of immunogenic peptides by MHC-I¹³⁻¹⁷. Biochemical characterization of peptide repertoires from TAP-deficient cells and their direct proficient counterparts indeed revealed unique complementing pools of peptides^{14,18,19}.

Two pathways for TAP-independent peptide loading are described thus far²⁰. One of these is active in the secretory route of the Golgi and is mediated by the protein convertase furin²¹⁻²³. The other concerns N-terminal leader sequences, which are liberated after arrival in the ER by the signal peptidase (SP). Cleavage by SP leaves small transmembrane leader peptide remnants in the ER membrane, which are removed by the intramembrane-cleaving signal peptide peptidase (SPP). The ER-directing parts of these peptides end up in the lumen of the ER and can be loaded unto MHC-I molecules^{24,25}. An immunogenic human tumor antigen from the leader sequence of calcitonin is released by this means and TAP is dispensable for its presentation on cancer cell lines^{26,27}.

In the current study we reveal the processing pathway of a C-terminal peptide from the ceramide synthase Trh4 (also known as CerS5). This protein resides in ER membranes and docks its C-terminal epitope-containing tail into the bilayer. Intramembrane proteolysis by SPP just in front of the epitope resulted in liberation and TAP-independent loading on D^b molecules. This represents a novel role for SPP, acting independently from the signal peptidase (SP). Our data indicate that SPP is able to directly liberate minimal peptide-epitopes from the C-terminus of proteins by intramembrane proteolysis and underlines the involvement of this enzyme in antigen processing.

| RESULTS

The TAP-independent processing pathway of the Trh4-derived peptide is common and conserved

The Trh4-derived peptide MCLRMTAVM is presented by the MHC class I molecule H-2D^b on TAP-deficient tumor cells¹⁷. We were interested in the nature of its TAP-independent processing pathway that leads to the generation and surface presentation of the Trh4 peptide in D^b, and therefore, analyzed the generality of this antigen processing pathway in different mouse and human tumor cell lines (Fig 1). Four mouse tumor cell lines of different origin and containing a TAP defect (a lymphoma, a fibrosarcoma, a colon carcinoma and a melanoma) were analyzed for recognition by a previously established MCLRMTAVM-specific CD8⁺ T-cell clone. These T cells efficiently recognized all TAP-deficient tumors, irrespective of their tissue origin (Fig 1A). Importantly, the different mechanisms underlying the TAP impediment in these tumors, being TAP2 mutation (in RMA-S), TAP1 gene loss (in MCA), expression of a small viral inhibitor (in MC38) or TAP2 gene loss (in B78H1), all led to efficient presentation of the Trh4-derived peptide, indicating that presentation of the Trh4 peptide is related to TAP-deficiency as such and not to a specific molecular mechanism. The fact that TAP-positive counterpart tumors presented much lower Trh4 peptide at their cell surface was not surprising, since we previously showed that restoration of TAP function decreases Trh4/D^b complexes at the cell surface, most likely due to competition²⁸. Therefore, the Trh4 peptide presentation is TAP-independent and operates in different tumor types.

The degree of CTL recognition of the tumor cell lines varied and we wondered if these differences could be explained by Trh4 expression levels. Trh4 transcripts were quantified by qPCR and indeed revealed higher expression in the lymphoma RMA-S compared to the solid tumors (Fig 1B). Expression of a splice variant of Trh4, which does not contain the D^b-binding peptide, showed less variety in the tumor panel. We concluded that surface display of Trh4/D^b complexes is determined by TAP function, Trh4 protein levels and possibly other factors like the protease responsible for peptide liberation.

We also wanted to know if human cells were equipped with the TAP-independent processing pathway. The mouse Trh4 gene was introduced into the human cell lines HeLa and HEK293T, together with the mouse MHC class I gene H-2D^b that presents

this peptide. The Trh4-derived peptide was efficiently presented at the cell surface of both human lines, indicating that the involved processing pathway was indeed also active in human cells (Fig 1C). Although these human cells have intact TAP function and thereby impede Trh4 peptide presentation, the high expression levels of Trh4 protein, from strong heterologous promoters overcomes the competition of peptides from the proteasome-TAP pathway, as illustrated by efficient CTL recognition of TAP-positive RMA lymphoma cells in which Trh4 is expressed eight hundred times higher than the endogenous level²⁸. Together, these data show that the TAP-independent processing

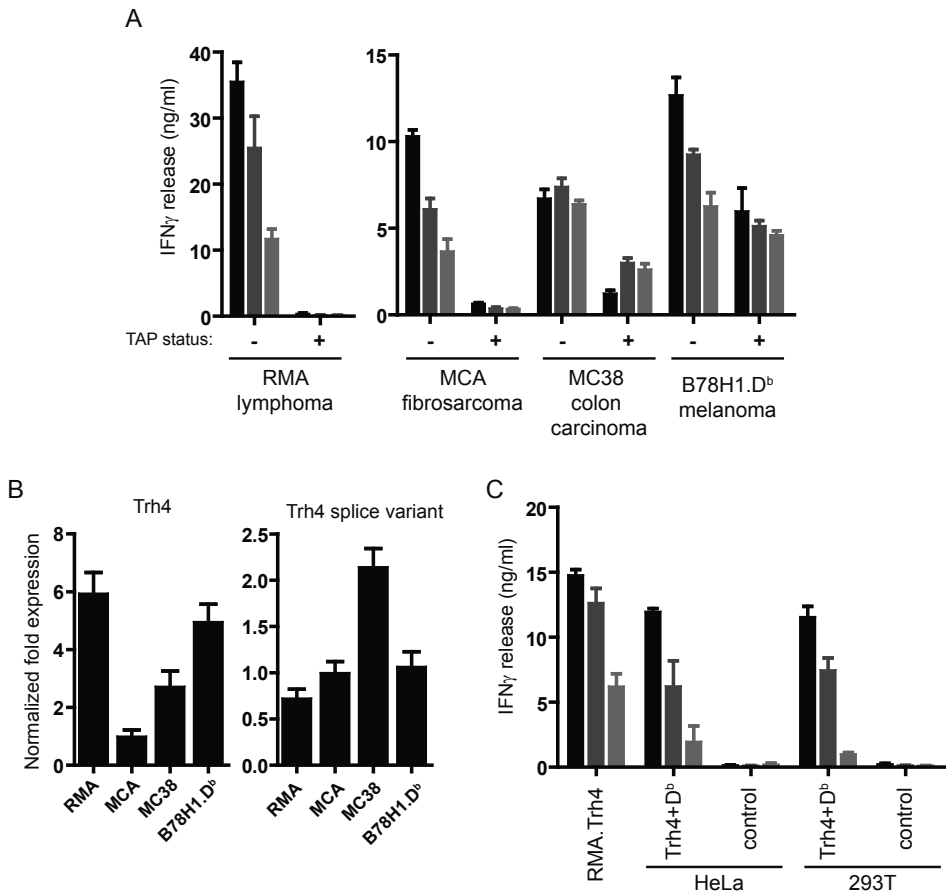


Figure 1. The Trh4 processing pathway is conserved and broadly active. (A) Presentation of Trh4-derived peptide by MHC-I was evaluated on lymphoma (RMA), fibrosarcoma (MCA), colon carcinoma (MC38) and melanoma cells (B78H1.D^b). Activation of Trh4-specific T cells is enhanced by the absence on peptide transporter TAP. (B) mRNA expression of Trh4 and a natural Trh4 splice variant was determined by quantitative PCR. The splice variant does not encode the Th4 peptide sequence due to a frame-shift. One out of three comparable experiments is shown. (C) Human 293T and HeLa cells were transfected with mouse Trh4 and H-2D^b genes and used as targets. Mean and standard deviation of triplicates are shown from one out of three experiments.

pathway that generates the Trh4 peptide is a common and conserved mechanism operational in mouse and human tumor cell lines of different histological origins.

The Trh4 protein is localized in the membrane of the endoplasmic reticulum

To unravel the processing pathway of the Trh4-derived peptide, we next determined the cellular localization of the Trh4 protein. The Trh4 protein (also known as CerS5) belongs to a family of ceramide synthases (CerS1 to CerS6) and exists in two isoforms as a result of alternative splicing of the transcript¹⁷. The shorter protein variant (UniProt Q9D6K9-2) contains the D^b-presented peptide MCLRMTAVM at the complete end of its C-terminus. The longer protein (UniProt Q9D6K9-1) lacks this peptide sequence due to a frame shift at the last intron-exon boundary¹⁷. Although the longer isoform was described to be a multi-transmembrane spanning protein in the endoplasmic reticulum³², no data were available concerning the Trh4 variant comprising the peptide-epitope. Algorithm programs predicting topology of transmembrane proteins anticipated that the short Trh4 protein would transgress membranes seven times with the C-terminal amino acids 373-384 located within the lipid bilayer, directed towards the lumen (Fig 2A). The V5 antibody epitope was introduced at two distinct sites of Trh4 gene constructs (C-terminal after the epitope and N-terminal at amino acid 32) and subcellular localization studies were performed using confocal microscopy. We observed a strong and clear co-localization with ER markers, but not with the Golgi with both constructs (Fig 2B for N-terminal tag and Suppl Fig 1A for C-terminal tag). ER co-localization was also observed in TAP-negative cells (Suppl Fig 1B). An intermediate degree of co-localization with mitochondria was found (Fig. 2B). It is known that the CerS1, CerS2 and CerS6 ceramide synthases can indeed be detected in mitochondria³³, but, alternatively, this mitochondria co-localization might merely reflect ER membranes that are in close proximity of mitochondria. These data indicate that Trh4 is a multimembrane spanning protein in ER membranes and that its C-terminal tail, comprising the epitope, docks into this membrane directing towards the ER lumen.

Inhibitors of intramembranous aspartyl proteases prevent peptide liberation

Next, we investigated which family of proteases was responsible for the proteolytic cleavage of Trh4 resulting in liberation of the C-terminal peptide. An array of inhibitors blocking hydrolysis by different classes of proteases were applied in a recovery assay in which TAP-deficient RMA-S cells were briefly acid stripped to remove cell surface MHC-I and allow for re-emergence of Trh4/D^b peptide complexes. Complete recovery of Trh4/D^b was reached within six hours, as determined with a peptide-specific T cell clone (Fig 3A). This highlighted a continuous and efficient supply of Trh4 peptide from this alternative processing pathway. Two enzymes were likely candidates, since they were described to result in TAP-independent peptide loading: signal peptidase and furin^{20, 21}. However, the specific inhibitors DCI and Dec-RVKR-CMK, respectively, failed to impair the generation of Trh4/D^b complexes (Fig 3B), indicating that Trh4 processing is mediated by a novel mechanism. In line with our previous data, inhibition of the proteasome rather

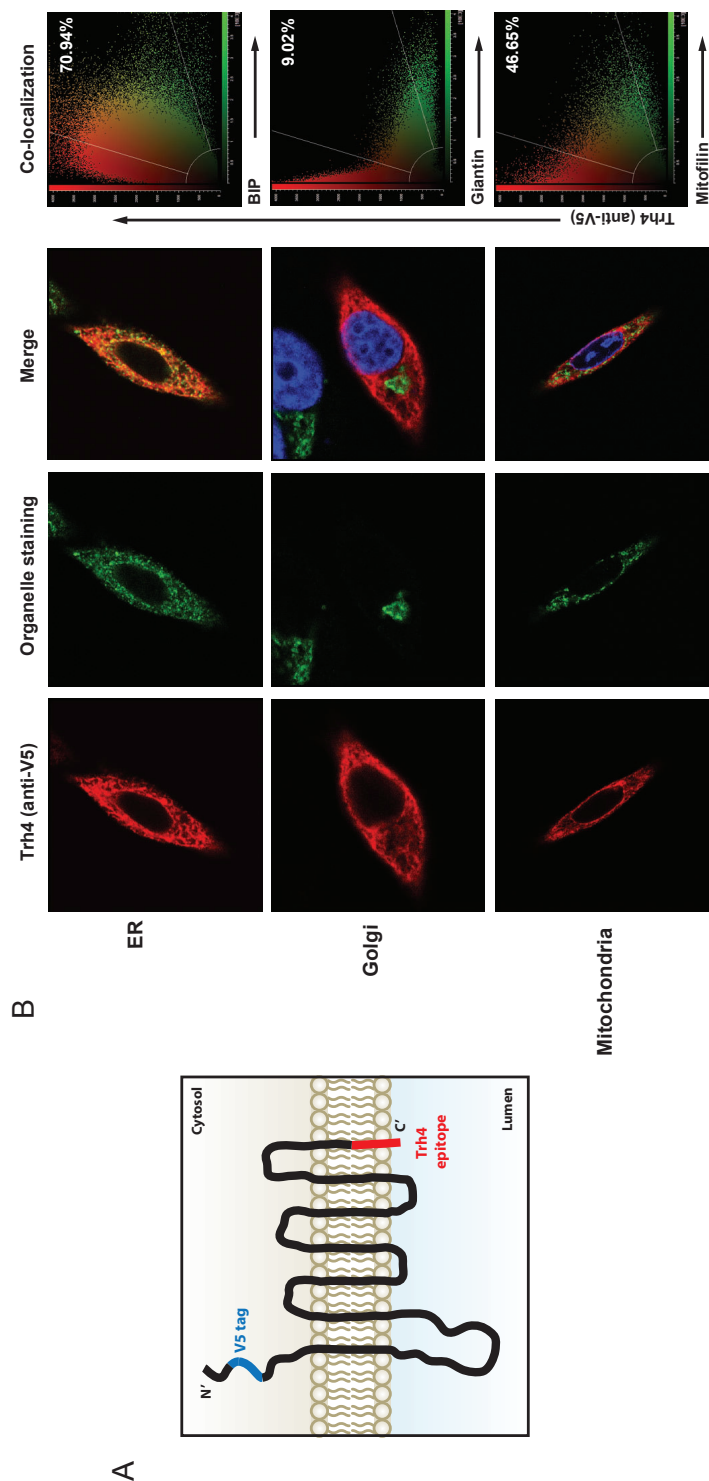


Figure 2. The C-terminal tail of Trh4 is localized within the ER membrane. (A) Prediction of the Trh4 topology in membranes according to algorithm software (www.predictprotein.org). The C-terminal peptide-epitope is indicated in red. (B) Confocal microscopy imaging of V5-tag at the N-terminus of Trh4 protein in combination with staining for endoplasmic reticulum (BIP), Golgi (giantin) or mitochondria (mitofilin). Mean percentages of colocalization were calculated on ten images of at least three independent experiments.

increased Trh4/D^b complexes (Fig 3B), suggesting that the proteasome somehow acts as a competitor for the novel processing mechanism²⁸.

Next, we observed that the common calpain inhibitor calpeptin partly decreased Trh4 presentation at relatively high concentrations (> 20 μ M) (Fig 3C and Suppl Fig 2A). The main targets for calpeptin are calpain 1 and calpain 2. We determined the expression of these two calpain members in RMA-S cells and observed that calpain 2 was expressed but calpain 1 was not detectable (Suppl Fig 2B). Therefore, we silenced the expression of calpain 2, but this did not lead to reduced Trh4/D^b peptide complexes at the cell surface

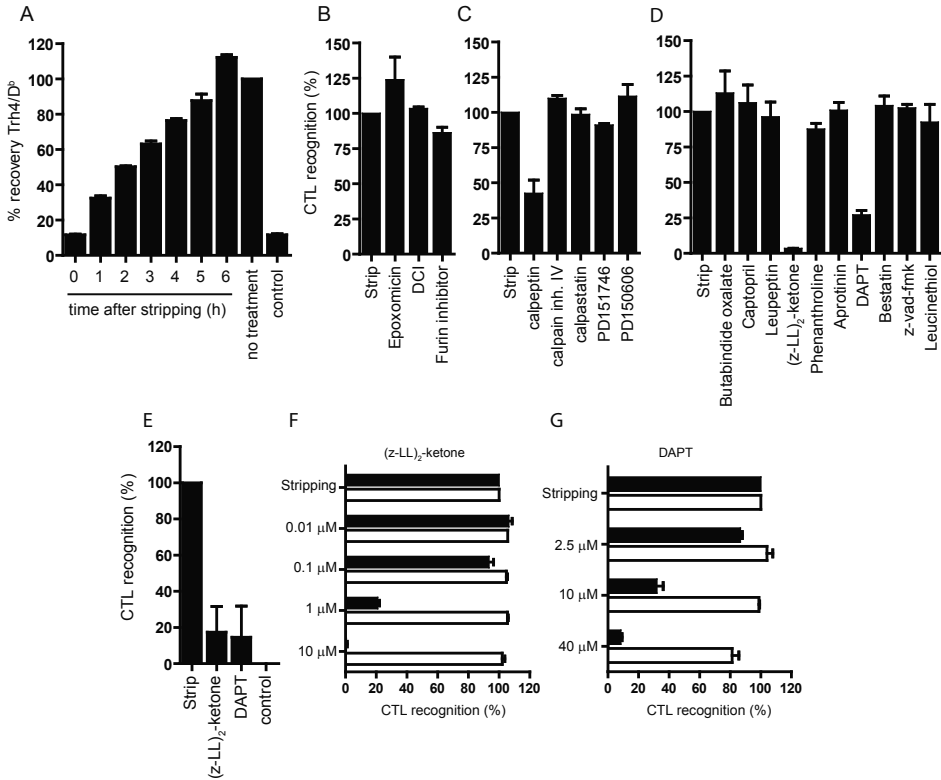


Figure 3. The D^b-presentation of the Trh4-derived peptide is generated by intramembrane-cleaving aspartyl proteases. (A) Recovery kinetics of Trh4/D^b complexes on TAP-deficient RMA-S cells after brief stripping with mild acid buffer. Cells were tested with Trh4-specific T cell clone in intracellular IFN- γ staining. (B) Recovery efficiency of Trh4/D^b complexes was tested in the presence of an array of protease inhibitors. Inhibitors of proteasome and enzymes known to be involved in TAP-independent processing pathways, (C) inhibitors of calpain family, (D) all other tested inhibitors. (E) TAP-deficient fibrosarcoma MCA cells were tested with inhibitors for aspartyl proteases. (F) Concentration range of the functional inhibitor (z-LL)₂-ketone and (G) DAPT on RMA-S cells. Trh4-specific T cells (filled bars) were used and control T cells (open bars) that recognize an independent K^b-presented peptide on RMA-S. All panels show compiled means and standard deviations from at least three independent experiments, setting the recovered cells at 100%.

(Suppl Fig 2C-D). Furthermore, more selective inhibitors for the calpain family did not affect the CTL response, collectively suggesting that the marginal blocking effect by calpeptin was based on cross-reactivity to other proteases (Fig 3C).

We then continued the screen with several other classes of chemical inhibitors targeting serine-, cysteine-, aspartyl- and metallo-proteases, and aminopeptidases (Fig 3D). The results revealed two inhibitors for aspartyl proteases, (z-LL)₂-ketone and DAPT, that strongly decreased presentation of the Trh4-derived peptide. Importantly, none of the other eight protease inhibitors influenced the Trh4/D^b presentation, suggesting that the involved aspartyl protease was necessary and sufficient. The inhibitory effect was also observed when we tested fibrosarcoma cells, indicating that the inhibition was not cell-type restricted (Fig 3E). Titration of the two active compounds demonstrated a 50% decrease at concentrations in the lower range in this cellular assay, 300 nM for (z-LL)₂-ketone and 5 μM for DAPT (Fig 3F and G). These concentrations were clearly not toxic for the target cells and, moreover, recognition of treated RMA-S cells by control T cell clones specific for another peptide epitope was unaltered, excluding a general defect in the MHC class-I presentation capacity by (z-LL)₂-ketone and DAPT (Fig 3F and G). These two compounds were known to target aspartic acid proteases that are located within membranes, particularly members of the signal peptide peptidase (SPP) family^{34, 35}. Interestingly, calpain inhibitors were shown to block the action of SPP at higher concentrations, corroborating our findings in the screen with calpeptin (Fig 3C)³⁶. Together, these results let us conclude that the liberation of the C-terminal Trh4 peptide within the ER membrane is part of a novel processing pathway for TAP-independent peptides and is distinct from the previously described routes.

Signal Peptide Peptidase is responsible for the processing of the C-terminal peptide

The SPP family is also known as intramembrane-cleaving proteases (I-CLiPs) of the aspartyl type and this family is involved in many biological processes, including cell proliferation, differentiation and immunity^{34, 37, 38}. SPP and SPPL3 were reported to be localized in ER membranes, forming catalytic cavities in which hydrolysis can take place within membranes³⁹. We first analyzed the expression of the family members at mRNA level in the tumor cell lines active in Trh4 peptide processing (Fig 4A). All family members were expressed to varying degree in this cell panel, except SPPL2c, which was hardly detectable. High levels of SPP were measured in all cells. Differences in expression levels in the tumor cell panel prompted us to profile transcripts of these genes in normal mouse tissues, especially since this information is lacking in the current literature (Suppl Fig 2E). RNA extracts from whole mouse organs were analyzed for these intramembrane aspartic peptidases and a general low expression for SPPL2b and SPPL2c was found. Similar expression profiles were seen for SPP, SPPL2a and SPPL3 in that thymus and brain contained low transcript numbers and lung was high (Suppl Fig 2E).

Next, we wanted to determine which family member was responsible for the liberation of the C-terminal Trh4 peptide in tumor cells and silenced each gene individually using

short hairpin RNA constructs. SPPL2c was not included in this analysis, since it was not expressed in the tumor cells (Fig 4A). Efficiency of gene silencing was approximately 50-60% for all genes and, importantly, the shRNA constructs were specific in that the other members were not targeted (Fig 4B). We then used this cell panel to determine effects on recognition by Trh4-specific CTL. Downregulation of SPP clearly decreased the recognition by these CTL (Fig 4C). Similar decrease of Trh4 peptide presentation was observed in TAP-negative MCA fibrosarcoma cells (Suppl Fig 2F-G). Silencing of the other SPP family members resulted in a marginal variation of CTL recognition (Fig 4C). The presentation of the Trh4 peptide was not completely blocked by SPP silencing, most likely due to a substantial residual expression of the protease. The residual CTL recognition could furthermore be blocked by (z-LL)₂-ketone, supporting this notion. Importantly, an independent other peptide-epitope presented by RMA-S was not affected by SPP silencing (Fig 4D). These data and the fact that SPP is expressed in all mouse cells that naturally display Trh4/D^b complexes and the ER localization of Trh4 substantiate the conclusion that the SPP enzyme is crucial for the processing of the C-terminal peptide-epitope of Trh4 in a TAP-independent way.

The transmembrane region directly upstream of the Trh4 peptide is crucial for Trh4 processing

To study the underlying mechanism of Trh4 peptide processing in detail and to determine critical regions for the proteolytic process, extensive mutagenesis analysis of Trh4 was performed. First, some larger segments at the N- and C-terminus of the protein were deleted. Removal of the first 32 aa did not diminish the processing efficiency of the peptide-epitope (Fig 5A), whereas we hypothesized that this region functioned as a leader sequence routing the protein to the ER, as indeed was predicted by the online SignalP 4.1 Server (www.cbs.dtu.dk). Confocal microscopy analysis however confirmed that this construct was still localized in ER (Suppl Fig 1C). We then removed segments located near the C-terminus, starting with the sequence corresponding to amino-acids 340-374, which is a cytosolic loop located between the two last transmembrane domains (see Fig 2A). We did a progressive deletion in this construct comprising the upstream amino-acids 315-330, which includes most of the second last transmembrane domain. However, none of these large deletions decreased Trh4 peptide processing (Fig 5A). In order to exclude the possibility that these altered constructs delivered defective and thus proteasome-targeted proteins, we confirmed that the Trh4 peptide presentation from these mutant constructs was still (z-LL)₂-ketone and DAPT dependent (Fig 5B). Then, nine amino-acids just upstream of the C-terminal peptide were removed. This resulted in a more than 60% impairment of CTL recognition (Fig 5A), indicating that this intramembrane stretch, just upstream of the peptide-epitope was critical for SPP cleavage. Furthermore, the C-terminus was extended by cloning small VS-containing tags of 14 and 47 amino-acids. Although these proteins were properly expressed, as tested by western blot (Suppl Fig 3A), and were localized in the ER (Suppl Fig 1A), they did not result in any Trh4 peptide presentation (Fig 5A), implying

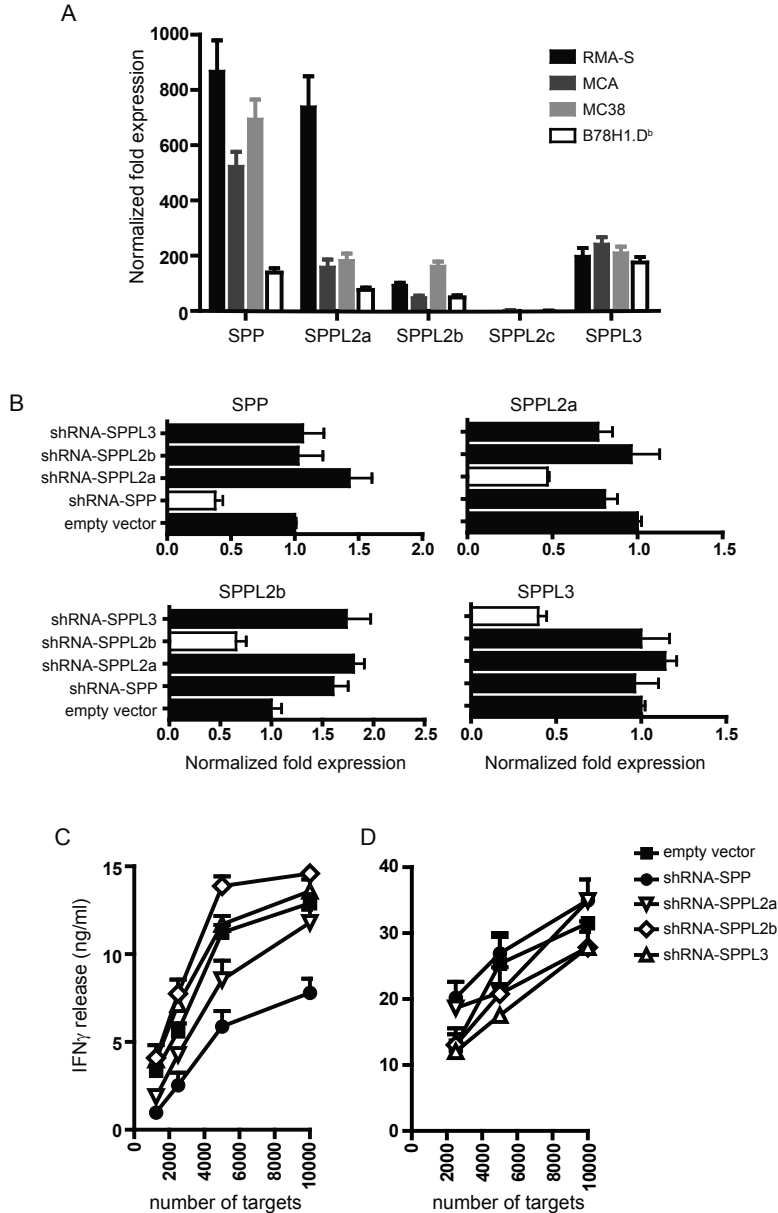


Figure 4. The intramembrane-cleaving signal peptide peptidase is involved in Trh4 processing. (A) mRNA expression of *SPP*, *SPPL2a*, *SPPL2b*, *SPPL2c* and *SPPL3* genes was determined by qPCR in tumor cell panel. (B) Silencing of SPP family members in RMA-S cells was reached by lentiviral transfer of shRNA constructs. Empty shRNA vector served as control. Efficiency and specificity of downregulated levels is shown as measured by qPCR. (C-D) Cell panel with silenced single SPP family members was tested for recognition by Trh4-specific T cells (C) or control T cells (D) reactive to the independent K^b-presented peptide mi3 by RMA-S. Mean and standard deviation from one out of three experiments is shown. Difference between SPP and control vector is statistically significant ($p < 0.001$, two way Anova test). Similar data were obtained in the fibrosarcoma cell line (Suppl Fig 2F-G).

that the C-terminal location of the peptide in the Trh4 protein is crucial for its liberation. Failure to process the Trh4 peptide when it was not at the complete C-terminus might be related to the absence of carboxyl-peptidases in the ER and suggests that the SPP cleavage products do not travel through the cytosol. Furthermore, we also tested if the C-terminal region would function as a leader sequence for the Trh4 protein, but removal of the last 20 amino-acids (aa368-387) did not change its subcellular localization (Supp Fig 1D). We therefore concluded that Trh4 peptide is directly liberated by SPP from the mature protein after which the peptide is available in ER for loading into MHC-I molecules.

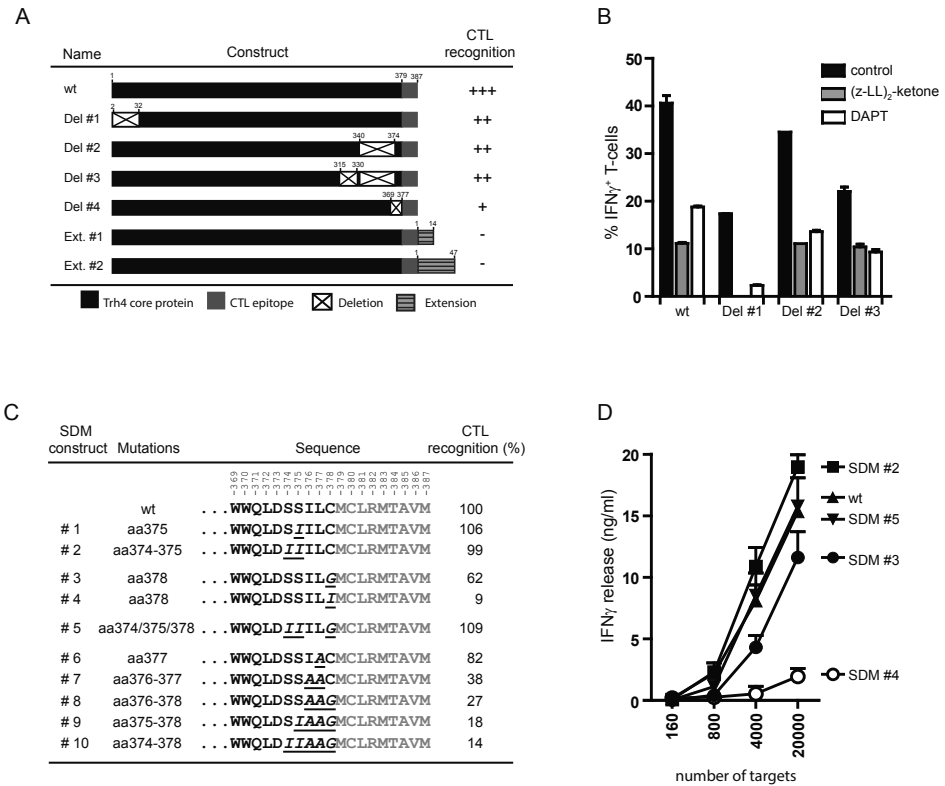


Figure 5. The intramembrane region directly upstream of the Trh4 peptide is crucial for processing. (A-B) Trh4 constructs with deletions or extensions were tested for liberation and MHC-I presentation of the C-terminal peptide-epitope (aa379-387). (A) DNA plasmid constructs were transiently transfected into HeLa cells together with the mouse D^b gene. Extend of IFN- γ release by T cells was quantified and scored as “+++” (wild-type construct, 100%), “++” (50-90%), “+” (10-50%) and “-” (<10%). (B) Processing from mutant constructs was still sensitive for the protease inhibitors (z-LL)₂-ketone and DAPT. All constructs were tested at least four times. (C-D) Site-directed mutagenesis directly upstream of the C-terminal peptide-epitope of the Trh4 gene. (C) The amino-acid substitutions and the position of the amino-acids in the protein are indicated. Degree of T cell recognition was quantified based on ‘area under the curve’ values of line graphs as shown in (D). Compiled means and standard deviations of at least three independent experiments is shown in Suppl Fig 3B.

The importance of the direct upstream amino-acids of the Trh4 peptide suggested that the SPP cleavage site was located within this last membrane region (aa374-387). Single amino acid substitutions were introduced using site-directed mutagenesis to determine the precise requirements for cleavage by SPP. Several publications demonstrated the strong preference of SPP for substrates with helix-destabilizing residues in their transmembrane domain⁴⁰⁻⁴². Amino-acids like asparagine, serine and cysteine disturb a perfect alpha-helical conformation of the transmembrane domain and are therefore referred to as 'helix-bending' or 'helix-breaking' residues. Three of such amino-acids are present in the Trh4 membrane region: two serines (aa374 and 375) and one cysteine (aa378) (Fig 5C). First, the two serines were replaced by isoleucines, which strongly contribute to a perfect alpha-helical structure. In contrast to our expectations, these mutations did not affect Trh4 presentation at all (Fig 5C and D; SDM #2). Second, the cysteine residue (aa378) in front of the epitope was substituted for a glycine (SDM #3), which still has some helix-breaking capacity, or an isoleucine (SDM #4), which removes the helix-breaking nature. These alterations had a very strong impact on peptide liberation and the C378I construct nearly completely failed to produce the Trh4 peptide. Third, we combined the cysteine mutation C378G with the serine mutations and surprisingly found that introduction of two isoleucines improved the Trh4 processing (Fig 5C and D; SDM #5), indicating that the more upstream serine residues were actually counterproductive for optimal SPP cleavage and support the importance of the cysteine residue directly upstream of the epitope. Fourth, we progressively substituted the complete stretch of amino-acids in this region also using alanines which are considered to promote helix-destabilization⁴³. Collectively, these constructs displayed a clear gradual decrease in peptide presentation (Fig 5C and Suppl Fig 3B). Together, these data point to the importance of the cysteine directly upfront of the epitope, most likely marking the actual SPP cleavage site and suggest that nearby flanking residues indirectly influence this process via conformational change in the intramembrane alpha-helix. In line with this hypothesis is the finding that presentation of the Trh4 peptide does not depend on the N-terminal trimming peptidase ERAAP (Fig 3D), suggesting that the minimal 9-mer epitope is directly generated by SPP cleavage without further need for C-terminal nor N-terminal trimming. The C-terminal liberation of this CTL peptide-epitope by the aspartyl protease SPP within the ER membrane exemplifies a novel way by which TAP-deficient cells are still able to present a diverse antigen repertoire.

Contribution of SPP protease family to MHC-I peptide repertoire of TAP-negative cells

We further examined the contribution of the SPP family of proteases to the overall MHC-I presentation of TAP-deficient cells. We analyzed the surface display of the two alleles of this mouse strain, D^b and K^b, on RMA-S cells in which individual SPP members were silenced by shRNAs. SPP and SPPL2a silencing resulted in reduction of K^b molecules to 77% and 65% of control, respectively (Fig 6). The presentation of D^b molecules on these cells and MHC-I surface display on RMA-S cells in which SPPL2b

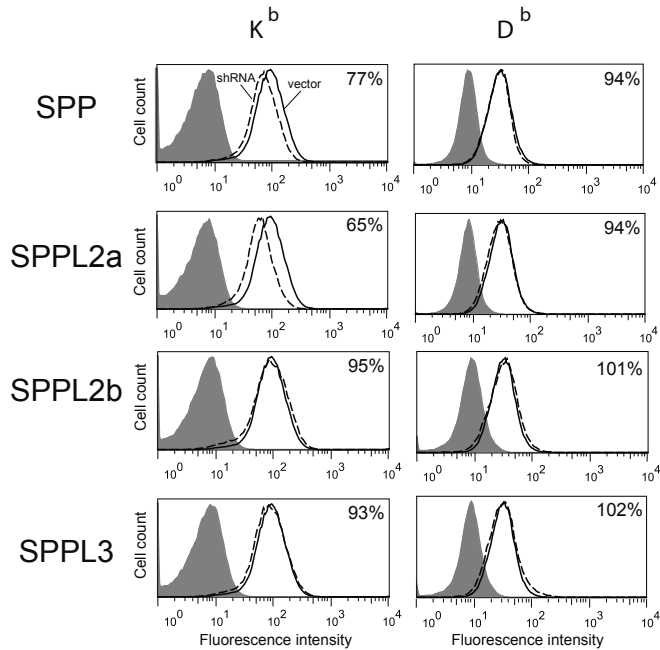


Figure 6. Silencing of SPP and SPPL2a leads to decrease in surface MHC-I. MHC-I surface display of RMA-S cells expressing shRNA constructs for SPP, SPPL2a, SPPL2b and SPPL3 was measured by flow cytometry (K^b and D^b molecules separately). Percentages in the upper right corners reflect the residual MHC-I expression compared to control cells ('vector'), based on mean fluorescence intensity. Data are representative for three independent experiments.

and SPPL3 were silenced, was not affected (Fig 6). These data underline the involvement of intra-membrane cleaving proteases in antigen processing, although precise underlying mechanisms remain to be elucidated.

DISCUSSION

Studies with TAP-deficient cells showed that peptides can still gain access to MHC-I molecules, although with much lower efficiency. This peptide repertoire is different from that of processing-intact cells, indicating that alternative processing pathways partly compensate for a defective conventional route based on proteasome and TAP^{14, 18, 19}. The processing mechanisms that lead to presentation of TAP-independent peptides are poorly characterized. Here we describe a new mechanism that leads to liberation of a C-terminal peptide-epitope from the ER membrane-spanning Trh4 protein. We found that the intramembrane-cleaving enzyme SPP was responsible for the cleavage event at the direct upstream region of the T cell epitope within the lipid bilayer. We speculate that SPP activity in the ER membrane is sufficient to liberate the minimal 9-mer peptide and release of this peptide into the ER lumen. Additional N-terminal trimming of the

cleavage product is unlikely, since inhibitors of amino-peptidases did not prevent Trh4 peptide presentation and the cysteine just upfront of the 9-mer epitope appeared to be critical in the SPP cleavage process (see Fig 3 and 5C). Moreover, direct release of the liberated peptide into the ER lumen is very likely, due to the type II transmembrane orientation of the Trh4 protein tail (see Fig 2A) and the fact that the epitope is located at the very C-terminal end of the protein. The incompetence of C-terminally extended versions of Trh4 to present the peptide (see Fig 5A) corroborate the idea that the peptide is directly released in the ER lumen where it needs a free C-terminus for loading into MHC-I molecules. The exact peptide loading mechanism of the Trh4 membrane peptide however remains to be determined.

The involvement of SPP in the C-terminal processing of a transmembrane protein initially surprised us. The activity of SPP was thus far largely related to the removal of small trunks of signal-sequence-derived transmembrane peptides from the ER membrane^{34, 37, 42}. The processing of leaders from nascent proteins requires a first cut by signal peptidase (SP), after which the remaining transmembrane stretch is cut in two parts by SPP. However, the liberation of the Trh4 peptide did not require SP activity, since its inhibitor DCI did not prevent the presentation of the epitope. Furthermore, the Trh4-peptide is not part of a signal sequence and removal of the N- or C-terminal domain did not route the Trh4 protein to a different subcellular localization (see Suppl Fig 1C-D). Nevertheless, the involvement of SPP with Trh4 cleavage fits with the strong preference for type II membrane segments and with the ‘helix-breaking’ residues that are often found in SPP target proteins^{25, 41, 42}. Therefore it seems that the role of SPP is more extended than the release of transmembrane leader-sequences from the ER membrane. Interestingly, it has been suggested that SPP is physically associated with misfolded membrane proteins^{44, 45} and as such might function as a chaperone to dispose membrane aggregates.

The Trh4-derived peptide is an example of TAP-independently processed peptides. This particular SPP-mediated processing pathway is operational in TAP-negative as well as TAP-positive cells (see Fig 1) and also in tumor as well as healthy cells²⁸. Therefore, the TAP-independent pathway described here seems to be part of the “normal activity” of cells and does not represent a disease-related feature. Over the years, some other TAP-independent pathways have been characterized. They comprise peptides generated in the secretory and vesicular compartments by the protein convertase furin^{22, 23}. Second, leader sequence derived peptides are frequently TAP-independently processed²⁴. Peptides that arrive in the ER via this way overcome the need of TAP transport. Interestingly, TAP-independent processing was observed earlier using several C-terminally tagged constructs of ER-targeted proteins, feeding the speculation on a common ‘C-terminal’ processing rule^{46, 47}. We hypothesize that SPP might be a central player for this category of TAP-independent antigen processing.

The family of intramembrane-cleaving aspartyl proteases (SPP family and presenilins) have important biological roles in signal transduction, cell differentiation and immunity^{25, 48}. For instance, SPPL2a and SPPL2b were shown to be responsible for the release of the

TNF- α intracellular domain, leading to expression of the pro-inflammatory cytokine IL-12 in dendritic cells³⁸ and also for the release of the intracellular domain of fasL in T cells⁴⁹. These proteases are mainly located in the vesicular pathway and at the cell surface. Recently, SPPL2a was reported to be involved in the proteolysis of the invariant chain (CD74) and thereby critical for the development and survival of B-cells and DCs⁵⁰⁻⁵², maybe explaining the effect we found on MHC class I expression (Fig 6). We now extend on the involvement of SPP family members in immunity by showing their role in alternative pathways of MHC-I antigen processing, thereby enabling T cell immune responses.

I MATERIAL AND METHODS

Cell lines

The mouse tumor cell lines RMA, RMA-S (TAP2-deficient), MCA (TAP1-deficient), MC38, B78H1 (TAP2-deficient) and human HeLa and 293T have been described previously^{14, 17, 28}. Where indicated, cell lines were transfected with H-2D^b or Trh4 genes (accession numbers: gene BC043059 (www.ncbi.nlm.nih.gov/nucore), protein UniProtKB Q9D6K9-2 (www.uniprot.org/uniprot) via retroviral transduction using the LZRS vector containing GFP behind an internal ribosome entry site¹⁶. The mouse TAP genes were introduced into MCA and B78H1 cells by gene transfer. MC38.UL49.5 cells contain the UL49.5 gene from the Bovine Herpes Virus-1 which blocks mouse TAP activity^{16, 29}.

Generation and culture of CD8⁺ T cell clones used in this study were previously described: D^b-restricted CTL clone B5 specific for the TAP-independent Trh4 peptide (MCLRMTAVM); K^b-restricted CTL clone mi3 specific for a yet unknown TEIPP peptide and the D^b-restricted CTL clone 1 specific for the TAP-dependent MuLV gag-leader peptide (CCLCLTVFL)^{16, 17, 30}.

All cells were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies, Rockville, MD), 2mM L-glutamine (Invitrogen) and 30 μ mol/L of 2-mercaptoethanol (Merck, NJ, USA) at 37°C in humidified air with 5% CO₂.

Protease inhibitor experiments

Cells were resuspended in X-vivo medium (Lonza) supplemented with 0,5% of BSA and incubated with protease inhibitors for 1h at 37°C, under continuous mixing. Cells were incubated for 2-4 min with mild acid citrate/phosphate buffer (pH 3.1) at room temperature to disrupt MHC class I/peptide complexes²⁸. Cells were washed twice and incubated with protease inhibitors for additional 6h under the conditions mentioned above and then treated with brefeldin A. Inhibitor-treated cells were then incubated with the indicated T cell clones for 18h in the presence of brefeldin A and intracellular stained for IFN γ production²⁸. Chemical inhibitors used in these assays are: epoxomicin (1 μ M, Sigma-aldrich), 3,4-Dichloroisocoumarin (5 μ M, Sigma-aldrich), Decanoyl-RVKR-CMK (10 μ M, Merck), calpeptin (30 μ M, Merck), calpain inhibitor IV (13 μ M, Merck), calpastatin

peptide (10 μ M, Merck), PD151746 (50 μ M, Santa Cruz Biothecnology), PD150606 (50 μ M, Santa Cruz Biothecnology), butabindide oxalate (400 μ M, TOCRIS Bioscience), captopril (100 μ M, Sigma-aldrich), leupeptin (100 μ M, Merck), (z-LL)₂-ketone (5 μ M, Merck), 1,10-Phenanthroline monohydrate (400 μ M, Sigma-aldrich), aprotinin (10 μ M, Merck), DAPT (10 μ M, Sigma-aldrich), Bestatin hydrochloride (100 μ M, Sigma-aldrich), z-vad-fmk (10 μ M, Merck), L-leucinethiol (60 μ M, Sigma-aldrich) and DL-Dithiothreitol (5mM, Sigma-aldrich). None of the inhibitors was toxic at the applied concentrations, as determined by trypan-blue counting and annexin-V staining.

T-cell activation assays and flow cytometry

Intracellular cytokine staining of IFN- γ in T cell clones was performed for the protease inhibitor screen and expressed as percentage positive cells³¹. IFN- γ secretion by T-cell clones was measured by ELISA as previously published²⁸. Data shown represent mean values obtained from triplicate test wells and error bars represent standard deviation of these values. Flow cytometry analysis of surface expression of D^b and K^b molecules was performed using directly labeled anti-D^b mAb (clone 28.14.8S; BioLegend, California, USA) and anti-K^b mAb (clone AF6-88.5, BioLegend). Cells were measured using a FACS Calibur and analysed with Flowjo software (Tree Star, Ashland, OR).

Quantitative PCR analysis

Total RNA isolation was performed using RNeasy Mini Kit (Qiagen, Maryland, USA). 500 ng of purified total RNA was used to synthesize cDNA using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, USA). Quantitative PCR on two different transcripts of Trh4 was done using unique forward primers. Forward primer for the Trh4-epitope containing transcript 5'-GCAGACCCCTTACTGGAAGCTGC-3' and for the short transcript 5'-TACATCACTGCGGTCATC-3'. Common reverse primer was 5'-CTGCGGTCATCCTTAGACACCTTTC-3'. The primers used to detect the expression of mouse transcripts from SPP, SPPLs and calpains were designed by us using the Beacon designer software and are listed in Supplementary Table 1 (calpain family) and Table 1 (SPP family). All primer sets were validated to be specific for the cognate genes by sequencing of the amplified PCR products. For the PCR reaction SensiMix™ SYBR No-ROX kit from GC Biotech Bioline (Alphen aan den Rijn, Netherlands) was used in a C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) and results were analysed using Bio-Rad CFX manager software.

Table I. Primer sequences for quantitative PCR for the mouse SPP family.

Name	Gene ID	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)
SPP	14950	CAGGATCTGCTGGAGAAG	GAAGTAGGTGTGCGTGTT	138
SPPL2a	66552	CTGATGGCATAAGGAACAAG	GAGGAAGGAATTAGGACACT	133
SPPL2b	73218	CATTGCCTTCTGCCTGTA	GATGCTGTTGCCACTCTT	142
SPPL2c	237958	TGCATTGCCTCTGCTGTGGGC	TGTGGCCCTCTGGACTCGTGG	181
SPPL3	74585	TCGCTATGACAACACTACAAGA	GCAGTGGAAGTAGGAGAC	93

Confocal microscopy

Human HeLa cells or mouse MCA cells were plated in 12-wells plates on glass coverslips and transfected the next day with the indicated Trh4 gene constructs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. After one day cells were stained with monoclonal antibodies: cells were fixed with methanol for 30 minutes at 4°C, washed with TBSB (TBS with blocking reagent from Boehringer Mannheim) and incubated for 1.5 hours at room temperature with the first antibody diluted in 500 µl TBSB. The antibodies used were anti-V5 (Invitrogen, R960-25), anti-Giantin (Abcam, ab24586) and anti-BIP (Abcam, ab21685). Cells were then washed again with TBSB and incubated with second antibodies; anti-rabbit-Alexa488 (Invitrogen, a11008) and anti-mouse-Alexa594 (Invitrogen, a11005) for 1.5 hours at room temperature. Finally, cells were washed with TBSB and the coverslips were put on microscope glass slides with 5µl of ProLong Gold antifade reagent (Invitrogen) containing Hoechst (Enzo, Hoechst 33342). The stained cells were imaged with a TCS SP5 fluorescent confocal microscope (Leica, Germany) using LAS AF software. Stained samples were observed at room temperature. All images were acquired with a 63X glycerol immersion objective lens NA 1.4 (Leica). Analysis of colocalization was performed with LAS AF software (Leica).

shRNA treatment

MISSION® shRNA constructs specific for mouse SPP, SPPL2a, SPPL2b, SPPL3 and calpain 2 were obtained from Sigma-Aldrich and lentiviral encapsulation was reached in HEK.293T cells via transfections with calcium phosphate (Promega) of pCMV-VSVG, pMDLg-pRRE, pRSV-REV and pLKO plasmid with puromycin resistance gene and a shRNA sequence or empty vector. RMA-S cells were incubated with lentivirus particles encoding shRNA and particles encoding GFP in a 10:1 ratio for 20h at 37°C. Then the cells were washed and resuspended in complete medium supplemented with 1µg/ml of puromycin and cultured for 4 days. GFP expressing cells were sorted and used in experiments.

Trh4 gene constructs

Length variants of Trh4 (deletions and extensions) were performed on the cloned cDNA (accession number BC043059) in pcDNA3.1 plasmid. C-terminal extensions were created by removing the stop codon of Trh4 in the pcDNA3.1/V5-His vector (Invitrogen, Life Technologies), resulting in the in frame encoding V5 antibody epitope. The deletion variants were based on an N-terminally tagged V5 construct in which the antibody site was introduced after nucleotide nr 96, which was supposed to be the leader sequence. We introduced a NOT-I restriction site at this place by creating two complementary PCR products, resulting in two altered amino acids at position 32-33 (from DL to AA). Then the 14 aa long V5 sequence was introduced at this NOT-I site using long synthetic oligonucleotides including NOT-I overhangs. Orientation and sequence of this insert was validated by sequencing. This 'N-terminal' tagged Trh4 construct was then used to create several deletion variants using PCR primer sets. All PCR amplifications were performed with Accuprime kit (Invitrogen).

Site-directed mutagenesis was done on the N-terminal V5 construct in pcDNA3.1 using USB Change-IT Multiple Mutation Site Directed Mutagenesis technology (Affymetrix). Generated clones were sequenced to validate the introduced changes.

Western blot

Cells were lysed in 1% Nonidet P-40 lysis buffer (50 mM Tris-HCl, 150 mM NaCl (pH 8.0), 1% NP40, 1mM leupeptin, 1mM 4-(2-aminoethyl)-benzene-sulfonyl fluoride hydrochloride (Sigma-Aldrich, The Netherlands). The samples were mixed 1:1 with sample buffer (0,1 M Tris (pH 8.0), 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.05 % bromophenolblue) and heat up to 95°C for 1 minute. The samples were loaded in pre-cast gels 4-15% (BioRad) using the Mini-PROTEAN® Tetra 4-gel vertical electrophoresis system (BioRad). The BenchMark Pre-Stained Protein Ladder (Invitrogen) was included. Gels ran for 25 min at 250V and blotted in Trans-Blot® Turbo™ Transfer System (BioRad) according to the manufacturer recommendations. After blotting, the membrane was incubated overnight at 4°C in 5% low-fat milk/TBST (10 mM Tris-base, 150 mM NaCl, 0.05% Tween 20, pH 7.4). The next day the blots were stained with anti-V5 monoclonal antibody 1:2500 (Invitrogen) in 1% low-fat milk/TBST for 2 hours at room temperature. After the staining, the membrane was washed three times for 5 min in TBST. Next, the membrane was incubated with HRP-labeled goat anti-mouse Ig antibody (1:2000) in 1% low-fat milk/TBST for 2 hours at room temperature, followed by three times washing in TBST and three times in TBS. Visualization of the blot was performed using ECL Plus Western blotting detection reagents (GE Healthcare) on films.

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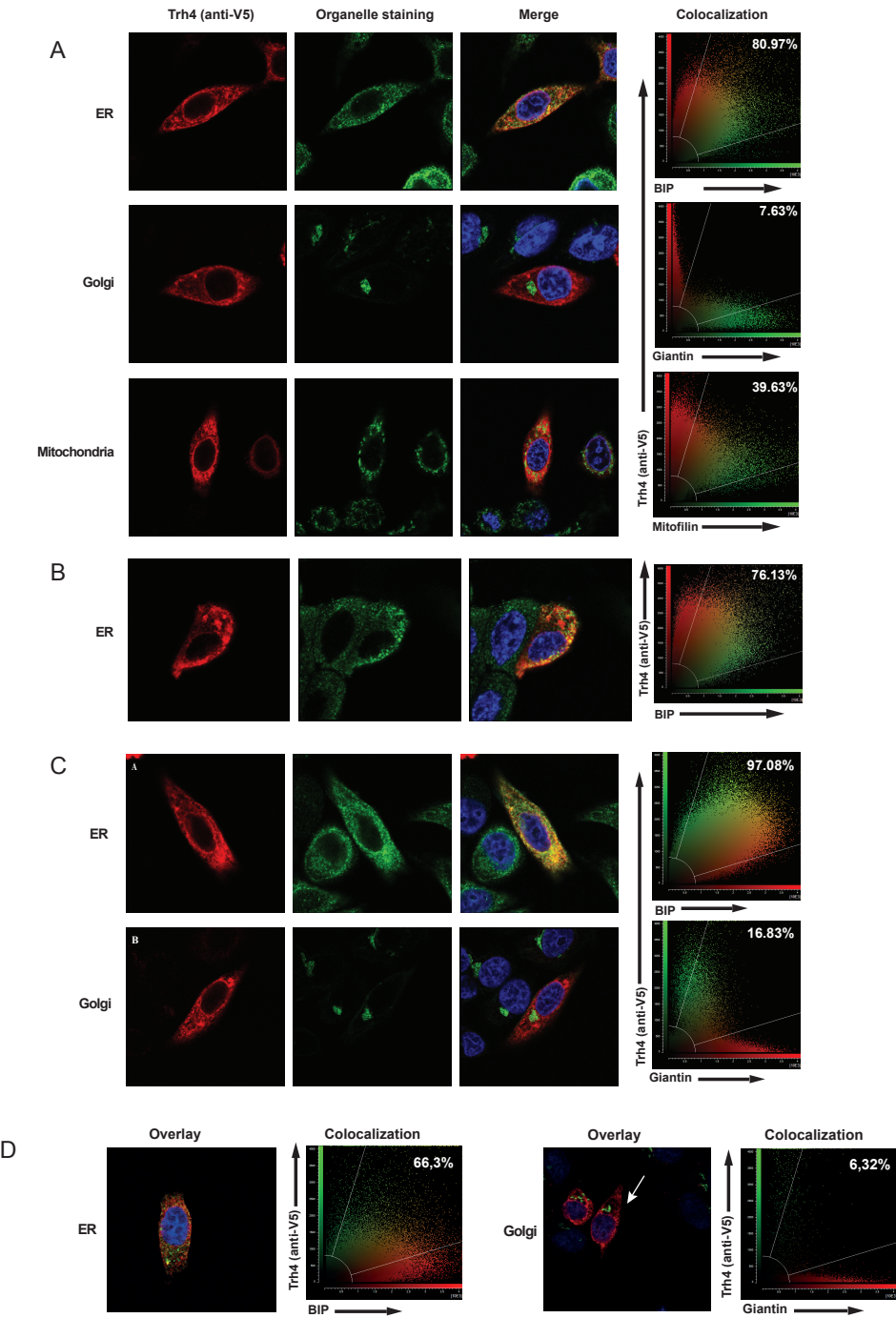
I REFERENCES

1. Cresswell, P., Ackerman, A.L., Giodini, A., Peaper, D.R. & Wearsch, P.A. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol. Rev.* **207**, 145-157 (2005).
2. Rock, K.L., Farfan-Arribas, D.J. & Shen, L. Proteases in MHC class I presentation and cross-presentation. *J Immunol* **184**, 9-15 (2010).
3. Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* **8**, 607-18 (2008).
4. Kessler, J.H. et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* **12**, 45-53 (2011).
5. Seifert, U. et al. An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat Immunol* **4**, 375-9 (2003).
6. Aldrich, C.J. et al. Positive selection of self- and alloreactive CD8+ T cells in TAP-1 mutant mice. *Proc. Natl. Acad. Sci. USA* **91**, 6525-6528 (1994).

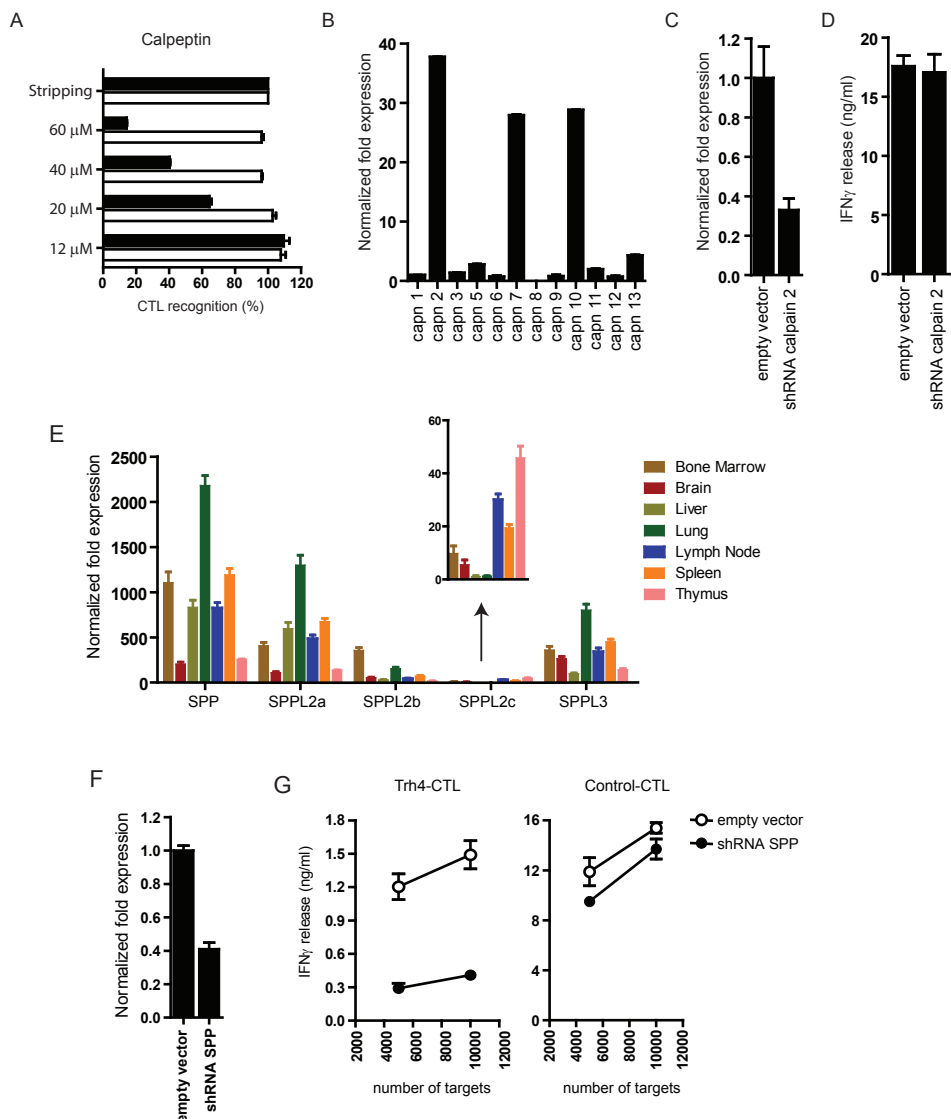
7. Sandberg, J.K., Chambers, B.J., Van Kaer, L., Karre, K. & Ljunggren, H.G. TAP1-deficient mice select a CD8+ T cell repertoire that displays both diversity and peptide specificity. *Eur. J. Immunol.* **26**, 288-293 (1996).
8. Van Kaer, L., Ashton-Rickardt, P.G., Ploegh, H.L. & Tonegawa, S. TAP1 mutant mice are deficient in antigen presentation, surface class I molecules, and CD4-8+ T cells. *Cell* **71**, 1205-14 (1992).
9. Cerundolo, V. & de la Salle, H. Description of HLA class I- and CD8-deficient patients: Insights into the function of cytotoxic T lymphocytes and NK cells in host defense. *Semin Immunol* **18**, 330-6 (2006).
10. Zimmer, J. et al. Clinical and immunological aspects of HLA class I deficiency. *QJM* **98**, 719-27 (2005).
11. de la Salle, H. et al. Human peptide transporter deficiency: importance of HLA-B in the presentation of TAP-independent EBV antigens. *J Immunol* **158**, 4555-63 (1997).
12. Lautscham, G. et al. Identification of a TAP-independent, immunoproteasome-dependent CD8+ T-cell epitope in Epstein-Barr virus latent membrane protein 2. *J Virol* **77**, 2757-61 (2003).
13. Chambers, B. et al. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer Res* **67**, 8450-5 (2007).
14. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-21 (2010).
15. Seidel, U.J., Oliveira, C.C., Lampen, M.H. & Hall, T. A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens. *Cancer Immunol Immunother* **61**, 119-25 (2012).
16. Van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b. *J Immunol* **178**, 657-62 (2007).
17. Van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Medicine* **12**, 417-424 (2006).
18. Lampen, M.H. et al. Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2. *Mol Immunol* **53**, 126-31 (2013).
19. Weinzierl, A.O. et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur J Immunol* **38**, 1503-10 (2008).
20. Oliveira, C.C. & van Hall, T. Importance of TAP-independent processing pathways. *Mol Immunol* **55**, 113-6 (2013).
21. Del Val, M., Iborra, S., Ramos, M. & Lazaro, S. Generation of MHC class I ligands in the secretory and vesicular pathways. *Cell Mol Life Sci* **68**, 1543-52 (2011).
22. Gil-Torregrosa, B.C., Raul Castano, A. & Del Val, M. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-Golgi network protease furin. *J Exp Med* **188**, 1105-16 (1998).
23. Medina, F. et al. Furin-processed antigens targeted to the secretory route elicit functional TAP1-/-CD8+ T lymphocytes in vivo. *J Immunol* **183**, 4639-47 (2009).
24. Martoglio, B. & Dobberstein, B. Signal sequences: more than just greasy peptides. *Trends Cell Biol* **8**, 410-5 (1998).
25. Weihofen, A. & Martoglio, B. Intramembrane-cleaving proteases: controlled liberation of proteins and bioactive peptides. *Trends Cell Biol* **13**, 71-8 (2003).
26. Durgeau, A. et al. Different expression levels of the TAP peptide transporter lead to recognition of different antigenic peptides by tumor-specific CTL. *J Immunol* **187**, 5532-9 (2011).
27. El Hage, F. et al. Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway. *Proc Natl Acad Sci U S A* **105**, 10119-24 (2008).
28. Oliveira, C.C. et al. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. *Eur J Immunol* **41**, 3114-24 (2011).
29. Verweij, M.C. et al. Structural and functional analysis of the TAP-inhibiting UL49.5 proteins of varicelloviruses. *Mol Immunol* **48**, 2038-51 (2011).
30. Van Hall, T. et al. Differential influence on cytotoxic T-lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J. Exp. Med.* **192**, 483-494 (2000).
31. van Stipdonk, M.J. et al. Design of agonistic altered peptides for the robust induction of CTL directed towards H-2Db in complex with the melanoma-associated epitope gp100. *Cancer Res* **69**, 7784-92 (2009).
32. Riebeling, C., Allegood, J.C., Wang, E., Merrill, A.H., Jr. & Futerman, A.H. Two mammalian longevity assurance gene (LAG1) family members, trh1 and trh4, regulate dihydroceramide synthesis using different fatty acyl-CoA donors. *J. Biol. Chem.* **278**, 43452-9 (2003).
33. Yu, J. et al. JNK3 signaling pathway activates ceramide synthase leading to mitochondrial dysfunction. *J Biol Chem* **282**, 25940-9 (2007).

34. Golde, T.E., Wolfe, M.S. & Greenbaum, D.C. Signal peptide peptidases: a family of intramembrane-cleaving proteases that cleave type 2 transmembrane proteins. *Semin Cell Dev Biol* **20**, 225-30 (2009).
35. Weihofen, A. et al. Targeting presenilin-type aspartic protease signal peptide peptidase with gamma-secretase inhibitors. *J Biol Chem* **278**, 16528-33 (2003).
36. Weihofen, A., Lemberg, M.K., Ploegh, H.L., Bogyo, M. & Martoglio, B. Release of signal peptide fragments into the cytosol requires cleavage in the transmembrane region by a protease activity that is specifically blocked by a novel cysteine protease inhibitor. *J Biol Chem* **275**, 30951-6 (2000).
37. Martoglio, B. & Golde, T.E. Intramembrane-cleaving aspartic proteases and disease: presenilins, signal peptide peptidase and their homologs. *Hum Mol Genet* **12 Spec No 2**, R201-6 (2003).
38. Friedmann, E. et al. SPPL2a and SPPL2b promote intramembrane proteolysis of TNFalpha in activated dendritic cells to trigger IL-12 production. *Nat Cell Biol* **8**, 843-8 (2006).
39. Li, X. et al. Structure of a presenilin family intramembrane aspartate protease. *Nature* **493**, 56-61 (2013).
40. Fluhrer, R. et al. The alpha-helical content of the transmembrane domain of the British dementia protein-2 (Bri2) determines its processing by signal peptide peptidase-like 2b (SPPL2b). *J Biol Chem* **287**, 5156-63 (2012).
41. Lemberg, M.K. & Martoglio, B. Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. *Mol Cell* **10**, 735-44 (2002).
42. Lemberg, M.K. & Martoglio, B. On the mechanism of SPP-catalysed intramembrane proteolysis; conformational control of peptide bond hydrolysis in the plane of the membrane. *FEBS Lett* **564**, 213-8 (2004).
43. McLauchlan, J., Lemberg, M.K., Hope, G. & Martoglio, B. Intramembrane proteolysis promotes trafficking of hepatitis C virus core protein to lipid droplets. *EMBO J* **21**, 3980-8 (2002).
44. Loureiro, J. et al. Signal peptide peptidase is required for dislocation from the endoplasmic reticulum. *Nature* **441**, 894-7 (2006).
45. Schrul, B., Kapp, K., Sinning, I. & Dobberstein, B. Signal peptide peptidase (SPP) assembles with substrates and misfolded membrane proteins into distinct oligomeric complexes. *Biochem J* **427**, 523-34 (2010).
46. Snyder, H.L. et al. Two novel routes of transporter associated with antigen processing (TAP)-independent major histocompatibility complex class I antigen processing. *J. Exp. Med.* **186**, 1087-98 (1997).
47. Snyder, H.L., Bacik, I., Yewdell, J.W., Behrens, T.W. & Bennink, J.R. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur. J. Immunol.* **28**, 1339-46 (1998).
48. Puente, X.S., Sanchez, L.M., Overall, C.M. & Lopez-Otin, C. Human and mouse proteases: a comparative genomic approach. *Nat Rev Genet* **4**, 544-58 (2003).
49. Kirkin, V. et al. The Fas ligand intracellular domain is released by ADAM10 and SPPL2a cleavage in T-cells. *Cell Death Differ* **14**, 1678-87 (2007).
50. Beisner, D.R. et al. The intramembrane protease Sppl2a is required for B cell and DC development and survival via cleavage of the invariant chain. *J Exp Med* **210**, 23-30 (2013).
51. Bergmann, H. et al. B cell survival, surface BCR and BAFFR expression, CD74 metabolism, and CD8- dendritic cells require the intramembrane endopeptidase SPPL2A. *J Exp Med* **210**, 31-40 (2013).
52. Schneppenheim, J. et al. The intramembrane protease SPPL2a promotes B cell development and controls endosomal traffic by cleavage of the invariant chain. *J Exp Med* **210**, 41-58 (2013).

| SUPPLEMENTARY FIGURES AND TABLES

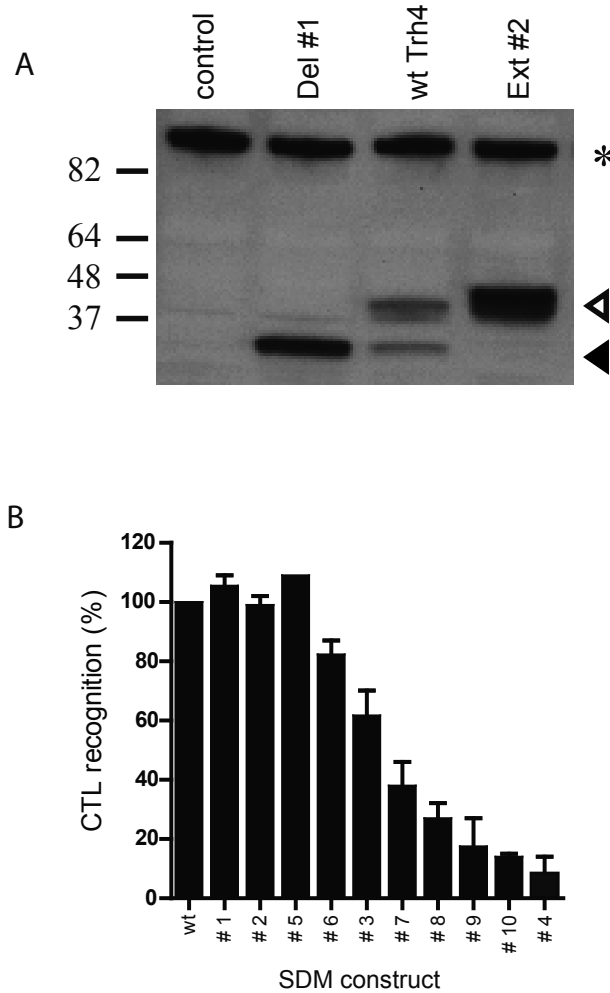


- ◀ **Supplementary Figure 1. Trh4 localizes in the ER of TAP-proficient and TAP-deficient cells.** Confocal microscopy images were taken from transfected cells with V5-tagged Trh4 constructs. Cells were co-stained with antibodies against BIP (ER), giantin (golgi) and mitofilin (mitochondria). Computer-assisted analysis was performed using LAS AF software to calculate colocalization. At least ten images were included of two independent experiments. (A) HeLa cells transfected with the C-terminally tagged Trh4 construct. (B) TAP-deficient MCA cells with the N-terminally tagged Trh4 construct. (C) HeLa cells expressing the Trh4 construct lacking the first 32 amino acids (Del #1 construct from Fig 5A), which was predicted to constitute the leader sequence by the SignalP 4.1 Server (www.cbs.dtu.dk). V5 antibody tag was located at the C-terminus of this construct. (D) HeLa cells with a Trh4 construct in which the C-terminal amino acids 368-387, including the peptide-epitope, were removed and the V5 tag was introduced at the N-terminus.



Supplementary Figure 2. Presentation of Trh4-derived peptide is not affected by calpain 2 silencing, but is SPP-dependent. (A) RMA-S cells were incubated with the protease inhibitor calpeptin at the indicated concentrations for 1h. Cells were washed and incubated with a mild acid solution to disrupt MHC-I/peptide complexes from the cell surface. After washing, cells were incubated again with calpeptin for additional 6h to allow recovery of MHC-I/peptide complexes and then incubated overnight with Trh4-specific CTL in the presence of Brefelding A, to prevent release of IFN- γ . Intracellular cytokine staining was performed and percentage of activated CTL was determined by flow cytometry. Control CTL recognized the MuLV gag-leader peptide (CCLCLTVFL) on RMA cells. At the given calpeptin concentrations no toxicity on target cells was detected. (B) Expression of the calpain family member genes was analysed in RMA-S cells with qPCR. Expression levels were normalized to GAPDH housekeeping gene and expressed relative to the calpain 1 gene. Primer sequences are given in Suppl Table I. (C) RMA-S cells were transduced with lentiviruses encoding shRNA sequences that target calpain 2 or an

- empty vector, leading to decreased mRNA levels of this gene. (D) CTL recognition of RMA-S cells with downregulated expression of calpain 2 was comparable to control RMA-S targets. Data are representative of three independent experiments. (E) Expression levels of SPP family members in mouse organs. RNA from complete mouse organs of wild type C57BL/6 mice was extracted and expression of SPP family members was determined by qPCR. Expression levels were normalized to GAPDH housekeeping gene and expressed relative to the SPPL2c sample in liver. (F) Silencing of SPP in TAP-deficient MCA cells was reached by lentivirus-mediated expression of shRNA constructs. Empty shRNA vector served as control. Efficiency of downregulated levels of transcripts was measured by qPCR. (G) Cells with silenced SPP were tested for recognition by Trh4-specific CTL or control CTL reactive to an independent K^b-presented peptide. Data are representative of three independent experiments.



Supplementary Figure 3. Detection of V5-tagged Trh4 constructs by western blot and CTL recognition efficiency of all side-directed mutagenesis constructs. (A) HeLa cells were transiently transfected with V5-tagged Trh4 constructs. The tag was introduced at the N-terminus of the protein (at aa position 33; 'Del #1' and 'wt Trh4') or as an extension at the C-terminus ('Ext #2'), see Fig 5A. Two days later the cells were lysed and proteins were separated on SDS-page gel. Western blot analysis was performed with anti-V5 antibodies to confirm expression of these constructs after transfection. Data are representative of three independent experiments. Open triangle indicates full length Trh4 protein, closed triangle represents Trh4 without the first 32aa, predicted to be a leader sequence and * indicates a non-specific band present in all HeLa lysates, serving as loading control. (B) The Trh4 gene was mutated at single positions using site-directed mutagenesis. The construct numbers refer to the constructs mentioned in Figure 5C. These Trh4 constructs were transiently transfected into HeLa cells together with the mouse MHC-I molecule D^b. Two days later HeLa cells were collected and incubated at different target concentrations with Trh4-specific CTL. CTL recognition was determined by IFN- γ release in the supernatants and quantified by ELISA and the efficiency of CTL recognition was calculated as a percentage compared to the wild type construct using 'area under the curve' results of original line graphs (see Figure 5D). Depicted data are compiled from three independent experiments and means plus standard error of the means are plotted.

Supplementary Table 1. Primer sequences for the quantitative PCR for the mouse Calpain family.

Name	Gene ID	Forward primer (5'→3')	Reverse primer (3'→5')	Prod. Size (bp)
Calpain 1	12333	TTCTTCCATTCTTCCTCTG	CTTCCTTCCTGTCTGAG	114
Calpain 2	12334	TTCGGCATCTATGAGGT	CGGAGGTAAATGAAGGTATC	116
Calpain 3	12335	GAGGACTCTGAGGTAATCTG	GCGAAGCCAATAGTGAAG	101
Calpain 5	12337	CACGTACGAGGCTAAGAG	C'TTGATGGAGGCACTGAT	85
Calpain 6	12338	GGAACGTGACCTTGGACAT	TTCAITGGCATACTTCITCTC	121
Calpain 7	12339	TACTCGGTGGGGCTCTG	CTCAITGTCTCCTCGTGTCTTAT	90
Calpain 8	170725	ACTACACCATCCAGAAGG	TCCAGTCCACAGAGTATGC	131
Calpain 9	73647	GGACGGAGACCTGAGTAG	CTGGAATCGCTCTGAGACT	97
Calpain 10	23830	AGTGGTATCTTCATTCCTTAGTG	TCTGCCTGCCCTAGTGTA	121
Calpain 11	268958	GGAGGTGAACAGCCATCT	AAGCAGGAAGTCAGCATCT	91
Calpain 12	60594	GCATATCCACATCTTCCA	C'TTCATCATCGTTCATCCT	143
Calpain 13	381122	GCTACTTGGCTCCTATTC	GAITGCGGTCTTCACTGT	136

4

THE PROTEASOME STRONGLY CONTRIBUTES TO THE TAP-INDEPENDENT PEPTIDE REPERTOIRE PRESENTED BY MHC-I

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| ABSTRACT

Tumors frequently display defects in the antigen processing pathway affecting molecules such as the peptide transporter TAP. TAP-deficiency results in decreased peptide/MHC-I complexes at the cell surface, but they still have residual presentation of those complexes. Part of this collection is surprisingly not presented by normal cells and we called these TEIPP (T-cell epitopes associated with impaired peptide processing), since they constitute immunogenic neo-antigens for CD8⁺ T-cells. Thus far, only a few enzymes have been identified that liberate peptide ligands for this alternative repertoire: signal peptidase (SP) and signal peptide peptidase (SPP) in the ER and furin in the trans-Golgi. Here we studied the presentation of a novel TEIPP peptide epitope presented by TAP-deficient lymphoma cells. We showed that the thus far known enzyme systems were not involved, but that the proteasome was required for the processing and presentation of this TAP-independent peptide. Interestingly, the proteasome was revealed as the major contributor to the alternative peptide repertoire, overruling the effect of SP, SPP and furin. The loading of the studied TEIPP peptide-epitope most likely occurred in the endocytic vesicular compartment. We conclude that TAP-deficient cells have a multitude of alternative pathways to generate and present their MHC-I ligands and that the proteasome has a surprisingly dominant role to shape this peptide repertoire.

| INTRODUCTION

The Major Histocompatibility complex class I (MHC-I) processing pathway is a mechanism by which cells can sample the pool of peptides produced by cellular catabolism and display them on the cell surface for scrutiny by CD8⁺ T cells. In the conventional MHC-I processing pathway, a substantial fraction of the peptides presented by MHC-I molecules results from proteolysis in the cytosol, mainly via the action of the proteasome. These peptides are transported into the endoplasmic reticulum (ER) by TAP transporters, where they meet nascent MHC class I molecules. However, cells can perform the task of antigen presentation even in cases of malfunctioning of some molecules of the conventional MHC-I processing pathway, such as the proteasome and TAP¹⁻⁸. Studies with TAP-deficient cells and animals have shown that MHC-I ligands are formed in the secretory and vesicular compartments. Initial peptides identified in cells lacking the TAP transporters were derived from leader sequences^{9, 10}. Leader sequences target fresh translational products to the ER and are cleaved by signal peptidases (SPase) in close proximity of the ER membrane and then by Signal Peptide Peptidase (SPP) in the intramembrane region, and thereby releasing these leader remnants from the ER membrane. After cleavage by SPP, partial domains of the signal peptides end up in the ER lumen and after further trimming, can be loaded into MHC-I molecules⁹⁻¹¹. In addition to this TAP-independent peptide loading pathway, two proprotein convertases have been shown to generate MHC-I ligands in TAP-deficient cells: furin, located in the trans-Golgi network and PC7, located in the trans-Golgi network and endocytic vesicles¹²⁻¹⁵. Furin cleaves at polybasic sites on protein substrates in the process of protein maturation. The action of furin frequently liberates long peptides and thus strongly depends on trimming peptidases working progressively from either end of the oligopeptides to form the most suitable MHC-I ligands.

We have recently shown a novel processing route for TAP-independent peptides by examining the liberation of a C-terminal peptide-epitope derived from the ceramide synthase Trh4 protein. The Trh4-epitope was liberated from the ER-membrane via the intramembrane-cleavage activity of SPP, independent from proteasome, SPase or furin activity (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press). We speculate on the existence of yet other processing pathways active in cells to load MHC-I peptides, since tumors with processing defects still present a broad repertoire of peptide antigens^{7, 16, 17}. The characterization of these peptides, which we called TEIPP – "T-cell epitopes associated with impaired peptide processing", showed that they derive from housekeeping proteins, are diverse in length and amino-acid composition, and are not presented on normal cells which makes them neo-antigens for CD8⁺ T-cells.

In this report we studied the processing pathway of a new TEIPP epitope recognized by a specific CD8⁺ T-cell clone capable of killing TAP-deficient tumor cells¹⁷. Surprisingly, the proteasome was involved in the generation of this epitope, although TAP-positive cells do not present this peptide by K^b at their cell surface. Furthermore, we found that the proteasome strongly contributed to the general surface display of peptide/MHC-I

in TAP-deficient cells. This multicatalytic protease is even the dominant provider of this alternative peptide repertoire. Our results suggest that MHC-I loading of the TEIPP epitope occurred in the vesicular compartments associated with the cellular process of autophagy. We speculate that the majority of TAP-independent peptides are proteasome-mediated peptides that might gain access to MHC-I loading compartments in the vesicular pathway.

I RESULTS

TAP-independent presentation of a novel T cell peptide-epitope

We wanted to unravel the intracellular presentation pathway of a peptide-epitope that we previously discovered in a mouse tumor model¹⁷. The peptide is presented by the classical MHC class I molecule K^b on several TAP-deficient tumor cells, including lymphoma, fibrosarcoma and melanoma, and is targeted by a CTL clone (mi3-CTL). Here, we studied the presentation of this mi3 peptide by non-transformed cells isolated from TAP1-knockout and wild-type mice. LPS-stimulated B-cell blasts and fresh B-cells from the spleen were prepared and incubated with mi3-CTL to test for recognition. The mi3 antigen was presented by these TAP-deficient targets, and the level of presentation by the activated blasts was comparable to that of TAP2-deficient lymphoma line RMA-S (Fig. 1). The TAP-positive targets from the wild-type mice, did not present the mi3 antigen. These results showed that the mi3 antigen is presented in tumor as well as freshly isolated non-transformed cells as long as these cells display deficiency in TAP function. We speculate that normal cells did not present the mi3 antigen due to competition by the conventional proteasome-TAP peptide

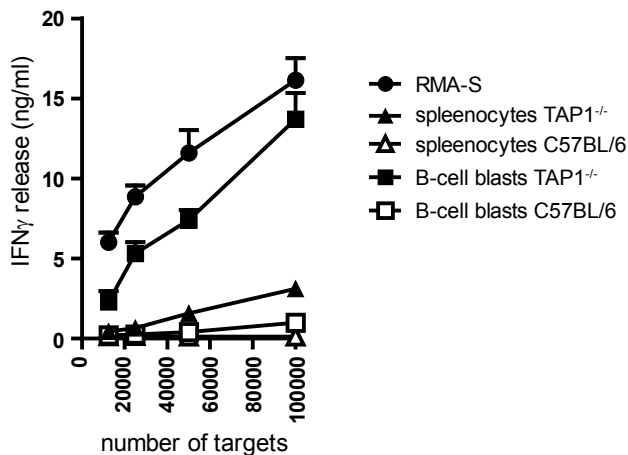


Figure 1. The TEIPP peptide mi3 antigen is presented by TAP-deficient targets. Presentation of the T-cell-epitope mi3 was evaluated by mi3-specific CTL. Activation of CTL was measured by IFN- γ release upon incubation with targets at different concentrations. Targets were composed by TAP2-deficient RMA-S tumor cells, freshly isolated B-cells and LPS-induced B-cell blasts from TAP1-deficient and wild-type mice as control. Mean and standard deviation of triplicates are shown from one out of two experiments.

repertoire, as previously reported for another TAP-independent peptide¹⁸. These data show that the mi3 antigen represents the category of TEIPP antigens in that it originates from a 'self' protein and is selectively presented by cells with processing defects.

mi3 antigen is a H-2K^b-bound peptide

Next, we aimed to identify the molecular nature of the mi3 antigen. Therefore, MHC-peptide complexes of mi3-positive RMA-S cells were purified by affinity chromatography using anti-Kb antibodies bound to sepharose beads. The peptide species were purified from heavy chains through 10kDa filters and eluted peptides were separated in fractions by SCX cation exchange reversed phase high-performance liquid chromatography (rp-HPLC) columns based on charge. Samples from the fractions were taken and tested for the presence of the mi3 antigen by mi3-CTL. One HPLC fraction was strongly recognized to comparable extent as an agonistic mimotope peptide SLGHQFAPV. This synthetic mimotope was identified through synthetic peptide library screen, but is most likely not related to the natural mi3 antigen (Fig 2). Therefore we concluded that the mi3 antigen is a peptide and successfully extracted from Kb molecules of RMA-S cells. The eluate samples will be submitted to further biochemical analysis by tandem mass spectrometry to identify the natural peptide sequence of this antigen.

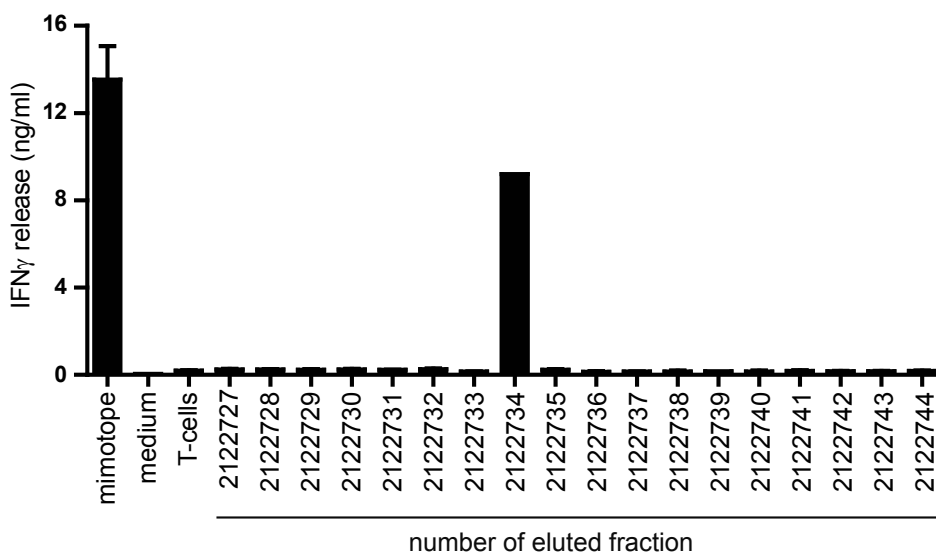


Figure 2. Eluted peptides from RMA-S cells were tested for presence of the mi3 antigen. K^b-peptide complexes were purified from RMA-S cells. Peptides were eluted from the heavy chains and separated by HPLC in fractions based on charge. Samples from the several fractions were taken and loaded on T2.K^b cells to test for the presence of the mi3 antigen using mi3-specific CTL. CTL activation was measured by IFN- γ release. An agonistic peptide that functions as a stimulator or mi3-CTL activity was used as a positive control. Mi3-CTL activity was detected against one of the peptide pools indicating presence of mi3 antigen in this pool. Mean and standard deviation of triplicates are shown from one out of two experiments.

The proteasome is involved in processing of the TAP-independent mi3 antigen

We were interested in the intracellular processing pathway of the TAP-independent mi3 antigen and investigated which family of proteases was involved in the generation of this epitope. RMA-S cells were incubated with an array of chemical inhibitors to block the activity of different classes of proteases in a peptide/MHC-I recovery assay. For this, cells were submitted to mild acid treatment to strip surface peptide/MHC-I complexes and then incubated with inhibitors during the time of peptide/MHC-I re-appearance at the cell surface (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press). Pathways thus far known for presentation of TAP-independent peptides include the signal sequence-derived peptides processed by signal peptidase (SP) and the C-terminal liberation by furin in the Golgi. However, the furin inhibitor did not affect mi3 presentation and the SP inhibitor even led to an increase of mi3 presentation (Fig 3A). We recently discovered a third way of TAP-independent peptide presentation, involving the SPP-mediated cleavage of C-termini in the ER (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press), but the SPP inhibitor ((z-LL)₂-ketone) did not have an inhibitory effect on mi3 presentation. We therefore concluded that the processing pathway of mi3 is distinct from all other previously described TAP-independent pathway.

Out of a list of fourteen inhibitors, bestatin decreased mi3-CTL recognition by ~40% (Fig. 3B). This broad inhibitor of aminopeptidases like the cytosolic leucine aminopeptidase and the secretory-vesicle associated aminopeptidase B¹⁹, was only effective at relative high concentrations. The highest tolerated dose of bestatin (200μM) induced approximately 60% of inhibition (Fig. 3B). To test if these results were due to cellular toxicity, treated RMA-S cells were incubated with control CTL that recognize another TEIPP antigen. Recognition by control CTL was unaltered indicating no toxicity that could compromise overall MHC-I peptide presentation. These results showed a moderate inhibitory effect of bestatin, suggesting an indirect role for aminopeptidases or that bestatin was cross-reactive to other enzymes.

Strikingly, the proteasome inhibitor epoxomicin was capable of efficient blocking of mi3 presentation (Fig. 3A). A dose titration of epoxomicin showed that a low dose of 40 nM still induced 60% inhibition (Fig. 3C). The presentation of the SPP-dependent Trh4 control peptide was even increased by treatment with epoxomicin at all concentrations tested, indicating that mi3 blocking was not due to toxicity of the target cells. This increase of Trh4 peptide presentation was previously reported by our group and was explained by the removal of competitor peptides from the convention proteasome-TAP pathway (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press). We concluded that the TAP-independent mi3 epitope needs proteasome cleavage for its processing and MHC-I presentation.

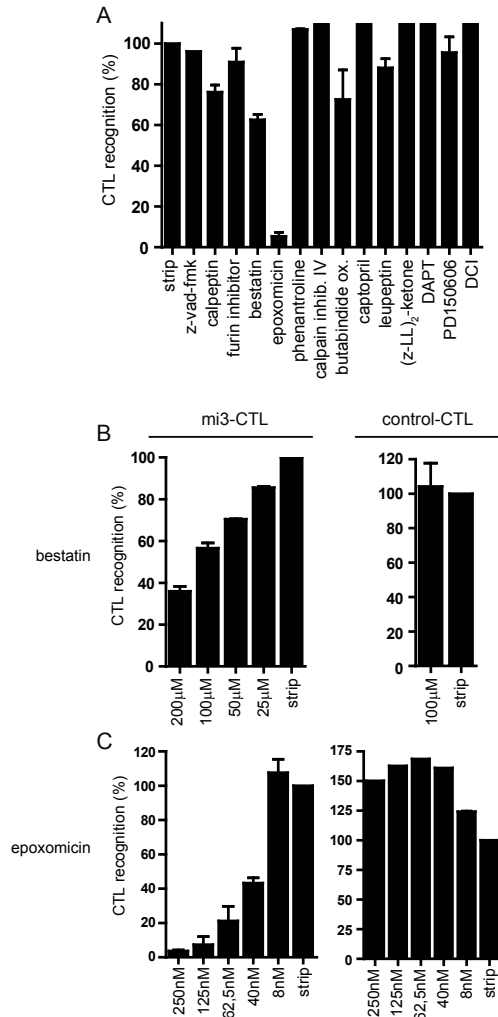


Figure 3. Identification of the processing pathway that leads to presentation of the mi3 antigen.

(A) The presentation of the mi3 antigen in RMA-S cells was tested in the presence of an array of protease inhibitors. RMA-S cells were briefly resuspended in mild acid buffer to disrupt surface MHC-I complexes and incubated with protease inhibitors. Re-emergence of peptide/MHC-I complexes at the cell surface occurred during this time (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press). Cells were then incubated with mi3-specific CTL to test for the presence of K^b-mi3 complexes. IFN- γ production by CTL was determined by intracellular cytokine staining. Inhibitors and concentrations used in the assay: epoxomicin (0.5 μ M), 3,4-Dichloroisocoumarin (5 μ M), Decanoyl-RVKR-CMK (100 μ M), calpeptin (60 μ M), calpain inhibitor IV (20 μ M), PD150606 (200 μ M), butabindide oxalate (400 μ M), captopril (100 μ M), leupeptin (200 μ M), (z-LL)₂-ketone (5 μ M), 1,10-Phenanthroline monohydrate (100 μ M), DAPT (10 μ M), Bestatin hydrochloride (100 μ M), z-vad-fmk (10 μ M). (B-C) Dose-dependent inhibition of mi3 antigen-presentation by the inhibitors bestatin (B) and epoxomicin (C) on RMA-S cells. Mi3-specific CTL was used to test the presentation of the mi3 antigen and control CTL to test for toxicity. Control CTL recognize the TAP-independent Trh4 antigen (MCLRMTAVM) presented by RMA-S cells¹⁸. CTL recognition was determined as described in (A). Data are representative of three independent experiments.

The proteasome is a major participant in peptide presentation of the TAP-independent repertoire

A few TAP-independent processing pathways that participate in MHC-I peptide presentation have been characterized. They comprise peptides generated by furin in the Golgi, the processing of leader sequences from proteins in the ER by SP and the SPP-dependent pathway for ER resident proteins¹¹⁻¹³ (Oliveira *et al.*, "Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation" in press). Although these pathways do not depend on proteasome activity, our data now suggested the existence of additional proteasome-dependent pathway. We wondered what the contribution of these different pathways was for the overall MHC-I peptide presentation in TAP-deficient cells. For that, we incubated cells with several chemical inhibitors of proteases to shut down the different pathways. Cells were treated with a mild acid solution to strip the surface MHC-peptide complexes and incubated with the chemical inhibitors similarly to what was described in figure 3. After the incubation period, cells were stained with specific antibodies for the MHC-I molecules D^b and K^b and analysed by flow cytometry. TAP2-deficient RMA-S lymphoma cells and the TAP1-deficient MCA fibrosarcoma cells were tested and the results were depicted as the inhibition of re-emerging MHC-I molecules at the cell surface in the presence of the inhibitors. The data revealed that the known TAP-independent pathways, based on furin, SP and SPP were active and indeed contributed to the overall MHC-I presentation in these cells but individually they only marginally contributed to MHC-I presentation (Fig 4). The most prevalent inhibitor was epoxomicin, the specific inhibitor for the proteasome. Approximately 50% recovery of the normal MHC-I levels could be blocked with epoxomicin. The K^b molecule was more sensitive than the D^b molecule. We concluded that the proteasome dominantly participate in the formation of the TAP-independent peptide repertoire.

Contribution of other proteases

The MHC-I levels were also firmly reduced with the inhibitor phenanthroline, which is known to block metalloproteases (Fig 4). The activity of metalloproteases has been implicated in the generation of specific CTL epitopes, such as derived from the PRAME tumor antigen and the HIV-1 ENV protein^{12, 20}. The presentation of these nardilysin-cleaved peptides followed a TAP-dependent pathway. However, metalloproteases have also been shown to participate as amino-terminal trimming enzymes in TAP-deficient microsomes^{21, 22}. These results suggested that the activity of phenanthroline-sensitive proteolytic enzymes such as metalloproteases complement the proteasome as dominant players in the overall TAP-independent processing pathway.

Comparison between TAP-positive and TAP-negative cells

We then analyzed if the TAP-independent pathways have different relative contributions in TAP-proficient cells. In TAP-proficient counterparts, the relative contribution of the proteasome was very comparable with that in the TAP-deficient cells (Fig 4). However,

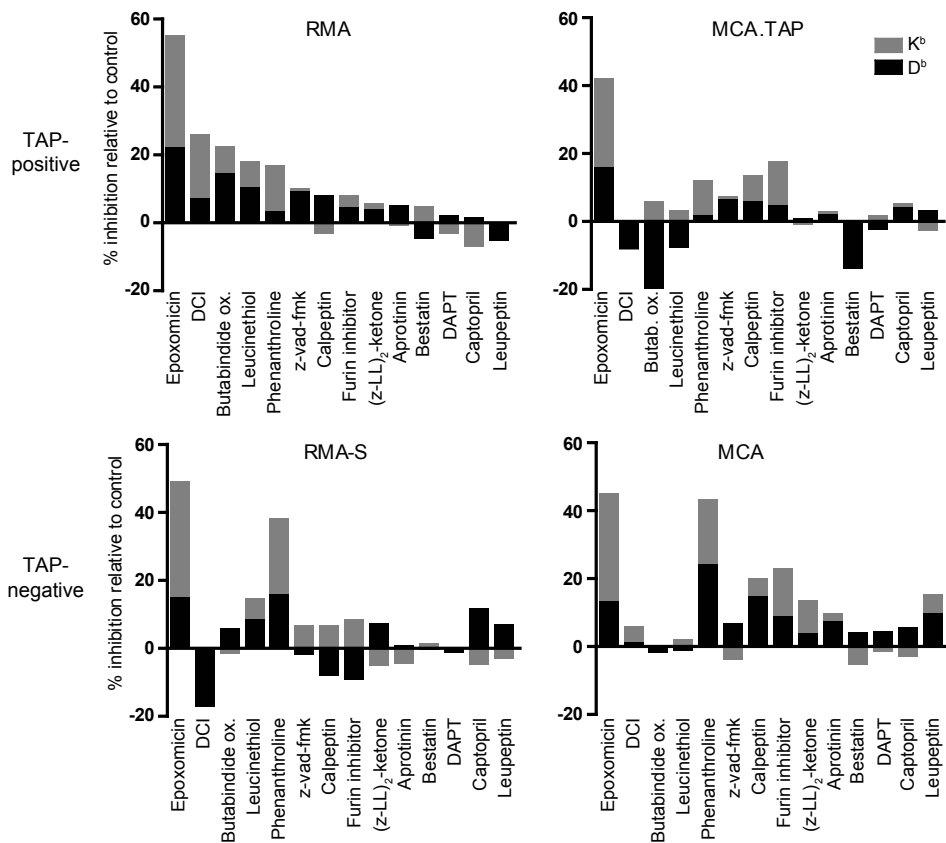


Figure 4. Contribution of different processing pathways for the overall MHC-I peptide presentation in TAP-deficient cells. MHC-I surface expression on stable cell lines was determined by flow cytometry after incubation with protease inhibitors (see procedure in Figure 3A). RMA is a mouse lymphoma cell line and RMA-S is a TAP-deficient variant of RMA. MCA is a mouse TAP1^{-/-} fibrosarcoma and MCA.TAP is TAP-transfectant variant of MCA. The mean fluorescence intensity (MFI) of D^b and K^b molecules in the samples was measured for the untreated sample (positive control), inhibitor-treated samples and brefeldin-A-treated sample (negative control). The effect of the chemical inhibitors in the expression of each MCH-I molecule was determined by calculating the difference between the untreated sample and the inhibitor-treated samples. This was expressed in percentage relative to the negative control. The expression of D^b and K^b was determined with the specific monoclonal antibodies clone 28.14.8S and clone AF6-88.5, respectively, that recognize peptide-bound MHC-I complexes. Protease inhibitors and concentrations used are: epoxomicin (0.4μM), 3,4-Dichloroisocoumarin (5μM), Decanoyl-RVKK-CMK (10μM), calpeptin (30μM), butabindide oxalate (400μM), captopril (100μM), leupeptin (200μM), (z-LL)₂-ketone (5μM), 1,10-Phenanthroline monohydrate (50μM), aprotinin (10μM), DAPT (10μM), Bestatin hydrochloride (200μM), z-vad-fmk (25μM), L-leucinethiol (15μM) and DL-Dithiothreitol (0.5M). Mean and standard deviation of triplicates are shown from one out of three experiments.

the other inhibitors ranked quite differently in the two sets of cells under influence of TAP status, indicating that other processing enzymes and pathways are involved. For instance, the metalloprotease inhibitor phenanthroline was much more dominant in TAP-deficient cells.

Furthermore, the influence of the SP leader peptide pathway seemed to be more pronounced in TAP-positive cells. DCI treatment even led to an increase of D^b molecules on RMA-S cells, suggesting that SP would destroy potential D^b-binding peptides. Interestingly, the furin pathway appeared to be more active in the fibrosarcoma cells than in the lymphomas.

The surprising finding of the major contribution of the proteasome to the MHC-I peptide presentation on TAP-deficient cells prompted us to confirm the TAP status of our cell panel. For that, we performed a TAP activity assay with this cell panel in which peptide transport efficiency is examined and found a normal TAP function in RMA and MCA.TAP cells and a complete loss in the TAP-negative counterparts (Fig 5). This confirmed the expected TAP function and we concluded that the proteasome is the major generator of peptides for the conventional processing pathway as well as for the alternative TAP-independent route.

How do cytosolic peptides get access to MHC-I in the absence of TAP?

We wanted to understand how proteasome-dependent peptides, such as the mi3 antigen, enter MHC-I loading compartments in the absence of TAP. Recently, it has been shown that autophagy promotes the processing of viral antigens for MHC-I presentation. Peptide loading seemed to occur within autophagic vesicles associated with lysosomes (autophagolysosomes)²³. We tested the involvement of the vacuolar pathway in the presentation of the mi3 antigen. For this, RMA-S cells were treated with bafilomycin A1 (BAF-A) which prevents acidification of lysosomes and fusion with autophagosomes. RMA-S cells were incubated with BAF-A in a peptide/MHC-I recovery assay and used as targets for mi3-specific and control CTL. BAF-A consistently led to an increase in antigen presentation of the mi3 epitope, but not of the control SPP-mediated Trh4 TEIPP antigen, both in lymphoma and fibrosarcoma tumor cells (Fig 6A and 6B). This

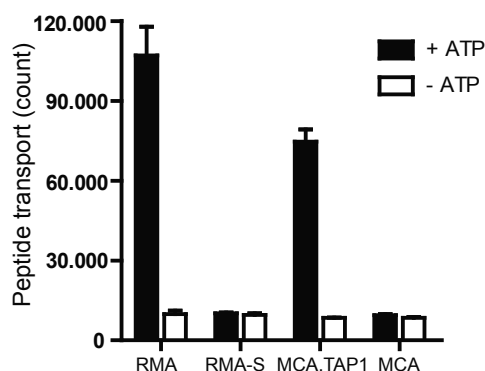


Figure 5. The activity of TAP was determined by the efficiency of peptide transport. Cell lines tested were the tumor cell panel of TAP-positive and TAP-negative cells described in figure 4. Peptide transport was measured in semipermeabilized cells using the fluorescently labeled peptide CVNKTERAY. ATP-dependent TAP transport was measured by fluorescence intensity and peptide transport is expressed as a percentage of translocation, which is relative to the translocation in control cells⁴. Mean and standard deviation of triplicates are shown from one out of two experiments.

suggests that acidification of endolysosomes is detrimental for the mi3 antigen, possibly by mediating acid-dependent enzymatic destruction of the epitope. Importantly, it also indicates that the mi3 antigen has access to vesicular compartments.

We asked the question of how the proteasome-liberated mi3 peptide gets access to the vesicular compartments. Cytoplasmic content, such as organelles and proteins, is compartmentalized in vesicles during the process of autophagy that transport it to lysosomal-mediated destruction. We hypothesized that the mi3 antigen or peptide precursors could be captured into autophagosomes after liberation by the proteasome. To test this, we treated RMA-S cells with a compound that inhibits the formation of autophagic vesicles, 3-methyladenine (3-MA), and incubated these with mi3-specific. The treatment with 3-MA induced approximately 50% inhibition of mi3 antigen presentation (Fig 6B). This result suggests that autophagy is involved in the presentation of the mi3 antigen that might provide access to MHC-I molecules. In conclusion, the mi3 antigen is presented by a proteasome- and TAP-independent pathway that might use the vesicular endocytic pathway for MHC-I antigen presentation.

I DISCUSSION

The currently known pathways of TAP-independent peptide processing involve leader peptides, which are liberated by SPase in the ER, and peptides liberated by furin, which cleaves C-terminal peptides in the trans-Golgi system^{11-13, 24}. We recently identified an additional pathway for C-terminal peptides of ER resident proteins and the SPP enzyme

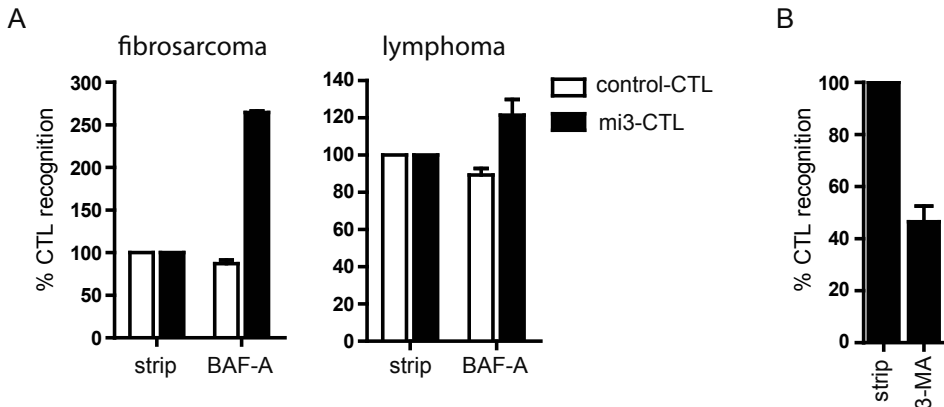


Figure 6. Involvement of the vacuolar pathway related to autophagy for the presentation of mi3 antigen. (A) RMA-S (lymphoma) and MCA (fibrosarcoma) cells were treated with bafilomycin (BAF-A) in peptide/MHC-I recovery assays performed as described in figure 3. These cells were then incubated with mi3-specific CTL or control CTL. Control CTL recognize the TAP-independent Trh4 antigen (MCLRMTAVM) presented by RMA-S cells¹⁸. CTL recognition was measured as described in figure 3. (B) RMA-S cells were treated with autophagy inhibitor 3-methyladenine (3-MA) and incubated with mi3-specific CTL. Inhibitors and concentrations used in the assay: 3-MA (5mM), BAF-A (200nM). Mean and standard deviation of triplicates are shown from one out of three independent experiments.

was responsible for the liberation of these peptides (Oliveira *et al.*, “Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation” in press). We here describe the intracellular processing of a T-cell epitope that requires proteasomal activity. This antigen, called mi3, was presented by several TAP-deficient cells, both from tumor origin or from primary tissues. These proteasome-mediated peptides strikingly gain access to MHC-I loading machinery in a TAP-independent fashion and our experiments indicate that endocytic vesicular compartments are involved. Surprisingly, the majority of the TAP-independent peptide repertoire appears to be generated by the proteasome, since inhibitors for the previously known pathways only marginally blocked the overall surface display of MHC-I.

MHC-I bound peptides from this processing category have been described before. Several epitopes from the LMP2 protein of the Epstein-Barr virus (EBV) are presented by a TAP-independent and proteasome-dependent manner²⁵. The authors found a good correlation between the hydrophobicity index of the peptides and TAP-dependence in that the TAP-independent epitopes had a high hydrophobicity index, proposing a mechanism by which these epitopes are produced in the cytosol and then can traverse the ER membrane to reach MHC-I loading compartments due to their highly hydrophobic characteristics. It remains to be determined if the mi3 antigen has such characteristics, and that will only be solved when the natural sequence is revealed. Moreover, we cannot exclude other possibilities for the access of the mi3 peptide into the ER. For instance, it could be transported into the ER via the Sec61 translocon or via other means²⁴.

Our data showed that the process of autophagy might be involved in the presentation of the mi3 antigen to CTL. During autophagy, large portions of cytoplasmic content, including proteins and organelles, are encapsulated in double membrane vesicles called autophagosomes²⁶⁻²⁹. The autophagosomes then fuse with acidic vesicles such as late endosomes and lysosomes to form the autolysosome. The autolysosomes are terminal digestive compartments where several proteases and lipases breakdown the encapsulated contents into its constituents such as amino-acids and lipids which are exported to the cytoplasm for recycling²⁷. The autophagosomal membrane has been proposed to originate from the ER^{30,31}. In addition, during ER stress and the unfolded-protein response, autophagy is induced and consumes portions of the ER, serving as an alternative to ER-associated degradation of proteins³²⁻³⁴. The MHC-I loading complex might therefore be present in the autophagosome and allow for loading of peptides in these compartments. Also, MHC-I molecules from the cell membrane internalized in endosomes can have contact with autophagosomes and return to the endocytic network. Although there is still much unknown about this, transit for membrane-associated proteins between autophagosomes and endosomes has been observed by live cell imaging³⁵. In fact, autophagy has recently been shown to enhance antigen processing for MHC-I presentation. This recent study demonstrated that the processing of viral glycoprotein B for antigen presentation on MHC-I was facilitated by autophagy in HSV-1 infected murine macrophages, which led to CD8⁺ T-cell activation³⁶. In our results, we

observed that the compound 3-MA partly inhibited presentation of the mi3 antigen. This compound inhibits the action of PI3-kinases which is necessary for the formation of the double membrane of the autophagosome and therefore inhibits the initial steps of autophagy. Another compound, bafilomycin, increased the presentation of the mi3 antigen. This compound inhibits the acidification of endosomes and lysosomes and therefore interferes with the maturation of autophagosomes by inhibiting the fusion with lysosomes³⁷. These two observations suggest that the mi3 antigen enters the autophagic pathway. Possibly, the mi3 antigen is produced in the cytosol by proteasomal activity and then engulfed into autophagosomal vesicles. In these vesicles, the peptide antigen could bind MHC-I molecules that are present in these vesicles and return to the secretory pathway to be presented at cell surface.

The question of how TAP-deficiency promotes the presentation of the mi3 antigen is interesting and might be related to the fact that TAP-deficiency leaves many peptide-receptive MHC-I molecules in the ER³⁸. These might be incorporated in autophagic compartments and sensitive for peptide loading in the endocytic pathway. Trafficking of MHC-I molecules to endolysosomal compartments have been described and the invariant chain seems to facilitate this process³⁹.

We were surprised to observe a strong role for the proteasome in the generation of TAP-independent peptides and our results implies the existence of new route of peptide loading into MHC-I in the vesicular pathway that awaits in-depth investigation.

| MATERIAL AND METHODS

Cell lines and mice

The mouse tumor cell lines RMA, RMA-S (TAP2-deficient), MCA (TAP1-deficient), and human TAP-deficient T2 cells have been described previously¹⁶⁻¹⁸. T2 cells were transfected with H-2K^b gene via retroviral transduction using the LZRS vector containing GFP behind an internal ribosome entry site⁴⁰. The mouse TAP genes were introduced into MCA cells by gene transfer. Generation and culture of CD8⁺ T cell clones used in this study were previously described: K^b-restricted CTL clone mi3 specific for a yet unknown TEIPP peptide, D^b-restricted CTL clone B5 specific for the TAP-independent Trh4 peptide (MCLRMTAVM);^{17,40,41}. All cells were cultured in complete IMDM medium (Invitrogen, Carlsbad, CA) containing 8% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin (Life Technologies, Rockville, MD), 2mM L-glutamine (Invitrogen) and 30 µmol/L of 2-mercaptoethanol (Merck, NJ, USA) at 37°C in humidified air with 5% CO₂.

C57BL/6 mice were purchased from Charles River Laboratories (France) and housed in the animal facility of the Leiden University Medical Center under specified pathogen-free conditions. TAP1-knockout mice were purchased from Jackson laboratories. Mice were used between 8 and 12 weeks of age. Spleens from C57BL/6 and TAP^{-/-} mice were harvested, mechanically disrupted with a cell strainer and depleted of CD3⁺ cells with anti-CD3 MACS beads (Miltenyi Biotec) according to manufacturer's recommendations.

B-cell blasts were obtained by culturing CD3-depleted spleen cells for 3 days with 10 µg/ml LPS (Sigma). Experiments were performed in accordance with national legislations and institutional guidelines and were approved by the local ethical committees.

Protease inhibitor experiments

Experiments were performed as previously described (Oliveira *et al.*, “Signal peptide peptidase liberates immunogenic C-terminal peptides for MHC class-I presentation” in press). Chemical inhibitors used in these assays are: epoxomicin (Sigma-aldrich), 3,4-Dichloroisocoumarin (Sigma-aldrich), Decanoyl-RVKR-CMK (Merck), calpeptin (Merck), calpain inhibitor IV (Merck), PD150606 (Santa Cruz Biothecnology), butabindide oxalate (TOCRIS Bioscience), captopril (Sigma-aldrich), leupeptin (Merck), (z-LL)₂-ketone (Merck), 1,10-Phenanthroline monohydrate (Sigma-aldrich), aprotinin (Merck), DAPT (Sigma-aldrich), Bestatin hydrochloride (Sigma-aldrich), z-vad-fmk (Merck), L-leucinethiol (Sigma-aldrich) and DL-Dithiothreitol (Sigma-aldrich), 3-methyladenine (Merck), bafilomycin-A (Merck). None of the inhibitors was toxic at the applied concentrations, as determined by trypan-blue counting and annexin-V staining.

T-cell activation assays and flow cytometry

Intracellular cytokine staining of IFN-γ in T cell clones was performed for the protease inhibitor screen and expressed as percentage positive cells⁴². IFN-γ secretion by T-cell clones was measured by ELISA as previously published¹⁸. Data shown represent mean values obtained from triplicate test wells and error bars represent standard deviation of these values. Flow cytometry analysis of surface expression of D^b and K^b molecules was performed using directly labeled anti-D^b mAb (clone 28.14.8S; BioLegend, California, USA) and anti-K^b mAb (clone AF6-88.5, BioLegend). Cells were measured using a FACS Calibur and analysed with Flowjo software (Tree Star, Ashland, OR).

Peptide elution and HPLC

Peptides were eluted out of purified K^b molecules from RMA-S cells. Immunoprecipitation of K^b molecules was performed with shepharose beads covalently coupled with anti-K^b mAb (clone AF6-88.5, BioLegend) from two independent lysates of 20.10⁹ RMA-S cells as previously described^{16,41}. The peptide pools were prefractionated on SCX cation exchange columns as previously described¹⁶.

TAP-transport assay

A peptide transport assay was done as described previously^{4, 43}. Briefly, cells were permeabilized and incubated with the fluorescein-conjugated synthetic peptide CVNKTERAY in the presence or absence of 10 mM ATP. Peptide translocation was terminated by adding ice-cold lysis buffer. After the removal of cell debris, isolated glycosylated peptides were incubated with ConA beads. The fluorescence intensity was measured, and peptide transport is expressed as a percentage of translocation, which is relative to the translocation in control cells.

I REFERENCES

1. Cerundolo, V. & de la Salle, H. Description of HLA class I- and CD8-deficient patients: Insights into the function of cytotoxic T lymphocytes and NK cells in host defense. *Semin Immunol* **18**, 330-6 (2006).
2. Weinzierl, A.O. et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur J Immunol* **38**, 1-8 (2008).
3. Lampen, M.H. & van Hall, T. Strategies to counteract MHC-I defects in tumors. *Curr Opin Immunol* **23**, 293-8 (2011).
4. Lampen, M.H. et al. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. *J Immunol* **185**, 6508-17 (2010).
5. van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat Med* **12**, 417-24 (2006).
6. Wolpert, E.Z. et al. Generation of CD8+ T cells specific for transporter associated with antigen processing deficient cells. *Proc Natl Acad Sci U S A* **94**, 11496-501 (1997).
7. Oliveira, C.C. & van Hall, T. Importance of TAP-independent processing pathways. *Mol Immunol* **55**, 113-6 (2013).
8. Glas, R., Bogoy, M., McMaster, J.S., Gaczynska, M. & Ploegh, H.L. A proteolytic system that compensates for loss of proteasome function. *Nature* **392**, 618-22 (1998).
9. Henderson, R.A. et al. HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science* **255**, 1264-1266 (1992).
10. Wei, M.L. & Cresswell, P. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* **356**, 443-446 (1992).
11. Martoglio, B. & Dobberstein, B. Signal sequences: more than just greasy peptides. *Trends Cell Biol* **8**, 410-5 (1998).
12. Gil-Torregrosa, B.C., Castano, A.R., Lopez, D. & Del Val, M. Generation of MHC class I peptide antigens by protein processing in the secretory route by furin. *Traffic* **1**, 641-51 (2000).
13. Gil-Torregrosa, B.C., Raul Castano, A. & Del Val, M. Major histocompatibility complex class I viral antigen processing in the secretory pathway defined by the trans-Golgi network protease furin. *J Exp Med* **188**, 1105-16 (1998).
14. Leonhardt, R.M. et al. Post-endoplasmic reticulum rescue of unstable MHC class I requires proprotein convertase PC7. *J Immunol* **184**, 2985-98 (2010).
15. Tiwari, N. et al. A transporter associated with antigen-processing independent vacuolar pathway for the MHC class I-mediated presentation of endogenous transmembrane proteins. *J Immunol* **178**, 7932-42 (2007).
16. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-21 (2010).
17. Van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Medicine* **12**, 417-424 (2006).
18. Oliveira, C.C. et al. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. *Eur J Immunol* **41**, 3114-24 (2011).
19. Hwang, S.R., O'Neill, A., Bark, S., Foulon, T. & Hook, V. Secretory vesicle aminopeptidase B related to neuropeptide processing: molecular identification and subcellular localization to enkephalin- and NPY-containing chromaffin granules. *J Neurochem* **100**, 1340-50 (2007).
20. Kessler, J.H. et al. Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes. *Nat Immunol* **12**, 45-53 (2011).
21. Fruci, D., Niedermann, G., Butler, R.H. & van Endert, P.M. Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity* **15**, 467-76 (2001).
22. Saric, T., Muller, D., Seitz, H.J. & Pavelic, K. Non-covalent interaction of ubiquitin with insulin-degrading enzyme. *Mol Cell Endocrinol* **204**, 11-20 (2003).
23. Tey, S.K. & Khanna, R. Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway. *Blood* **120**, 994-1004 (2012).
24. Del Val, M., Iborra, S., Ramos, M. & Lazaro, S. Generation of MHC class I ligands in the secretory and vesicular pathways. *Cell Mol Life Sci* **68**, 1543-52 (2011).
25. Lautscham, G. et al. Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell recognition reveals a proteasome-dependent, transporter associated with antigen processing-independent pathway. *J Exp Med* **194**, 1053-68 (2001).
26. Crotzer, V.L. & Blum, J.S. Autophagy and adaptive immunity. *Immunology* **131**, 9-17 (2010).
27. Kim, H.J., Lee, S. & Jung, J.U. When autophagy meets viruses: a double-edged sword with

- functions in defense and offense. *Semin Immunopathol* **32**, 323-41 (2010).
28. Levine, B. & Deretic, V. Unveiling the roles of autophagy in innate and adaptive immunity. *Nat Rev Immunol* **7**, 767-77 (2007).
 29. Munz, C. Autophagy and antigen presentation. *Cell Microbiol* **8**, 891-8 (2006).
 30. Mijaljica, D., Prescott, M. & Devenish, R.J. Endoplasmic reticulum and Golgi complex: Contributions to, and turnover by, autophagy. *Traffic* **7**, 1590-5 (2006).
 31. Yorimitsu, T. & Klionsky, D.J. Eating the endoplasmic reticulum: quality control by autophagy. *Trends Cell Biol* **17**, 279-85 (2007).
 32. Bernales, S., McDonald, K.L. & Walter, P. Autophagy counterbalances endoplasmic reticulum expansion during the unfolded protein response. *PLoS Biol* **4**, e423 (2006).
 33. Ogata, M. et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol* **26**, 9220-31 (2006).
 34. Yorimitsu, T., Nair, U., Yang, Z. & Klionsky, D.J. Endoplasmic reticulum stress triggers autophagy. *J Biol Chem* **281**, 30299-304 (2006).
 35. Jahreiss, L., Menzies, F.M. & Rubinsztein, D.C. The itinerary of autophagosomes: from peripheral formation to kiss-and-run fusion with lysosomes. *Traffic* **9**, 574-87 (2008).
 36. English, L. et al. Autophagy enhances the presentation of endogenous viral antigens on MHC class I molecules during HSV-1 infection. *Nat Immunol* **10**, 480-7 (2009).
 37. Tumbarello, D.A. et al. Autophagy receptors link myosin VI to autophagosomes to mediate Tom1-dependent autophagosome maturation and fusion with the lysosome. *Nat Cell Biol* **14**, 1024-35 (2012).
 38. Day, P.M., Esquivel, F., Lukszo, J., Bennink, J.R. & Yewdell, J.W. Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity* **2**, 137-47 (1995).
 39. Basha, G. et al. A CD74-dependent MHC class I endolysosomal cross-presentation pathway. *Nat Immunol* **13**, 237-45 (2012).
 40. Van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b. *J Immunol* **178**, 657-62 (2007).
 41. Van Hall, T. et al. Differential influence on cytotoxic T-lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J. Exp. Med.* **192**, 483-494 (2000).
 42. van Stipdonk, M.J. et al. Design of agonistic altered peptides for the robust induction of CTL directed towards H-2Db in complex with the melanoma-associated epitope gp100. *Cancer Res* **69**, 7784-92 (2009).
 43. van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b1. *J Immunol* **178**, 657-62 (2007).

5

THE NONPOLYMORPHIC MHC Qa-1^b MEDIATES CD8⁺ T CELL SURVEILLANCE OF ANTIGEN-PROCESSING DEFECTS

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| ABSTRACT

The nonclassical MHC Qa-1^b accommodates monomorphic leader peptides and functions as a ligand for germ line receptors CD94/NKG2, expressed by natural killer cells and CD8⁺ T cells. We here describe that the conserved peptides are replaced by a novel peptide repertoire of surprising diversity as a result of impairments in the antigen processing pathway. This novel peptide repertoire represents immunogenic neo-antigens for CD8⁺ T cells as we found that these Qa-1^b-restricted T cells dominantly participated in the response to tumors with processing deficiencies. A surprisingly wide spectrum of target cells, irrespective of transformation status, MHC background or type of processing deficiency was recognized by this T cell subset, complying with the conserved nature of Qa-1^b. Target cell recognition depended on T cell receptor and Qa-1^b interaction and immunization with identified peptide-epitopes demonstrated in vivo priming of CD8⁺ T cells. Our data reveal that Qa-1^b, and most likely its human homologue HLA-E, is important for the defense against processing deficient cells by displacing the monomorphic leader peptides, which relieves the inhibition through CD94/NKG2A on lymphocytes and by presenting a novel repertoire of immunogenic peptides, which recruits a subset of cytotoxic CD8⁺ T cells.

I INTRODUCTION

Qa-1^b and its human homolog HLA-E are conserved MHC class I like molecules, often categorized as ‘nonclassical’ or class Ib MHC. Similar to the classical MHC molecules, they accommodate small peptides in their binding grooves and present these on the cell surface^{1,2}. Crystal structures of HLA-E show that the overall fold of the peptide/MHC complex is very similar to that of the classical MHC class I molecules³. The unique feature of Qa-1^b and HLA-resides in the very limited number of alleles that is present in the population^{4,5}: as few as two alleles exist in the human population differing just in one amino acid and this has hardly any functional consequence⁶. The monomorphic nature of Qa-1^b and HLA-E is underlined by the finding that they are predominantly filled with one peptide ligand, which is derived from the signal sequence of classical MHC class I molecules, often referred to as ‘Qdm’ for ‘Qa-1 determinant modifier’^{7,8}. These signal peptides are strikingly conserved in all tested mammalian species⁹, pointing at an important function of this peptide/MHC combination for the immune system. A substantial body of evidence reveals that it regulates natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) via the heterodimeric receptors CD94/NKG2A and CD94/NKG2C^{2,10,11}. These germline-encoded receptors specifically engage the Qdm peptide when bound to Qa-1^b/HLA-E, since residues at peptide position 5 and 8 are part of the surface interface of the CD94/NKG2 receptors^{12,13}. Interestingly, this interaction is quite similar to the footprint of the hyper variable regions of the T cell receptor on peptide/MHC class I¹⁴. Absence of these Qdm side chain residues results in lack of receptor engagement^{15,16}, indicating that this innate receptor is truly peptide specific. Qdm/Qa-1^b complexes act as remote sensors of integrity of the MHC class I antigen processing machinery, because defects in proteasomal cleavage, TAP-mediated peptide transport, signal peptidases or tapasin-mediated peptide loading all result in failure to process and present Qdm peptides¹⁷⁻²⁰. The lack of Qdm/Qa-1^b complexes at the cell surface is sensed by NK cells expressing CD94/NKG2 receptors, and allows for immunosurveillance of such processing deficient cells²¹. Thus, this receptor/ligand system is a major molecular mechanism behind the ‘missing self’ principle of NK cell reactivity²².

In addition to their role in the innate immune system, Qa-1^b and HLA-E serve the adaptive response as well. T-cell receptor recognition of these nonclassical MHC has been described in the context of immunity against intracellular pathogens, e.g. *Listeria*, *Salmonella* and *Mycobacterium tuberculosis*²³⁻²⁵, suggesting that foreign antigens can replace the ‘self’ Qdm peptides and be recognized by CD8⁺ T cells. HLA-E-restricted T cells specific for Qdm do exist, but have only been reported in response to allogeneic MHC^{26,27}. Furthermore, Qa-1^b-restricted CD8⁺ T cells have been implicated in the regulation of experimental autoimmune diseases (reviewed in ^{21,28}). The antigenic targets of these regulatory T cells are most likely of ‘self’ origin, but different from Qdm. These data reveal that Qa-1^b/HLA-E-restricted T cells are present in the host, but that the specificity, diversity and function of this T-cell subset await further investigation.

Here we describe the existence of a surprisingly broad peptide repertoire that is presented by Qa-1^b on cells with impairments in the antigen processing machinery. These

peptides replace Qdm and are targeted by a unique population of CD8⁺ cytotoxic T cells. Normal cells with intact processing machinery were not recognized by these Qa-1^b-restricted CTL, but partial defects readily resulted in the appearance of the immunogenic 'self' peptides, which are derived from housekeeping proteins. Interestingly, we show that these Qa-1^b-restricted T cells are abundantly present in the immune response to processing deficient tumors. Our data show that the nonclassical Qa-1^b molecule plays a prominent role in the adaptive immune response as a restriction element for T cells and presents a much larger peptide repertoire than thus far anticipated.

I RESULTS

Qa-1^b-restricted T cells are frequently present in the immune response against TAP-deficient tumors

We previously described that the immune response against TAP-deficient tumors comprises Qa-1^b-restricted T cells among T cells with MHC restriction to the classical class I molecules²⁹. This prompted us to determine the relative contribution of this Qa-1^b-restricted subset to the overall response. Mice were immunized with syngeneic TAP-deficient RMA-S cells expressing the co-stimulatory molecule CD80. *Ex vivo* cultured spleens were examined for IFN γ production against the parental RMA-S lymphoma, the β 2m-negative C4.4-25 line and a panel of single class I expressing EC7.1 cells, which are MHC class I-loss variants of RMA-S³⁰ (Table I). All polyclonal *ex vivo* cultures responded to RMA-S, but not against the β 2m-negative lymphoma, indicating that they exhibited specific T-cell reactivity against TAP-deficient tumors, and not merely reflected natural killer (NK) cell reactivity. Dissection of these IFN γ responses revealed a co-dominance of Qa-1^b-restricted activity compared to those restricted by the classical D^b- and K^b-molecules, as the majority of the examined twelve independent cultures produced high IFN γ levels when incubated with EC7.1.Qa-1^b (Table I). These data suggested that the

Table I. Detection of Qa-1^b-directed IFN γ responses in *ex vivo* cultures.

Target cells ^a	Independent T-cell culture ^b											
	# 1	# 2	# 3	# 4	# 5	# 6	# 7	# 8	# 9	# 10	# 11	# 12
RMA-S	5,723 ^c	10,113	1,204	6,582	12,199	4,896	2,894	10,389	1,359	11,593	4,320	1,567
C4.4-25	97	84	92	82	164	61	23	18	106	61	47	14
EC7.1	364	712	310	216	1,311	799	273	81	485	564	418	38
EC7.1.K ^b	5,886	11,270	538	7,184	16,031	2,526	441	8,309	405	11,111	1,552	837
EC7.1.D ^b	888	7,507	645	690	10,113	2,125	1,129	604	648	8,314	1,561	446
EC7.1.Qa-1 ^b	2,053	5,579	3,330	6,677	16,265	3,108	2,999	272	1,410	8,827	2,697	7,039
EC7.1.control	380	700	386	250	1,370	1,065	418	63	591	620	401	56
Medium	287	120	287	152	276	156	177	38	218	110	74	13

^a RMA-S is TAP2-deficient; C4.4-25 is β 2m-deficient; EC7.1 is a MHC class I-deficient variant of RMA-S. Single class I genes were reconstituted in EC7.1.

^b Twelve mice were immunized with RMA-S.B7 and spleens were stimulated twice *in vitro* with RMA-S.B7 before testing.

^c Concentration of IFN γ released by *ex vivo* cultures (pg/ml)

nonclassical MHC class I Qa-1^b frequently acts as an antigen presentation molecule for T cells, and even can overrule the classical MHC class I molecules.

Critical involvement of Qa-1^b and the T-cell receptor in target cell recognition

To characterize these Qa-1^b-restricted T cells, we isolated several T-cell clones from the aforementioned cultures and extensively explored the expression and function of surface receptors. All clones displayed a classical CTL phenotype and expressed CD3, CD8αβ and TCRαβ (Fig S1). Different rearranged TCR Vβ segments were used (not shown), validating the independent origin of the clones and indicating that Qa-1^b recognition does not constrain the TCR repertoire. In addition to the T-cell lineage receptors, the T-cell clones also expressed CD94, NKG2A, NKG2C and NKG2D, receptors frequently found on Natural Killer (NK) cells, which interact with nonclassical MHC class I molecules, including Qa-1^b. Importantly, NK lineage markers NK1.1 and DX5, and CD16, CD32, CD244 (2B4) were not expressed, neither were receptors of the lectin Ly49 family, which interact with classical MHC class I (Fig S1 and data not shown). We concluded that our Qa-1^b-restricted T cells are indistinguishable at the phenotypic level from 'conventional' CD8⁺ T-cells restricted by classical MHC³¹, but are clearly distinct from the reported Qa-1-rerecognizing intestinal γδ⁺ T cells³² and Qa-1-restricted regulatory CD8αα⁺ T-cell subset³³.

To carefully determine the Qa-1^b-restriction of our isolated T-cell clones, we examined their reactivity against two cell panels, based on the TAP-negative EC7.1 lymphoma and TAP-deficient B78H1 melanoma^{30, 34} (Fig 1). Both cell lines are also devoid of MHC class I proteins and single class I molecules were reconstituted by gene transfer. All isolated T-cell clones exhibited cytolytic activity (Fig 1A) and produced IFNγ (Fig 1B and Fig S2) against the Qa-1^b-expressing cells, but not against cells in which only D^b or K^b were introduced. These data convincingly demonstrated the Qa-1^b-restriction of our T cells and excluded cross-reactivity to the classical class I molecules. Qdm-specific control CTL did not recognize the TAP-deficient tumor targets, since the presentation of Qdm in Qa-1^b is TAP-dependent⁷. Exogenous loading of Qdm conferred reactivity for this CTL clone (Fig 1, right panels). A direct interaction with Qa-1^b was revealed by the finding that anti-Qa-1^b antibody strongly blocked the recognition by our CTL (Fig 2A).

Next, we examined the involvement of antigen-receptors in target cell recognition. Although CD94/NKG2A and CD94/NKG2C do engage Qa-1^{b2} and were expressed by our isolated T-cell clones, their involvement was not likely, because TAP-negative cells are devoid of peptide ligands for these receptors³⁵. Indeed, blocking with NKG2A/C-specific antibodies did not alter the response, whereas anti-CD3 and -CD8 antibodies clearly inhibited the recognition of EC7.1.Qa-1^b cells (Fig 2B). A role for NKG2D was also not likely, since our RMA-based lymphoma panel lacks NKG2D ligands³⁶. Together these data indicated that the TCR governed the reactivity of our Qa-1^b-restricted CTL.

Furthermore, exogenous loading with excessive amounts of competing Qdm peptides inhibited T-cell recognition of EC7.1.Qa-1^b cells (Fig 2C). Similar competition was found when the Qdm variant L8K was used (AMAPRTLKL), but not with irrelevant control peptide

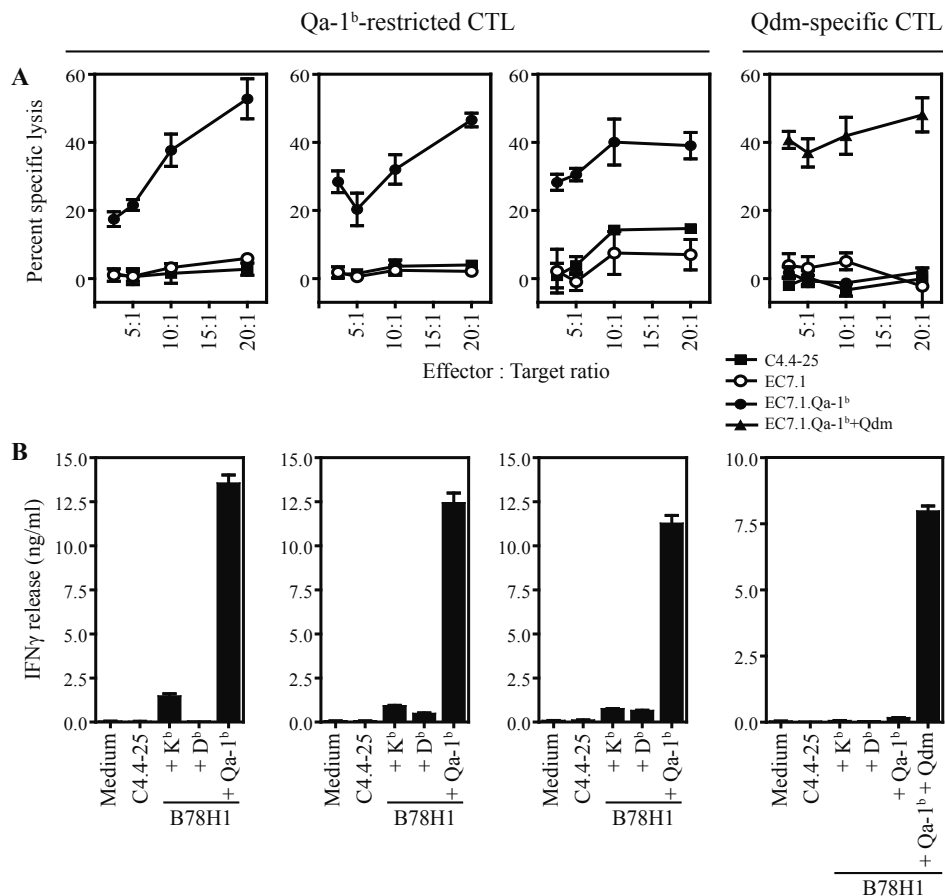


Figure 1. Selective recognition of Qa-1^b-expressing target cells. Isolated T-cell clones were tested against panels of TAP-deficient lymphoma (EC7.1) and melanoma (B78H1) cells. Both cell lines are also deficient in MHC class I^{30,34} and were reconstituted with constructs encoding H-2D^b, -K^b or Qa-1^b. (A) Cytotoxic activity against Qa-1^b-expressing EC7.1 cells and (B) IFNγ production against Qa-1^b-expressing B78H1 cells by three independent T-cell clones. Control Qdm-specific CTL failed to recognize the Qa-1^b expressing targets, due to the absence of TAP, unless pulsed with the Qdm peptide (right panels). Means and standard deviations of triplicate wells are shown for one out of three comparable experiments.

(Fig 2C, left panel). Qdm-specific control CTL confirmed that both peptides are efficiently loaded on the target cells (Fig 2C, right panel). The fact that the position 8 substitution in the peptide L8K prevents engagement to CD94/NKG2A receptors³⁷, but retained the capacity to inhibit T-cell reactivity in our assays, substantiated our findings that target cell recognition is regulated by TCR-mediated interaction with Qa-1^b and not by NK-receptors.

TAP- and MHC heavy chain-deficiencies are targeted by Qa-1^b-restricted CTL

To evaluate the appearance of the peptide-epitopes on target cells that express natural levels of Qa-1^b, we tested B-cell blasts from spleens of wild type, TAP1-deficient and

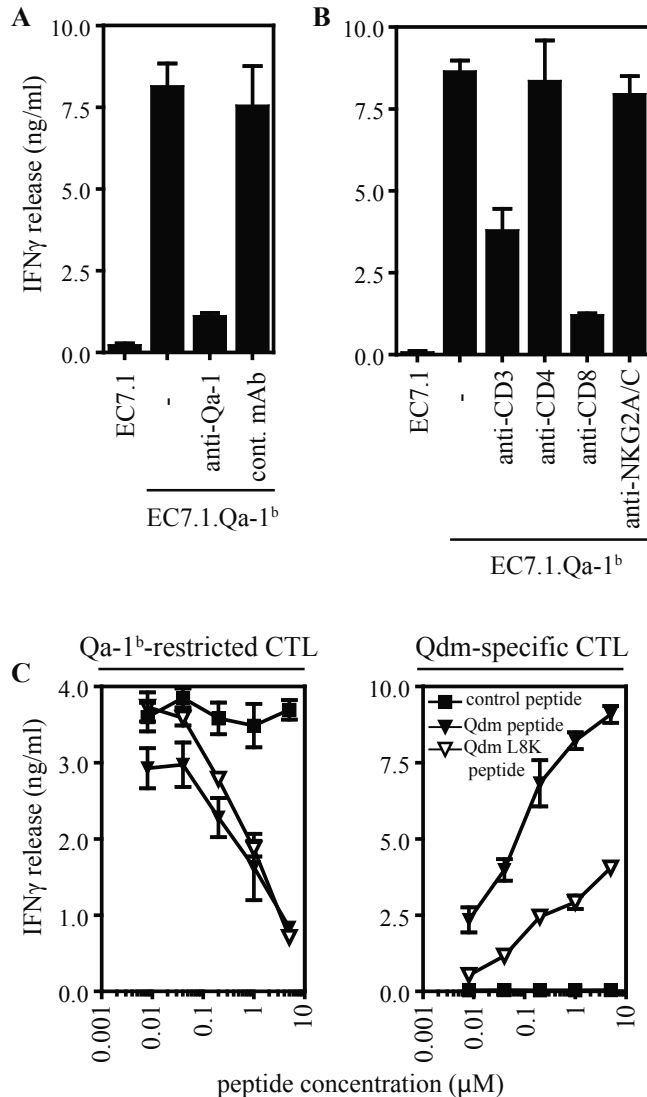


Figure 2. The T-cell receptor, but not NKG2A/C, mediates reactivity of the T cells. (A) Blocking antibody against Qa-1^b demonstrated a direct role of this molecule for T-cell recognition. Experiment was performed using EC7.1.Qa-1^b target cells. (B) Blocking CD3 or CD8 with monoclonal antibodies decreased T-cell reactivity against TAP-deficient EC7.1.Qa-1^b target cells. Antibodies against NKG2A/C did not alter the T-cell response, indicating that only the T-cell receptor is critically involved in mediating reactivity. (C) Exogenous peptide loading competes with endogenously presented epitopes on EC7.1.Qa-1^b cells and inhibits the recognition of these target cells (left panel). Qdm (AMAPRTLTL) or Qdm L8K (AMAPRTLKL) peptides were loaded exogenously at the indicated concentrations on EC7.1.Qa-1^b cells and IFN γ release by CTL was measured. Control peptide was the K^b-binding 8-mer SIINFELK from OVA. The Qdm L8K mutant peptide/Qa-1^b complexes fail to interact with CD94/NKG2A or CD94/NKG2C³⁷, indicating that the loaded peptides interfere with T-cell receptor mediated reactivity. Qdm-specific control CTL (right panel) was activated by the peptides. Means and standard deviations of triplicate wells are shown for one out of three comparable experiments.

β 2m-deficient mice as targets for three independently derived CTL clones (Fig 3A). TAP1-deficient cells were efficiently recognized by all three clones, but wild type and β 2m-deficient cells were not. Qdm-specific control CTL selectively responded to wild type cells, indicating that the absence of Qdm peptides due to the TAP defect promoted the presentation of the new peptide-epitopes. Qa-1^b surface levels on TAP1-deficient cells were lower than on wild type cells, but were clearly higher than the background levels on β 2m-deficient cells (not shown).

We also analyzed CTL reactivity against MHC class I heavy chain deficiencies with the use of B-cell blasts from H-2D^b- and K^b-knockout mice. Qdm peptides are derived from the leader sequence of H-2D^b and loss of this allele resulted in recognition by two of the three CTL clones, whereas deficiency in K^b heavy chains did not result in recognition (Fig 3B). These subtle differences between the CTL clones suggested that the cognate peptides are differentially influenced by loss of MHC class I heavy chains. Strikingly, all three CTL clones strongly responded to cells devoid of both alleles (H-2D^bK^b-knockout) (Fig 3B). Control blasts lacking β 2m in addition to H-2D^bK^b (triple knockout) were not recognized. These results revealed an unexpected influence of the K^b molecule on the Qa-1^b-presented antigenic peptides, in that removal of K^b molecules from H-2D^b-deficient cells strongly enhanced epitope display (Fig 3B). The underlying mechanism of this effect is currently elusive. The degree of CTL response to H-2D^bK^b-knockout cells was comparable to TAP-deficient cells (Fig 3A), indicating that both defects led to optimal presentation of the new Qa-1^b peptides. Interestingly, Qdm-specific CTL were able to differentiate between these two target populations, confirming previous findings that this CTL clone is also reactive to TAP-dependent peptides other than Qdm^{35, 38}. Together, these results demonstrated that our Qa-1^b-restricted CTL interact with peptides on activated B-cells with defects in the antigen presentation route.

CTL reactivity against tumors with partial processing impairments

We then determined CTL reactivity against tumor cells with TAP-dysfunction and tumor cells with undefined deficiencies in the processing machinery. Ad5-transformed Mouse Embryo Cells (Ad5MEC) and chemically-induced fibrosarcoma cells (MCA), both derived from TAP1-knockout mice, were recognized by the Qa-1^b-restricted CTL (Fig 4A). Correction of the TAP1 defect by gene transfer resulted in decreased recognition by our CTL and strongly increased recognition by the Qdm-specific control CTL (Fig 4A). Thus, the alternative Qa-1^b-binding peptide antigens are expressed in a wide array of hematopoietic and non-hematopoietic tissues of normal and transformed cells, including B-cells, fibroblasts, lymphoma, melanoma and sarcoma, and emerge at the cell surface of cells with genetic disruption of the TAP peptide transporter.

However, human tumors mostly display partial deficiencies in their processing pathway that originate at the transcriptional level, resulting in impaired, but not a complete shutdown in the generation of the human counterpart of Qdm/Qa-1^b complexes. To assess whether the CTL were also capable to recognize such tumor cells, we tested four chemically-induced

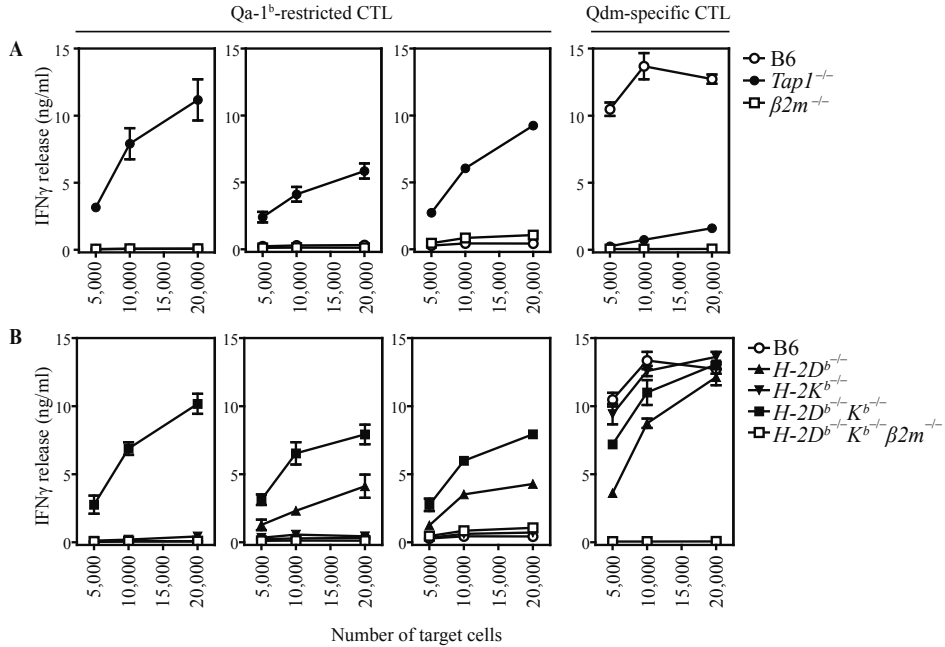


Figure 3. Qa-1^b-restricted CTL are reactive against cells with defined processing deficiencies. (A) LPS-stimulated B-cell blasts from wild type (B6), *TAP1*^{-/-}, and $\beta 2m$ ^{-/-} mice were used as targets for Qa-1^b-restricted CTL (left panels) or control Qdm-specific CTL (right panel). (B) LPS-stimulated B-cell blasts from wild type (B6), MHC class I-knockout and MHC class I/ $\beta 2m$ -knockout mice were tested for recognition by Qa-1^b-restricted CTL (left panels) or control Qdm-specific CTL (right panel). Panels display representative experiments out of four performed. Means and standard deviations of triplicates are shown.

mouse colon carcinomas, which are known to possess peptide-presentation capacity^{39, 40}. Qdm-specific control CTL indeed recognized all four cell lines, demonstrating a functional processing machinery (Fig 4B, right panel). Interestingly, our Qa-1^b-restricted CTL responded to all carcinoma lines, albeit to a varying extent (Fig 4B, left panel). Treatment of the carcinomas with the immunostimulatory cytokine IFN γ , which strongly improves the processing and presentation capacity of cells, resulted in decreased recognition, pointing at partial processing defects in the carcinomas that were corrected by IFN γ . In line with this idea, gene transfer of the viral TAP-inhibitor UL49.5⁴¹ led to a strongly increased recognition by our CTL. As expected, the Qdm-specific control CTL clone exhibited the opposite reactivity profile (Fig 4B, right panel). Of note, the four colon carcinoma lines were derived from two mouse strains with different MHC typing, BALB/c with H-2^d alleles (C26 and CC36) and C57BL/6 with H-2^b alleles (MC38 and CMT93), nicely illustrating the conserved nature of Qa-1, which is the same in these strains.

We concluded that our Qa-1^b-restricted CTL recognize peptide-epitopes that even appear on the surface of cells with mild or partial processing impairments, implying that a mixture of Qdm and TAP-independent peptides can co-exist in Qa-1^b.

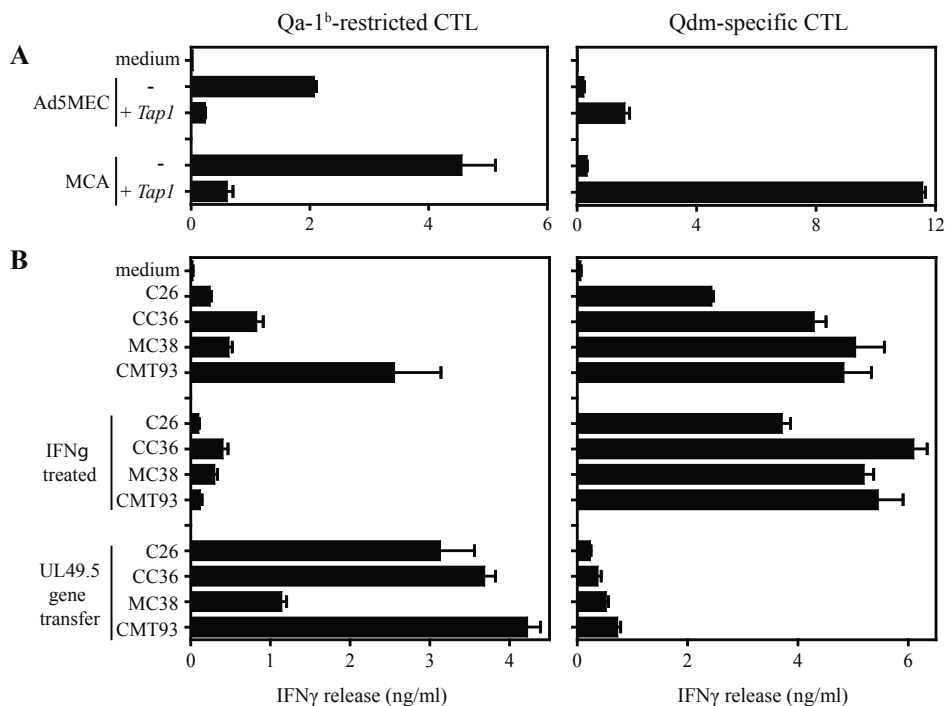


Figure 4. Partial deficiencies in the processing pathway of tumor cells induce the novel Qa-1^b-presented peptide-epitopes. (A) Qa-1^b-restricted CTL respond to Ad5-transformed mouse cells (Ad5MEC) and fibrosarcoma cells (MCA) that are derived from TAP1-knockout mice. Recognition was lost upon gene transfer of mouse TAP1 in these cell lines ('+ TAP1'). Qdm-specific control CTL displayed opposing specificity, indicating that the Qdm peptide is only presented on TAP-proficient tumor cells (right panel). (B) Four colon carcinoma cell lines from BALB/c (C26 and CC36) or C57BL/6 (MC38 and CMT93) background were used as targets for both CTL types. Pre-treatment with IFN γ to boost the antigen processing and presentation machinery resulted in decreased reactivity by the Qa-1^b-restricted CTL, whereas gene transfer of the viral TAP-inhibitor UL49.5⁴¹ led to strongly increased recognition. Again, Qdm-specific control CTL displayed opposing specificity (right panel). Means and standard deviations of triplicates are shown from one representative experiment out of four.

Determination of the Qa-1^b-presented peptide repertoire

We set out to determine the nature of the TAP-independent peptides that are presented by Qa-1^b via biochemical purification and tandem mass-spectrometry. Due to lack of suitable Qa-1^b specific antibodies, we made use of a chimeric MHC class I molecule in which the peptide binding domains (alpha 1 and 2) of Qa-1^b were coupled to the constant alpha 3 domain of D^b, against which good precipitating antibodies are available. This chimeric Qa-1^b/D^b molecule was introduced into TAP2-negative EC7.1 cells and in order to compare the corresponding TAP-dependent peptide repertoire, we also created a TAP2-reconstituted variant of EC7.1.Qa-1^b/D^b cells. Flow cytometry data confirmed that EC7.1.Qa-1^b/D^b cells only expressed this chimeric molecule on the cell surface and not the endogenous D^b molecule, excluding the risk that we would purify D^b-binding peptides

with our alpha 3-directed antibody (Fig 5A). Analysis of TAP2 expression in EC7.1.Qa-1^b/D^b and in the TAP-transfected variant confirmed the absence of TAP2 protein in the first and successful reconstitution in the other (Fig. 5B). Analysis with our Qa-1^b-restricted CTL and Qdm-specific CTL confirmed that our EC7.1 cell lines represented two extremes of the antigen processing status, in that the TAP negative line did not present Qdm and that reconstitution of TAP2 led to a disappearance of the newly defined peptides and that the chimeric molecule functionally presented Qa-1^b-bound peptides (Fig 5C).

Mass spectrometry of peptide species from purified Qa-1^b molecules demonstrated an evident difference between the two peptide repertoires. The peptide repertoire of TAP-positive cells was strikingly dominated by the Qdm peptide AMAPRTLLL (Table II). Six other peptide sequences were identified, but these represented only minor constituents (together less than 5% of the total ion count). In contrast, Qa-1^b from TAP-negative cells was filled with a remarkable diversity of peptides from endogenous proteins (Table II). No peptide clearly stood out in terms of quantity. More than 150 tandem mass fragmentation profiles sufficiently matched with the mouse IPI protein database (<http://www.ebi.ac.uk/IPI/IPImouse.html>) and analysis of their corresponding synthetic peptides confirmed the amino acid sequence of 84 peptides. The other peptide masses could not be matched with a known peptide sequence, but did not correspond to Qdm or previously defined Qa-1^b-binding peptides². The lengths of the TAP-independent peptides ranged from 8 to 18 amino acids, but the majority was 9 amino acids long (Fig 5D). Seven peptides were found in length variants (Table II, numbers 65 to 84) and six of these families varied at their C-terminus, reminiscent of leader peptide processing⁴². However, only one of these families was actually encoded in the leader domain, suggesting that amino acid trimming at the C-terminus might be a feature of TAP-independent peptides in general. Strikingly, no clear binding motif could be elucidated in our peptide repertoire, although previous studies with synthetic peptide libraries showed a dominant role for position 2 (M or L)³⁷. The C-terminus of the peptides frequently contained an aliphatic amino acid (L, I, M, A and F, see Table II), as found for most mouse MHC class I alleles.

We concluded that the presence of the high affinity binding peptide Qdm in the groove of Qa-1^b prevents the accommodation and display of a broad array of alternative peptides and that this new repertoire only emerges upon processing defects.

Immunogenicity of the Qa-1^b-binding peptides

A direct consequence of the absence of this alternative peptide repertoire under physiological conditions is that this repertoire may comprise neo-antigens for the CD8⁺ T-cell system. To estimate the immunogenicity of the identified Qa-1^b-binding peptides we tested them in a matrix-based screening approach in which each peptide was systematically represented in two different peptide pools containing five peptides each. None of our Qa-1^b-restricted T-cell clones recognized peptides from the identified repertoire, so polyclonal responder T cells from mice immunized with TAP-deficient EC7.1.Qa-1^b. B7 tumors were applied, assuring that the peptide-directed responses were induced by

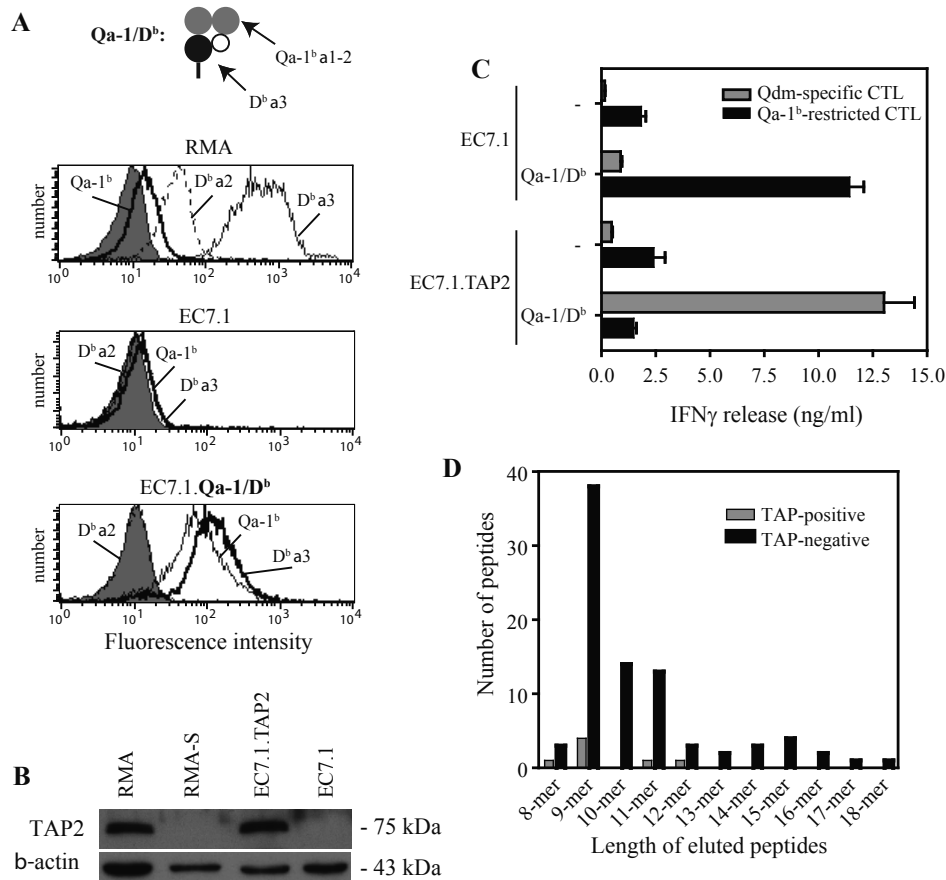


Figure 5. Identification of the TAP-independent peptide repertoire of Qa-1^b. (A) The chimeric Qa-1^b/D^b class I molecule was expressed in EC7.1 cells, which are TAP- and MHC class I-negative. TAP-positive counterparts were generated by introduction of the TAP2 gene. Surface display on 'EC7.1.Qa-1^b/D^b' cells of the chimeric molecule and absence of the endogenous D^b molecule was determined by flow cytometry with, respectively, Qa-1^b- and D^ba3-specific antibodies, and D^ba2-specific antibody, as indicated in the histogram plots (clone KH95 and also H131-31 (not shown)). Staining of TAP-reconstituted cells gave comparable results. Control TAP-proficient RMA cells displayed endogenous Qa-1^b and D^b molecules. (B) TAP expression was analyzed on RMA, RMA-S, EC7.1 and EC7.1.TAP2 cells by Western blot using the antibody TAP2.688 against mouse TAP2. Results confirmed the lack of TAP2 expression on EC7.1 and RMA-S cells. (C) The chimeric construct was recognized by CTL clones: the TAP-negative variant only by Qa-1^b-restricted CTL (black bars) and the TAP-positive variant only by Qdm-specific CTL (grey bars). Means and standard deviations of triplicate wells are shown from one representative experiment out of four. (D) Peptide purification and mass spectrometry analysis revealed the wide diversity of the TAP-negative peptide repertoire, whereas the TAP-positive peptide repertoire was mainly limited to the Qdm peptide. Data were collected from four independent experiments in the case of TAP-negative repertoire and two independent experiments for TAP-positive repertoire. Number of different peptides with indicated length is depicted of 84 identified peptides that are listed in Table II.

Table II. List of Qa-1^b-binding peptides isolated from TAP-positive and TAP-negative tumor cells

Peptide number	Peptide sequence	Peptide Location	Protein length	UniProtKB or SwissProt number	Protein description
TAP-positive					
1	AMAPRTLLL	3-11	362	P01899	H-2 class I histocompatibility antigen, D-B alpha chain
2	AQAERTPEL	691-699	1274	A2RT67	DENN domain-containing protein 3
3	IINTHTLLL	1302-1310	1657	Q80UW7	IQ motif containing GTPase activating protein 1
4	PKFEVIDKPQS	98-108	108	P97450	ATP synthase-coupling factor 6, mitochondrial
5	PTEEESPV	486-493	493	P53986	Monocarboxylate transporter 1
6	QAIPQGAIQ	246-254	461	Q8BG99	Homeobox protein PKNOX2
7	QLQPQQPLPQPQ	125-136	672	Q9VWH4	Forkhead protein FKHR2
TAP-negative					
1	AAIENIEHL	1193-1201	1392	Q6PB66	Leucine-rich PPR motif-containing protein, mitochondrial
2	AALKLGQEL	799-807	1271	Q9JJ28	Protein flightless-1 homologue
3	AAPTANANSLNSTF	454-466	575	Q8BT14	CCR4-NOT transcription complex subunit 4
4	AAPTSPDHSPA	699-709	709	Q66L44	Protein Dos
5	AAVIAHDFL	153-161	292	P30282	G1/S-specific cyclin-D3
6	AGIENDEAF	44-52	248	B1AWD9	Clathrin light polypeptide
7	AGPENSSKI	383-346	2075	Q80XK6	Autophagy-related protein 2 homologue B
8	AGQFNQDYL	45-53	503	Q921F1	Annexin A11
9	AGVRNPQQHL	515-524	636	Q8BN32	Pabpc1 protein
10	ASLQNFNISNL	2103-2113	2128	B2RRJ7	Wnk1 protein
11	KSISNPPGSNL	2115-2125	2128	B2RRJ7	Wnk1 protein
12	ASQQNSEEM	202-210	210	Q9CXE2	B-cell CLL/lymphoma 7 protein family member A
13	ASVLNVNHI	2195-2203	2603	Q99NH0	Ankyrin repeat domain-containing protein 17
14	ASYRAQPSVSL	270-280	573	Q62019	16 kD protein
15	ATPGRLIDFL	256-265	648	Q5U222	Ddx5 protein
16	AVSEGTKAVTKYTSK	111-126	126	Q8CGP1	Histone H2B type 1-K
17	FAPLRLPTL	17-26	156	Q9CR21	Acyl carrier protein, mitochondrial
18	FAPVNVTEVKSV	280-293	462	P10126	Elongation factor 1-alpha 1
19	FAYEGRDYI	137-145	184	Q62143	Qa-2 cell surface antigen
20	FGPVNHEEL	33-41	147	P46414	Cyclin-dependent kinase inhibitor 1B
21	FQIVNPHLL	634-642	792	Q6NZB3	Ribonucleoside-diphosphate reductase
22	FQVTHTV	113-121	361	Q9QYA2	Mitochondrial import receptor subunit TOM40 homologue

Table II. Continued.

Peptide number	Peptide sequence	Peptide Location	Protein length	UniProtKB or SwissProt number	Protein description
23	GGPINPATA	1036-1044	1107	Q80XS0	Ubiquitin-associated protein 2-like
24	GLGVLLAF	5-12	113	Q8QZT4	Crumbs protein homologue 3
25	HSIQNSQDM	61-69	210	Q91YN9	BAG family molecular chaperone regulator 2
26	IQKTPQIQVY	21-30	119	P01887	Beta-2-microglobulin
27	KAPPLPPLVVF	27-38	499	P16277	Tyrosine-protein kinase BLK
28	KAPTNEFYA	190-198	198	O35988	Syndecan-4
29	KCSVSIQYVDV NDNYPEL	328-345	794	Q91XZ8	Protocadherin beta 22
30	KSAVGHEYV	96-107	486	Q922I8	Hematopoietic cell specific Lyn substrate 1
31	LAIRNDEEL	93-101	137	Q64426	Histone H2A
32	LVRPGTALEL	2294-2303	2883	NP_076331	desmoplakin
33	METLTATPQ	976-984	1241	B2RXW8	Ppf1a1 protein
34	NSIRNLDTI	100-108	475	P28658	Ataxin-10
35	PADIVKNLK	12-20	341	Q8VDZ8	Calcium binding protein 39
36	PDTGISSKA	52-60	126	Q8CGP2	Histone H2B type 1-P
37	PEAFPALA	385-392	392	Q9CY58	Plasminogen activator inhibitor 1 RNA-binding protein
38	PNKLVELNK	138-146	943	Q6DFV7	Nuclear receptor coactivator 7
39	PPSAKAAID	97-105	323	Q5SSG6	TATA box binding protein (TBP)-associated factor
40	PPTAKAAVE	425-433	661	Q5SUS9	Ewing sarcoma breakpoint region 1
41	PVKAVEIEI	155-163	930	Q7TSZ1	Xeroderma pigmentosum, complementation group C
42	RSPENPPSKEL	171-181	181	Q9CQA0	Centromere protein M
43	RSPGNSPTPM	186-195	469	Q8CI61	BAG family molecular chaperone regulator 4
44	SALINLSSF	7-15	161	Q8VE65	Transcription initiation factor TFIID subunit 12
45	SAPENAVRM	28-36	126	O35127	Protein C10
46	SAPSNFEHR	12-20	593	Q8BTW9	Serine/threonine-protein kinase PAK 4
47	SAPTGSGKTL	227-236	639	Q6P9R1	ATP-dependent RNA helicase DDX51
48	SAVISLEGKPL	156-166	166	P18760	Cofilin-1
49	SAVSNNYIQT	168-178	911	P30999	Catenin delta-1
50	SHRKFSAPR	2-10	403	Q3U9L3	ribosomal protein L3P family member
51	SLGINPHVL	966-974	1170	B2RQL0	Nup98 protein
52	SLGKNPTDAYL	60-70	172	Q3THE2	Myosin regulatory light chain MRLC2
53	SQPESKV FYL	110-119	245	P63101	Protein kinase C inhibitor protein 1

Table II. Continued.

Peptide number	Peptide sequence	Peptide Location	Protein length	UniProtKB or SwissProt number	Protein description
54	SSITNPKLSTL	726-736	959	Q5NBZ5	Eukaryotic translation initiation factor 4E nuclear import factor 1
55	STIRLLTSL	458-466	545	P80318	T-complex protein 1 subunit gamma
56	TNPESKV FYL	155-160	284	P68254	14-3-3 protein theta
57	TQGQNIQHL	431-439	2326	Q80YT7	Myomegalin
58	VAVTNGPRS	371-379	393	Q9D6J1	LAG1 longevity assurance homologue 4
59	VPPVQVSPLIKFGRY	2-16	71	Q5EBI8	ATP synthase, H ⁺ transporting, mitochondrial F1F0 complex
60	VQVSNFKSGKGDSTL	254-268	579	Q80WJ7	Metastasis adhesion protein
61	VSLLDIDHL	473-481	679	Q8BQM4	HEAT repeat-containing protein 3
62	VSLLNPPETL	411-420	422	Q8BRG1	Cyclin A2, isoform CRA
63	VTLVNHGSTF	15-24	640	P59110	SUMO-1 protease 2
64	YGYSNRVVDLM	316-326	333	P16858	Glyceraldehyde-3-phosphate dehydrogenase
65	AAPRSGPSV	611-619	641	Q923D5	WW domain-binding protein 11
66	AAPRSGPSVA	611-620	641	Q923D5	WW domain-binding protein 11
67	AGIENKFGL	648-656	676	Q9QXX4	Calcium-binding mitochondrial carrier protein Aralar2
68	AGIENKFGLYL	648-658	676	Q9QXX4	Calcium-binding mitochondrial carrier protein Aralar2
69	AGIENKFGLYLP	648-659	676	Q9QXX4	Calcium-binding mitochondrial carrier protein Aralar2
70	TKAVTKYTSSK	117-127	127	A0JNS9	Histone H2B
71	GTKAVTKYTSSK	116-127	127	A0JNS9	Histone H2B
72	SEGTKAVTKYTSSK	114-127	127	A0JNS9	Histone H2B
73	VSEGTKAVTKYTSSK	113-127	127	A0JNS9	Histone H2B
74	AVSEGTKAVTKYTSSK	112-127	127	A0JNS9	Histone H2B
75	PEPAKSAPAPK	2-12	126	Q64478	Histone H2B type 1-H
76	PEPAKSAPAPKKG	2-14	126	Q64478	Histone H2B type 1-H
77	LGVKNSEPA	59-67	576	Q80UG5	Septin-9
78	LGVKNSEPAA	59-68	576	Q80UG5	Septin-9
79	SSPANISLEFEDA	583-596	773	P51125	Calpastatin
80	SSPANISLEFEDAK	583-597	773	P51125	Calpastatin
81	SSPANISLEFEDAKLS	583-599	773	P51125	Calpastatin
82	SSPANISLEFEDAKLSA	583-600	773	P51125	Calpastatin
83	KPLFEILNG	1138-1146	1735	Q9IZU3	Endogenous mouse mammary tumor virus Mtv1
84	KPLFEILNGD	1138-1147	1735	Q9IZU3	Endogenous mouse mammary tumor virus Mtv1

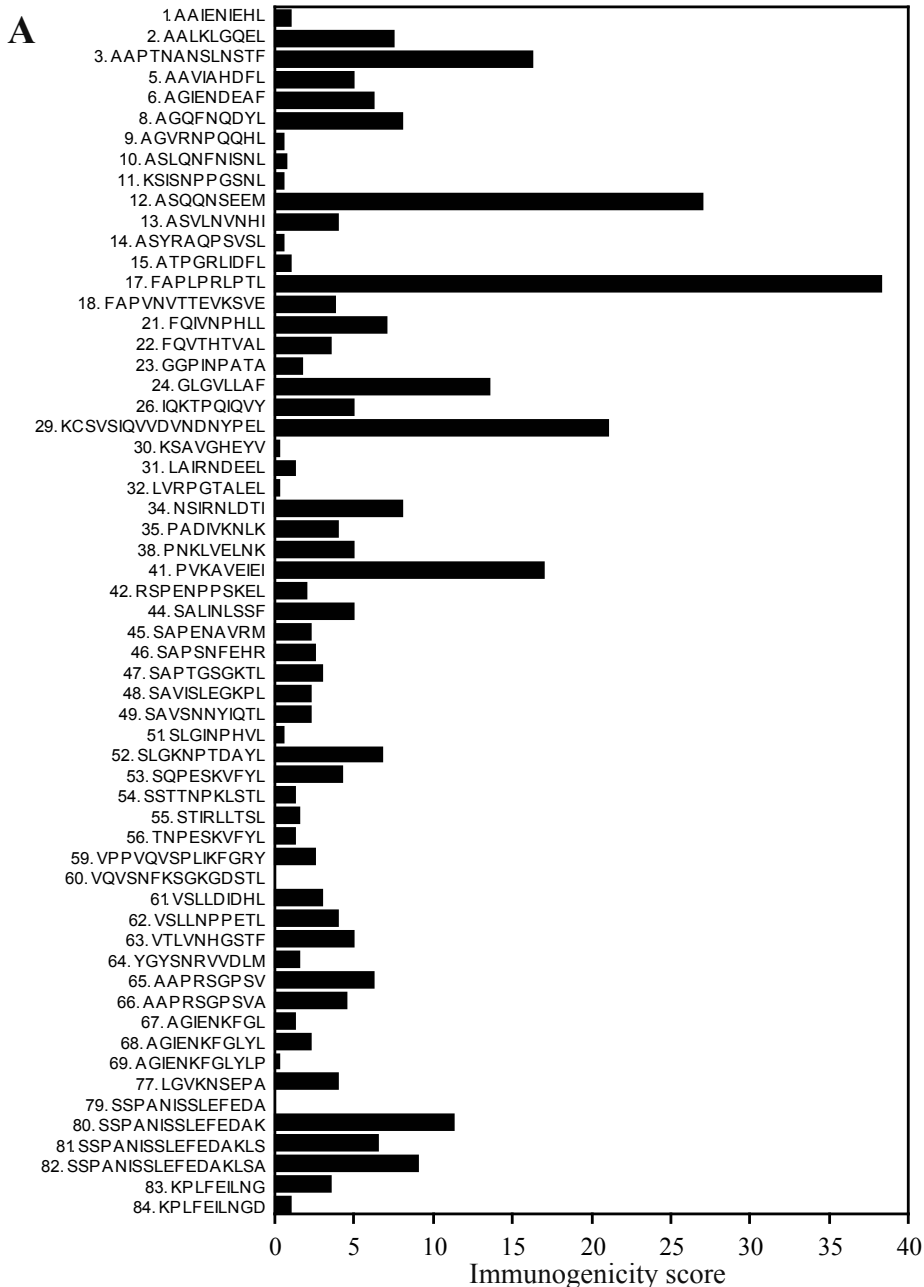


Figure 6. Immunogenicity of Qa-1^b-presented peptides *in vitro* and *in vivo*. (A) Immunogenicity of 59 peptides was determined by compiling the IFN γ response of twenty-five T-cell cultures, which were induced by immunization with irradiated TAP-negative EC7.1.Qa-1^b.B7 cells. Responses were measured using a matrix-based approach in which each peptide was represented in two independent pools. T-cell responses against each pool were scored from zero to three and compiled scores from the two pools were multiplied. (B-D) Percentage of IFN γ producing CD8⁺ T-cells in the blood of mice that were naïve

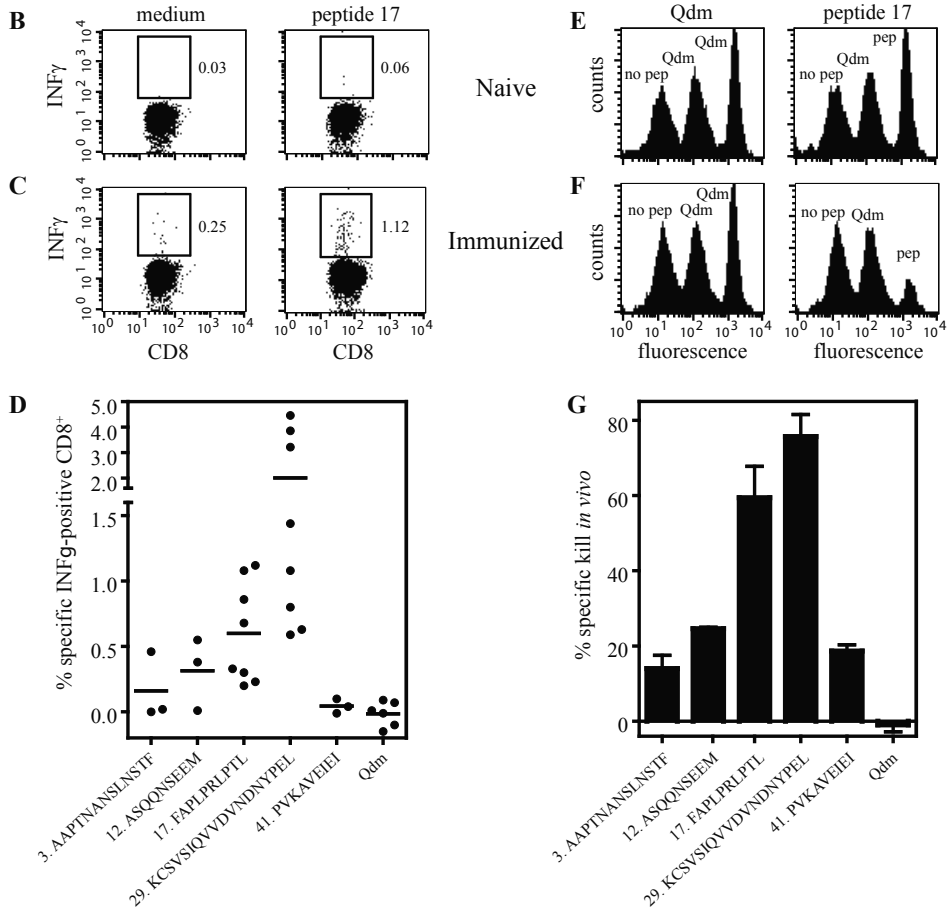


Figure 6. Continued. (B) or immunized with the indicated peptide (C). Blood samples were taken and cells were stimulated overnight with medium or specific peptide, stained with antibodies and analyzed by flow cytometry. Collected data from peptide-specific frequencies of CD8⁺ cells from mice are depicted in (D). Each data point represents one mouse, and data from two independent experiments is shown. (E-G) Cytotoxic reactivity *in vivo* in the same mice as shown in (D). Specific killing was determined in naïve (E) or immunized mice (F) by comparing the numbers of CFSE^{high} targets, which were loaded with relevant peptides, to CFSE^{low} targets, which were not loaded with peptide. CFSE^{intermediate} targets were loaded with Qdm and were always comparable to targets without peptide. The means of percentage *in vivo* killing is depicted with standard deviations (G).

naturally presented antigens. Responses of twenty-five independent T-cell cultures were compiled and final immunogenicity scores revealed a number of immunogenic peptides (Fig 6A). These peptide antigens had no common amino acid motif and were derived from different housekeeping proteins (see corresponding numbers in Table II), again pointing at the broad diversity of this antigen repertoire. To assess the potency of the five most immunogenic peptide sequences (number 3, 12, 17, 29 and 41) to induce CD8⁺ T cell

responses *in vivo*, we immunized mice with the peptides in conjunction with imiquimod as adjuvant. After two vaccinations, clear populations of peptide-specific CD8⁺ T cells were detectable in the blood (Fig 6B-D). The peptides with the largest T cell populations also scored high in the immunogenicity screen in which tumor-stimulated T cells were applied (Fig 6A). Interestingly, peptide immunizations in H-2D^{b/-}K^{b/-} mice did not lead to a population of IFN γ -positive CD8⁺ T cells, indicating that the T cell repertoire in these knockout mice is tolerant for the novel Qa-1^b binding peptides (Fig S3). This is in line with the finding that H-2D^{b/-}K^{b/-} target cells efficiently presented these peptides (Fig 3B), which are part of the normal Qa-1^b peptide repertoire that induces central tolerance in the thymus. Importantly, the vaccine-induced CD8⁺ T cells in wild type mice exhibited strong cytolytic reactivity *in vivo* against target cells that were loaded with the relevant peptide (Fig 6E-G). Unloaded target cells and cells loaded with Qdm peptide were not killed *in vivo*. Vaccination with the Qdm peptide did not trigger any T-cell immunity, illustrating that the T-cell repertoire for this sequence does not exist or is functionally tolerant.

In conclusion, a broad repertoire of Qa-1^b-binding peptide-epitopes of 'self' origin emerges at the surface of cells with impairments in the antigen processing pathway and that this novel repertoire replaces the TAP-dependent Qdm. These results underscore a role of Qa-1^b in adaptive immunity and infer that T cell responses are evoked via this nonclassical MHC molecule to prevent outgrowth of processing-deficient cells in the body.

I DISCUSSION

The nonclassical MHC class I molecule Qa-1^b normally presents monomorphic Qdm peptides derived from the leader sequences of classical MHC. This Qdm peptide/MHC complex functions as a remote sensor of the class I processing pathway integrity through detection by CD94/NKG2A receptors on NK cells. Failure to present Qdm, due to impairments in proteolysis, TAP or other means, increases the susceptibility of cells for attack by these lymphocytes. We here show that Qdm under such conditions is replaced by a surprisingly broad repertoire of alternative 'self' peptides. This novel peptide repertoire comprises neo-antigens for which the T-cell system is not hampered by immunological tolerance and can therefore induce a vigorous response of Qa-1^b-restricted CD8⁺ CTL. This antigen presenting function of Qa-1^b places this conserved MHC molecule at the intersection of innate and adaptive immunity. On one hand, it plays a role as a monomorphic inhibiting ligand when Qdm is presented, and, on the other hand, as a display system of an immunogenic 'self' peptide repertoire. Our data suggest that the immune system is equipped with multiple mechanisms to remove processing deficient cells from the body.

The finding that Qa-1^b is capable of presenting other peptides than Qdm is supported by previous work from several groups. The leader of the CMV protein UL40 was found to be presented, as well as peptides from *Salmonella*, *Listeria*, EBV and *Mycobacterium tuberculosis*². Furthermore, an increase of Hsp60 in the cellular stress response was shown to result in replacement of the human Qdm homolog with the Hsp60 signal peptide⁴³.

The unique feature, however, of the peptide repertoire we have elucidated in our current work is the fact that these peptides are derived from endogenous housekeeping proteins and only emerge in Qa-1^b on the cell surface when the processing pathway is hampered. We demonstrated the display of these peptides in cells with partial and mild deficiencies that do not completely prevent Qdm from appearance, but a complete block in the processing pathway, e.g. loss of TAP1 or TAP2, resulted in the most efficient presentation of the novel peptides, demonstrating that TAP-independent processing mechanisms are responsible for the replacement of Qdm. Surprisingly, cell surface expression of Qa-1^b is less affected by TAP deficiency than classical MHC class I molecules⁴⁴, and HLA-E surface expression has also been described in the absence of functional TAP proteins or class I-derived signal peptides^{45, 46}. Qa-1^b and HLA-E, indeed, seem to efficiently egress from the ER in the absence of TAP^{47, 48}. These data implicate that our mouse studies are relevant for the human setting. Two alternative processing routes have been described by which peptides can bypass the TAP transporter: N-terminal signal peptides, which are processed by signal peptide cleaving enzymes⁴² and C-terminal ends of ER-resident proteins can also be loaded without TAP function, although the responsible proteolytic enzymes have not been identified for this pathway⁴⁹. Forty percent of the 84 identified peptides in our search was indeed located at the N- or C-terminus. Interestingly, one of the most immunogenic Qa-1^b peptide-epitopes FAPLPRLPTL is encoded in the N-terminal signal sequence of a mitochondrial carrier protein (UniProt Q9CR21). The fact that sixty percent of the peptides cannot be explained by these two processing mechanisms suggests the existence of other yet uncharacterized processing pathways for MHC class I antigen presentation. It has been proposed that autophagy might contribute to MHC class I-restricted antigen presentation⁵⁰. Autophagy is a crucial cellular mechanism responsible for the clearance of old or damaged cellular components, including organelles and macromolecules. This pathway might lead to the generation of peptides susceptible for MHC class I loading. Previous characterizations of peptides bound to classical MHC class I from TAP-deficient cells revealed similar features: predominance of signal peptides, increased peptide lengths, and selective presentation on TAP-negative variants^{51, 52}. The Qa-1^b peptide repertoires present on TAP-positive versus TAP-negative cells were extremely different (Table II), in that the high affinity binding monomorphic Qdm strongly dominated the TAP-positive pool for more than 95%, whereas a broad diversity of other peptides was present in the TAP-negative pool. Previous comparisons of peptide repertoires presented by classical MHC class I molecules showed much more overlap between the TAP-positive and -negative pools^{51, 52}. This indicates that the high affinity binding Qdm competes with the TAP-independent peptides and thereby prevents their presentation under normal circumstances. However, even mild or partial processing deficiencies in tumor cells already led to the presentation of both type of peptides, as illustrated by the fact that Qdm-specific CTL as well as our Qa-1^b-restricted CTL were able to recognize the four colon tumors (Fig 4). Importantly, Qdm is not immunogenic due to the widespread presentation in the normal host, including thymus⁵³, and thereby

prevent tolerance mechanisms for CTL specificities that recognize the new emerging peptide-epitopes. We indeed found that T cells specific for these novel Qa-1^b peptides are deleted in mice that do present these peptides in Qa-1^b molecules during T-cell development, as in H-2D^b-/-K^b-/- mice (Fig S3).

Our Qa-1^b-restricted T-cells exhibit a normal phenotype for CD8⁺ T-lymphocytes (Fig S1) and use rearranged αβTCRs and thereby distinguish themselves from reported Qa-1-recognizing intestinal γδ⁺ T-cells³² and the Qa-1-restricted regulatory CD8αα⁺ T-cell subset³³. Target cell recognition was mediated by the TCR and this receptor did not cross-react with classical class I molecules, indicating that also at the functional level this subset is not different from conventional CD8⁺ T-lymphocytes. This is in line with data from mice lacking classical MHC class I molecules arguing that approximately 10% of the total CD8⁺ T-cell subset is restricted by nonclassical molecules^{23,54-56}. This T cell repertoire is heterogeneous, as witnessed by a diverse usage of TCRVβ segments^{54, 56}. Selection of Qa-1^b-restricted T-cells in the thymus indeed does occur⁵³, but it remains puzzling how a monomorphic Qdm/Qa-1^b complex can mediate positive selection resulting in such a broad repertoire. Furthermore, the relationship between the suppressive Qa-1^b-restricted T-cells^{21, 28} and the tumor-reactive T-cells described here remains elusive.

We speculate that comparable T-cell subsets exist in human beings, especially because Qa-1^b and HLA-E are rather conserved in structure and function². TCR-mediated recognition of HLA-E has been described²⁵⁻²⁷ and it is highly likely that a similar subset as our Qa-1^b-CTL, which responds against tumors with processing impairments, is present in humans. HLA-E surface expression is ubiquitous in the body and even detected in the absence of the human Qdm homolog^{45, 46}, although MHC class I leaders strongly stimulate HLA-E display^{19, 57}. The lack of polymorphism in the human population infers that identified neo-antigens presented in HLA-E constitute universal epitopes that might be exploited for the therapy of frequently occurring tumor immune escape variants and persistent infections by viruses encoding immune evasion proteins.

| MATERIAL AND METHODS

Cell lines and mice

The origin and culturing of most cell lines used in this study was described previously²⁹. C4.4-25⁺ is a β2m deficient variant of EL4. RMA-S.B7-1 is a CD80 transfectant of RMA-S. EC7.1 is a K^b- and D^b-negative variant of RMA-S³⁰. B78H1 is a TAP-deficient and MHC class I deficient melanoma³⁴. Tap1^{-/-} and wild type mouse embryo fibroblasts were immortalized by the adenovirus type 5 E1 gene (Ad5MEC, clone XC3). Tap1^{-/-} fibrosarcoma was induced with methylcholantrene (MCA, clone MCB6TAP). Gene transfer of mouse TAP1, mouse TAP2, H-2D^b, H-2K^b and Qa-1^b (T23 gene) was performed with retroviruses based on the MuLV vector LZRS. Generation of CTL clones was performed according to previous description²⁹. Briefly, syngeneic C57BL/6 mice were immunized with irradiated RMA-S.B7-1 cells and in vitro stimulated weekly

with a mixture of RMA-S.B7-1 and EC7.1.Qa-1^b.B7-1 cells, naïve splenocytes and IL-2. Qa-1^b-restricted CTL clone B12i that is reactive with the TAP-dependent AMAPRTLLL peptide was isolated from a B6.Tla mouse³⁸.

All mice were purchased from Iffa Credo (France) and housed in the animal facility of the Leiden University Medical Center under specific pathogen-free conditions and used between 8 and 12 weeks of age. TAP1-knockout mice were purchased from Jackson laboratories and MHC class I-knockout animals were obtained from Dr. F. Lemonnier⁵⁶. Experiments were performed in accordance with Dutch national legislation and institutional guidelines and were approved by the local ethical committee.

CTL assays and flow cytometry

CTL activity was measured by chromium (⁵¹Cr) release assay or IFN- γ ELISA as described before⁵⁸. Data shown represent mean values obtained from triplicate test-wells and the error bars represent standard deviation of these values. For antibody blocking, CTL were pre-treated with 20 μ g/ml antibodies, washed and added as responders to target cells for IFN- γ release. The following antibodies were used in functional blocking assays: hamster anti-CD3 (Fab₂ fragments of clone 145-2C11), control hamster anti-TNP, rat anti-CD4 (clone GK1.4), rat anti-CD8 (clone 2.43) anti-NKG2A/C (clone 20d5) and anti-Qa-1^b (clone 6A8.6F10.1A6). For flow cytometry analysis, the following monoclonal antibodies were purchased from Becton Dickinson: Ly49A^{B6} (clone A1), Ly49C/I (clone 5E6), Ly49G (clone LGL-1), Qa-1^b (clone 6A8.6F10.1A6), NKG2A^{B6} (clone 16a11), CD94 (clone 18d3), CD3 (clone 145-2C11), CD8 α (clone Ly2), CD8 β (clone Ly-3.2.), CD4 (clone GK1.4), CD16/CD32 (clone 2.4G2), NK1.1 (clone PK136), CD49b (clone DX5), CD244 (clone 2B4). From eBioscience we purchased NKG2D (clone CX5).

Peptide elution, HPLC and mass spectrometry

Peptides were eluted out of purified chimeric Qa-1^b/D^b molecules from EC7.1 cells. The genomic H-2D^b construct in which the alpha 1 and alpha 2 domains were exchanged with those of Qa-1^b was kindly provided by Dr. PJ Dyson (United Kingdom)³⁸. This genomic construct was expressed in HeLa cells and cDNA was then cloned into the retroviral construct LZRS. EC7.1 cells transduced with this construct were twice sorted by FACS. TAP-positive counterparts were generated by retroviral gene transfer of the mouse TAP2 gene. Immunoprecipitation of Qa-1^b/D^b molecules was performed with protein A beads covalently coupled with anti-D^b mAb 28-14-8S from two and four independent lysates of 40.10⁹ TAP-positive and TAP-negative EC7.1 cells, respectively, as previously described⁵⁸. The peptide pools were prefractionated on a C18 RP-HPLC system (200 μ m x 15 cm; Reprosil-C18-AQ 3 μ m (Dr. Maisch GmbH, Ammerbuch, Germany)). Fractions were reduced to near dryness, diluted in 95/3/0.1 v/v/v water/acetonitril/formic acid and subsequently analyzed by tandem mass spectrometry. Peptides were analyzed by nanoflow liquid chromatography using an Agilent 1100 HPLC system (Agilent Technologies), as previously described²⁹, coupled on line to a 7-tesla LTQ-FT mass spectrometer (Thermo Electron, Bremen, Germany). The end of the nanocolumn was drawn to a tip

(ID approximately 5 μm), from which the eluent was sprayed into the mass spectrometer. Peptides were trapped at 5 $\mu\text{l}/\text{min}$ on a 1-cm column (100- μm ID, ReproSil-Pur C18-AQ, 3 μm) and eluted to a 15 cm column (50- μm ID, ReproSil-Pur C18-AQ, 3 μm) at 150 nl/min in a 60-min gradient from 0 to 50% acetonitrile in 0.1% Formic acid.

The mass spectrometer was operated in data-dependent mode, automatically switching between MS and MS/MS acquisition. Full scan MS spectra were acquired in the FT-ICR with a resolution of 25,000 at a target value of 3,000,000. 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/protein identification, MS/MS data were submitted to the mouse IPI database using Mascot Version 2.1 (Matrix Science) with the following settings: 5 ppm and 0.8-Da deviation for precursor and fragment masses, respectively; no enzyme was specified. All peptides with a mascot ion score above 30 were manually inspected. This resulted in an initial set of 150 peptides. These peptides were synthesized and their MS/MS spectra compared with the MS/MS spectra of the Qa-1^b-eluted peptides. This resulted in 84 confirmed peptide identifications.

Immunogenicity screen of Qa-1^b presented peptides

C57BL/6 mice were immunized twice with irradiated EC7.1.B7-1 cells with stable expression of Qa-1^b. Spleens of immunized mice were cultured with the same cells for one to two weeks and reactivity of the cultures was tested against the EC7.1 cell panel to confirm Qa-1^b restriction of the T-cells. Reactivity against the identified peptides was assessed using a matrix-based system with pools of 5 different peptides and in which each peptide was represented in two independent pools. The peptide pools were loaded on spleen cells and IFN γ production by T-cell was measured with sandwich ELISA after 18 h. Reactivity against each pool was arbitrarily scored from zero to three, according to the mean background response. Twenty five independent cultures were assayed in this way and the scores of each pool compiled. The final immunogenicity score for each peptide was then obtained by multiplying the scores of two pools containing that particular peptide.

Peptide immunizations and in vivo cytotoxicity assay

For peptide immunizations, C57BL/6 and H-2D^b/K^b mice were injected s.c. at the flank with 50 μg peptide mixed with 50 μg helper peptide from MuLV (H19²⁹) in PBS on day 0 and 7. Immediately following injection, 60 mg Aldara[™] cream (3M Pharmaceutical) containing 5% imiquimod was applied to the skin at the injection site. Mice received i.p. injections of 500,000 IU recombinant human IL-2 (Novartis) on the day of the second vaccination and on the day thereafter. T-cell frequencies were determined from blood lymphocytes after four days and from spleens after seven days with intracellular cytokine staining, as previously described⁵⁹. In short, cells were cultured overnight with medium or 5 $\mu\text{g}/\text{ml}$ of peptide and stained for CD8 and intracellular IFN γ . Killing capacity of

the peptide-induced T-cells was determined in immunized animals using differentially CFSE-labeled and peptide-pulsed splenocytes, as described⁴⁹. Briefly, spleens from CD90.1 congenic C57BL/6 mice were passed through nylon wool and labeled with 5 μ M CFSE and pulsed with indicated peptides, labeled with 0.5 μ M CFSE and pulsed with control Qdm peptide or labeled with 0.05 μ M CFSE without peptide. These three target cell populations were washed, mixed and 1×10^7 cells were injected i.v. per recipient mouse. The spleens of recipient mice were harvested after two days, stained with CD90.1-specific antibodies and analyzed with flow cytometry. Percentage killing was calculated as the ratio between the numbers of peptide pulsed targets and control pulsed targets.

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REFERENCES

1. Jensen, P.E., Sullivan, B.A., Reed-Loisel, L.M. & Weber, D.A. Qa-1, a nonclassical class I histocompatibility molecule with roles in innate and adaptive immunity. *Immunol. Res.* **29**, 81-92 (2004).
2. Rodgers, J.R. & Cook, R.G. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5**, 459-471 (2005).
3. O'Callaghan, C.A. et al. Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E. *Mol. Cell* **1**, 531-541 (1998).
4. Grimsley, C. et al. Definitive high resolution typing of HLA-E allelic polymorphisms: identifying potential error in existing allele data. *Tissue Antigens* **60**, 206-212 (2002).
5. Hermel, E. et al. Polymorphism and conservation of the genes encoding Qa1 molecules. *Immunogenetics* **56**, 639-649 (2004).
6. Strong, R.K. et al. HLA-E allelic variants. Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. *J. Biol. Chem.* **278**, 5082-90 (2003).
7. Aldrich, C.J. et al. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* **79**, 649-658 (1994).
8. DeCloux, A., Woods, A.S., Cotter, R.J., Soloski, M.J. & Forman, J. Dominance of a single peptide bound to the class Ib molecule, Qa-1b. *J. Immunol.* **158**, 2183-2191 (1997).
9. Kurepa, Z., Hasemann, C.A. & Forman, J. Qa-1(b) binds conserved class I leader peptides derived from several mammalian species. *J. Exp. Med.* **188**, 973-978 (1998).
10. Braud, V.M. et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795-799 (1998).
11. Vance, R.E., Jamieson, A.M. & Raulet, D.H. Recognition of the class Ib molecule Qa-1b by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* **190**, 1801-1812 (1999).
12. Petrie, E.J. et al. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med* **205**, 725-735 (2008).
13. Kaiser, B.K., Pizarro, J.C., Kerns, J. & Strong, R.K. Structural basis for NKG2A/CD94 recognition of HLA-E. *Proc Natl Acad Sci U S A* **105**, 6696-6701 (2008).
14. Rudolph, M.G. & Wilson, I.A. The specificity of TCR/pMHC interaction. *Curr. Opinion Immunol.* **14**, 52-65 (2002).
15. Vales-Gomez, M., Reyburn, H.T., Erskine, R.A., Lopez-Botet, M. & Strominger, J.L. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2A and the activating receptor CD94/NKG2C to HLA-E. *EMBO J.* **18**, 4250-4260 (1999).
16. Miller, J.D. et al. Analysis of HLA-E peptide-binding specificity and contact residues in bound

- peptide required for recognition by CD94/NKG2. *J Immunol* **171**, 1369-1375. (2003).
17. Bland, F.A., Lemberg, M.K., McMichael, A.J., Martoglio, B. & Braud, V.M. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. *J. Biol. Chem.* **278**, 33747-52 (2003).
18. Lemberg, M.K., Bland, F.A., Weihofen, A., Braud, V.M. & Martoglio, B. Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. *J. Immunol.* **167**, 6441-6 (2001).
19. Braud, V.M., Allan, D.S.J., Wilson, D. & McMichael, A.J. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* **8**, 1-10 (1997).
20. Li, L. et al. Differential requirement for tapasin in the presentation of leader- and insulin-derived peptide antigens to Qa-1b-restricted CTLs. *J. Immunol.* **173**, 3707-15 (2004).
21. Lu, L., Werneck, M.B.F. & Cantor, H. The immunoregulatory effects of Qa-1. *Immunol Rev* **212**, 51-59 (2006).
22. Ljunggren, H.G. & Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11**, 237-244 (1990).
23. Seaman, M.S., Perarnau, B., Fischer Lindahl, K., Lemonnier, F.A. & Forman, J. Response to *Listeria* monocytogenes in mice lacking MHC class Ia molecules. *J. Immunol.* **162**, 5429-5436 (1999).
24. Lo, W.-F. et al. Molecular mimicry mediated by MHC class Ib molecules after infection with Gram-negative pathogens. *Nat. Medicine* **6**, 215-218 (2000).
25. Heinzel, A.S. et al. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J. Exp. Med.* **196**, 1473-1481 (2002).
26. Romagnani, C. et al. Identification of HLA-E-specific alloreactive T lymphocytes: a cell subset that undergoes preferential expansion in mixed lymphocyte culture and displays a broad cytolytic activity against allogeneic cells. *Proc Natl Acad Sci U S A* **99**, 11328-11333 (2002).
27. Garcia, P. et al. Human T cell receptor-mediated recognition of HLA-E. *Eur. J. Immunol.* **32**, 936-944 (2002).
28. Jiang, H. & Chess, L. The specific regulation of immune responses by CD8+ T cells restricted by the MHC class Ib molecule, Qa-1. *Annu. Rev. Immunol.* **18**, 185-216 (2000).
29. Van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Medicine* **12**, 417-424 (2006).
30. Howell, D. et al. Heterogeneity of RMA-S cell line: derivatives of RMA-S cells lacking H2-Kb and H2-Db expression. *Immunogenetics* **52**, 150-154 (2000).
31. McMahon, C.W. & Raulet, D.H. Expression and function of NK receptors in CD8+ T cells. *Curr. Opinion in Immunol.* **13**, 465-470 (2001).
32. Davies, A. et al. Infection-induced expansion of a MHC class Ib-dependent intestinal intraepithelial gd T cell subset. *J. Immunol.* **172**, 6828-6837 (2004).
33. Tang, X. et al. Regulation of immunity by a novel population of Qa-1-restricted CD8aa+ TCRab+ T cells. *J Immunol* **177**, 7645-7655 (2006).
34. Chiang, E.Y., Henson, M. & Stroynowski, I. Correction of defects responsible for impaired Qa-2 class Ib MHC expression on melanoma cells protects mice from tumor growth. *J. Immunol.* **170**, 4515-4523 (2003).
35. Gays, F. et al. Functional analysis of the molecular factors controlling Qa1-mediated protection of target cells from NK lysis. *J Immunol* **166**, 1601-1610 (2001).
36. Diefenbach, A., Jamieson, A.M., Liu, S.D., Shastri, N. & Raulet, D.H. Ligands for the murine NKG2D receptor: expression by tumor cells and activation of NK cells and macrophages. *Nat. Immunol.* **1**, 119-126 (2000).
37. Kraft, J.R. et al. Analysis of Qa-1(b) peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J. Exp. Med.* **192**, 613-624 (2000).
38. Cotterill, L.A. et al. Qa-1 interaction and T cell recognition of the Qa-1 determinant modifier peptide. *Eur. J. Immunol.* **27**, 2123-2132 (1997).
39. Rodolfo, M. et al. Cytotoxic T lymphocytes recognize tumor antigens of a murine colonic carcinoma by using different T-cell receptors. *Int J Cancer* **57**, 440-447 (1994).
40. Yang, J.C. & Perry-Lalley, D. The envelope protein of an endogenous murine retrovirus is a tumor-associated T-cell antigen for multiple murine tumors. *J Immunother* **23**, 177-183. (2000).
41. Van Hall, T. et al. The varicellovirus-encoded TAP inhibitor UL49.5 regulates the presentation of CTL epitopes by Qa-1b. *J Immunol* **178**, 657-662 (2007).
42. Martoglio, B. & Dobbertin, B. Signal sequences: more than just greasy peptides. *trends in Cell Biol.* **8**, 410-415 (1998).
43. Michaelsson, J. et al. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J. Exp. Med.* **196**, 1403-14 (2002).

44. Kambayashi, T. et al. The nonclassical MHC class I molecule Qa-1 forms unstable peptide complexes. *J. Immunol.* **172**, 1661-9 (2004).
45. Furukawa, H. et al. Cell surface expression of HLA-E molecules on PBMC from a TAP1-deficient patient. *Tissue Antigens* **53**, 292-5 (1999).
46. Palmisano, G.L. et al. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum. Immunol.* **66**, 1-12 (2005).
47. Robinson, P.J., Travers, P.J., Stackpoole, A., Flaherty, L. & Djaballah, H. Maturation of Qa-1b class I molecules requires b2-microglobulin but is TAP independent. *J. Immunol.* **160**, 3217-3224 (1998).
48. Monaco, E.L. et al. HLA-E: strong association with b2-microglobulin and surface expression in the absence of HLA class I signal sequence-derived peptides. *J. Immunol.* **181**, 5442-5450 (2008).
49. Snyder, H.L., Bacik, I., Yewdell, J.W., Behrens, T.W. & Bennink, J.R. Promiscuous liberation of MHC-class I-binding peptides from the C termini of membrane and soluble proteins in the secretory pathway. *Eur. J. Immunol.* **28**, 1339-46 (1998).
50. Vyas, J.M., Van der Veen, A.G. & Ploegh, H.L. The known unknowns of antigen processing and presentation. *Nat. Rev. Immunol.* **8**, 607-618 (2008).
51. Weinzierl, A.O. et al. Features of TAP-independent MHC class I ligands revealed by quantitative mass spectrometry. *Eur J Immunol* **38**, 1-8 (2008).
52. Suri, A., Walters, J.J., Levisetti, M.G., Gross, M.L. & Unanue, E.R. Identification of naturally processed peptides bound to the class I MHC molecule H-2Kb of normal and TAP-deficient cells. *Eur J Immunol* **36**, 544-557 (2006).
53. Sullivan, B.A., Kraj, P., Weber, D.A., Ignatowicz, L. & Jensen, P.E. Positive selection of a Qa-1-restricted T cell receptor with specificity for insulin. *Immunity* **17**, 95-105 (2002).
54. Ureta-Vidal, A., Firat, H., Perarnau, B. & Lemonnier, F.A. Phenotypal and functional characterization of the CD8+ T cell repertoire of HLA-A2.1 transgenic, H-2KbDbo double knockout mice. *J. Immunol.* **163**, 2555-2560 (1999).
55. Vugmeyster, Y. et al. Major histocompatibility complex (MHC) class I KbDb-/- deficient mice possess functional CD8+ T cells and natural killer cells. *Proc. Natl. Acad. Sci. USA* **95**, 12492-12497 (1998).
56. Perarnau, B. et al. Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+ T cell repertoire and anti-lymphocyte choriomeningitis virus cytolytic responses. *Eur. J. Immunol.* **29**, 1243-1252 (1999).
57. Lee, N., Goodlett, D.R., Ishitani, A., Marquardt, H. & Geraghty, D.E. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* **160**, 4951-4960 (1998).
58. van Hall, T. et al. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J. Exp. Med.* **192**, 483-494 (2000).
59. Bijker, M.S. et al. CD8+ CTL priming by exact peptide epitopes in incomplete Freund's adjuvant induces a vanishing CTL response, whereas long peptides induce sustained CTL reactivity. *J Immunol* **179**, 5033-40 (2007).

| SUPPLEMENTARY FIGURE LEGENDS

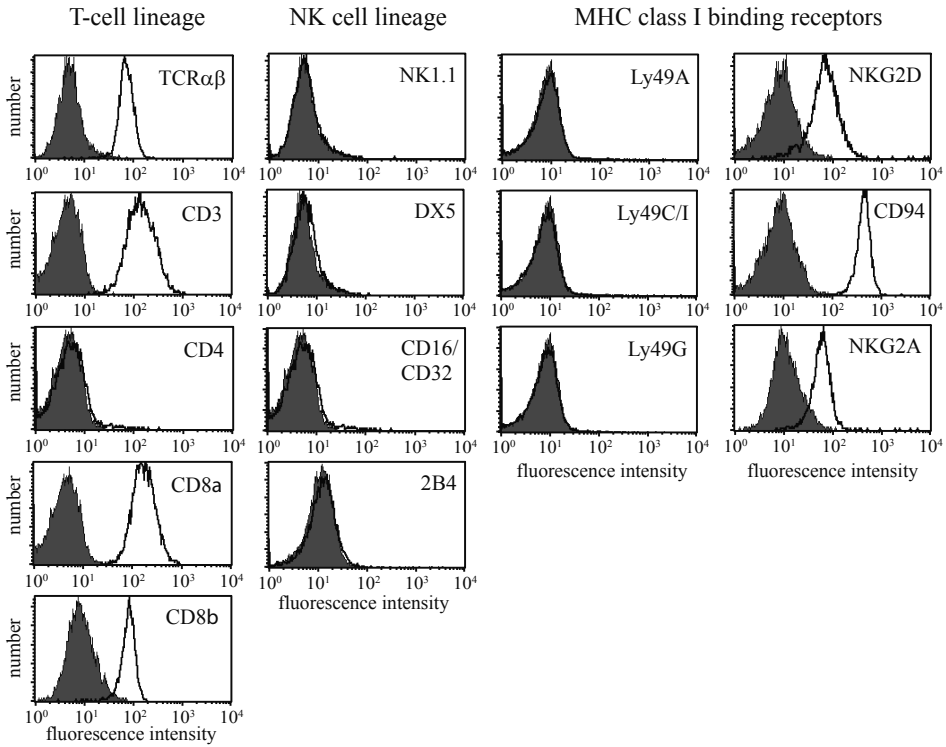


Figure S1. Qa-1^b-restricted T-cells display a conventional CD3⁺CD8⁺ phenotype. Flow cytometry analysis of Qa-1^b-restricted CTL revealed expression of T-cell lineage markers, but not NK cell markers. Conventional anti-tumor T-cells restricted by classical MHC class I molecules show comparable phenotype (not included in this graph). All markers were tested twice. One Qa-1^b-restricted T-cell clone is depicted, but all isolated T-cell lines and clones displayed similar phenotype.

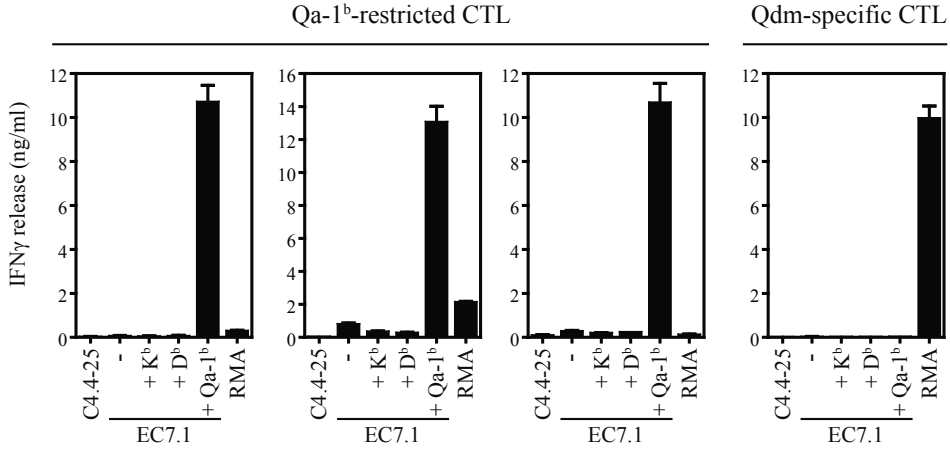


Figure S2. Selective recognition of Qa-1^b-expressing target cells. Isolated T-cell clones were tested against a panel of TAP-deficient lymphoma (EC7.1) cells. EC7.1 cells are also deficient in MHC class I³⁰ and were reconstituted with constructs encoding H-2D^b, -K^b or Qa-1^b. IFN γ production against Qa-1^b-positive EC7.1 cells by three independent T-cell clones. Control Qdm-specific CTL failed to recognize the Qa-1^b expressing targets, due to the absence of TAP. TAP-positive lymphoma cells (RMA) were recognized by this CTL clone (right panel). Means and standard deviations of triplicate wells are shown for one out of three comparable experiments.

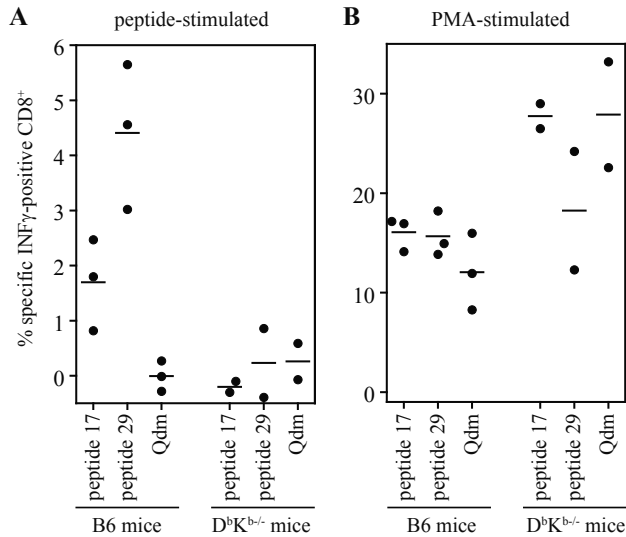


Figure S3. Immunogenicity of Qa-1^b-presented peptides in MHC class I knockout mice. H-2D^b/⁻K^b/⁻ and control B6 mice were immunized with two TAP-independent peptides FAPLPRLTL (peptide 17) and KCSVSIQVVDVNDNYPEL (peptide 29) and Qdm. PBL from blood samples were incubated overnight with selected peptides (panel A) or PMA/Ionomycin mitogens (panel B) and intracellular IFN γ production in CD8⁺ T cells was determined by flow cytometry. Frequencies of IFN γ producing T-cells were observed against Qa-1^b peptides in control B6 mice but not in MHC class I knockout mice. PMA induced activation of T cells from all mice. Each data point represents one mouse.

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THE OTHER JANUS FACE OF Qa-1 AND HLA-E: DIVERSE PEPTIDE REPERTOIRES IN TIMES OF STRESS

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| ABSTRACT

The non-polymorphic MHC molecule Qa-1 and its human counterpart HLA-E present monomorphic signal peptides to innate receptors and thereby regulate lymphocyte activity. Under stress, this peptide content is replaced with a surprisingly diverse repertoire of novel peptides that are associated with heat shock proteins, infectious agents or antigen processing defects.

| INTRODUCTION

MHC class I molecules serve to pickup and present intracellular antigens to circulating lymphocytes, which recognize these target structures after physical interaction with their T cell receptors (TCR). The immune system is thereby enabled to recognize and remove pathogens and transformed cells from the body. MHC class I molecules can be divided into classical Ia and non-classical Ib. The most striking feature of MHC class Ib molecules is their highly conserved nature. Whereas MHC class Ia genes display extensive polymorphism among their family members, the prototypic mouse MHC class Ib Qa-1 and its direct human homolog HLA-E are essentially nonpolymorphic. In this review we will refer to Qa-1 and HLA-E molecules as Qa-1/HLA-E. It is of interest that the Qa-1/HLA-E bound peptide content is normally highly restricted, comprising mostly of signal peptides derived from MHC Ia proteins. The resulting non variable peptide/MHC-Ib complexes constitute a ligand for the germ line encoded receptors CD94/NKG2. This function of Qa-1/HLA-E molecules is well established. However, in times of cellular stress, Qa-1/HLA-E shows its other face, like the Roman god Janus, typically portrayed with two faces in opposite directions (figure 1). This alternative face of Qa-1/HLA-E is associated with the replacement of bound signal peptides by a novel much more diverse repertoire of peptides, which can be sensed by $\alpha\beta$ TCRs. We have recently described two such novel peptide repertoires that were presented by Qa-1/HLA-E molecules to specific CD8⁺ T cells, which were related to sensing intracellular infection with mycobacteria, or to antigen processing defects in tumors^{1,2}. These two situations of intracellular stress illustrate that Qa-1/HLA-E probably serve a much broader function in adaptive immunity than thus far anticipated.



Figure 1. The Roman god Janus is always portrayed with two faces in opposite directions. Thereby, he represented an intermediary between two worlds. Qa-1/HLA-E molecules have an important function in innate immunity through interacting with innate, invariable CD94/NKG2 receptors. In addition, these conserved MHC class I molecules have a novel function in adaptive immunity since they can present diverse intracellular stress related peptide repertoires to hypervariable T cell receptors.

I Qa-1/HLA-E NORMALLY PRESENT MONOMORPHIC PEPTIDES IN THEIR BINDING GROOVES

The mouse Qa-1 protein is a nonclassical (class Ib) MHC molecule, encoded by the T23 locus and constitutes the mouse homolog of HLA-E. Like its human counterpart, Qa-1 is virtually nonpolymorphic, as sequencing of multiple mouse strains and even outbred mice revealed only two dominant allelic variants: Qa-1^a and Qa-1^b³. Strikingly, while human HLA-E and mouse Qa-1 share 73% homology at the protein level, their function and the non-variable peptides that they bind are surprisingly similar^{4,5}. Both MHC class Ib molecules accommodate signal peptides in their hydrophobic peptide binding groove, mostly derived from the leader sequences of classical MHC class Ia molecules. In the mouse, this peptide comprises the amino acid sequence AMAPRTLTL and has been called Qdm for 'Qa-1-determinant-modifier', since this factor had a strong influence on Qa-1 stability. It was a surprise-discovery that the Qa-1 bound signal peptide appeared to be encoded within the MHC I region⁶ and was derived from the classical class Ia molecules H-2D and H-2L. The other MHC class Ia protein in the mouse, H-2K, does not encode Qdm signal peptides and thus does not contribute to stabilization of Qa-1. Peptide elution studies of Qa-1 revealed that this Qdm peptide represented up to 70% of the total peptide content of Qa-1⁷. We recently confirmed the dominance of this one particular peptide using state-of-the-art mass-spectrometry². Although we also found other peptide sequences in Qa-1, these represented only minor amounts compared to Qdm. Not surprisingly, the AMAPRTLTL peptide seems to have an amino acid sequence optimal for binding and stabilization in the hydrophobic groove of Qa-1. Changes in amino acid residues in this 9-mer peptide invariably lead to decreased binding affinity^{8,9}. Compared to classical peptide/MHC complexes, though, the Qdm/Qa-1 complex is relatively unstable at the cell surface, and its expression therefore probably reflects the actual Qdm availability in the cell¹⁰.

As mentioned, humans share a nearly identical Qa-1 like MHC-Ib molecule, HLA-E. Only one single amino acid difference has been identified between the two HLA-E subtypes; this coding variation is located at position 107 (HLA-E*0101 has an arginine and E*0103 carries a glycine) on the loop between β -strands in the $\alpha 2$ domain of the heavy chain, outside the peptide binding groove^{11,12}. Both variants are indistinguishable in their structure and peptide binding features, although HLA-E^G homozygous cells seem to express higher levels of HLA-E at the cell surface^{5,12,13}. The frequency of both variants is equal amongst different populations suggesting balanced selection at the population level¹⁴. HLA-E molecules are filled with peptides with striking similarity to the mouse Qdm: some classical HLA class Ia proteins comprise signal peptides with the sequence VMAPRTLTL, differing at only one amino acid position from Qdm. Due to the extensive repertoire of human HLA class I, which comprises more than hundred allelic variants, the exact sequence of the human Qdm counterpart differs somewhat between different HLA class Ia backgrounds, especially at positions 2, 7 and 8 (Table I). Most of these signal peptides indeed efficiently

Table I. Qdm peptide sequences from HLA class I molecules

Locus	sequence	examples of HLA types	HLA-E binding
HLA-A	VMAPRTLIL	A*01, A*03	yes
	VMAPRTLVL	A*02, A*24	yes
HLA-B	VMAPRTLIL	B*07, B*08	yes
	VTAPRTLIL	B*13, B*27	no
HLA-C	VMAPRTLIL	Cw*02, Cw*15	yes
	VMAPRTLIL	Cw*03, Cw*04 ^a	yes
HLA-E	MVDGTLIL	E*01	no
HLA-F	MAPRTLIL	F*01	no
HLA-G	VMAPRTLFL	G*01	yes

^a except Cw*0402 subtype

bind to HLA-E, implying that they all support cell surface display of HLA-E. Exceptions are Qdm-like peptides from some HLA-B alleles, which carry a threonine amino acid at position 2, and HLA-E itself, which is too short and, in contrast to HLA-Ia molecules, does not contain the Qdm motif (Table I). A direct consequence is that HLA-E can not support its own stabilization at the cell surface, but is dependent on the *de novo* production of classical HLA Ia proteins¹⁵. All individuals in the human population carry at least one allele that provides a HLA-E-binding Qdm peptide. Interestingly, the leader sequence of the class Ib molecule HLA-G, which shows a highly restricted expression in placenta tissue, is one of the most optimal binding peptides¹⁶. HLA-G and HLA-E are amongst the few HLA molecules expressed in human trophoblasts, which invades the maternal part (the decidua) of the placenta¹⁷. Increased regulatory T cell numbers are observed in the placenta, mostly at this site of fetomaternal contact, maybe as a result of HLA-E. In any case, expression of HLA-G and HLA-E is associated with immunosuppression, needed to prevent rejection of the immunologically 'foreign' fetus¹⁷.

Another interesting aspect of the variations of Qdm-like peptides in the human population is the finding that these peptides behave as minor histocompatibility antigens (mHAGs). In mixed lymphocyte cultures, in which alloreactive T cell responses are induced, HLA-E restricted T cells can be detected that are specific for a 'non-self' Qdm peptide variant¹⁸⁻²⁰. These T cells might have been induced by viruses, as CMV has been shown to encode exactly the same Qdm-like peptide variants⁵.

I Qa-1/HLA-E AS REMOTE SENSORS OF THE INTRACELLULAR ANTIGEN PROCESSING MACHINERY

Qdm-like peptides are encoded by the N-terminal regions of MHC I molecules, which serve as hydrophilic segments to co-translationally target the newly synthesized proteins to the ER. Once in the ER, the hydrophilic N-terminus is released from the class I proteins by the dedicated enzyme signal peptidase (SPase)²¹. The native class I protein is further

chaperoned by calnexin and incorporated into a protein complex responsible for peptide loading, the so-called peptide loading complex (PLC)²². The chopped signal peptide of MHC I molecules, which is in most cases 24 amino acids long, remains in the ER membrane due to its hydrophobicity and needs liberation from this location by a second cleavage mediated by the signal peptide peptidase (SPPase), an aspartic protease, which acts within the ER membrane²³. This SPPase mediated cleavage results in release of the Qdm-containing part of the leader into the cytosol²¹. This cytosolic part is not just degraded, but gets a second life as peptide ligand of Qa-1/HLA-E. For this loading process, the precursor peptide is further sculpted by the proteasome to make it suitable for transport back into the ER by the peptide transporter TAP²⁴. In the ER, the PLC facilitates loading of the signal peptide into the groove of Qa-1/HLA-E, followed by transport to the cell surface^{15, 25, 26}. This complicated intracellular processing pathway of MHC class I leader peptides thus implicates a large set of proteins that is necessary for proper display of the class Ia-derived peptides in Qa-1/HLA-E: classical HLA Ia molecules, SPase, SPPase, proteasome, TAP and tapasin (figure 2). Deficiencies in each of these enzymes and chaperones have been demonstrated to result in failure to load and bind Qdm-like peptides. In other words, if one of these components is absent or disabled, the Qdm peptides are not presented anymore at the cell surface by Qa-1/HLA-E. This way, the Qa-1/HLA-E system elegantly reveals the integrity of the whole MHC I antigen processing pathway and is therefore often called 'remote sensors of proper class I presentation'. Malfunctioning cells, in cases of stress,

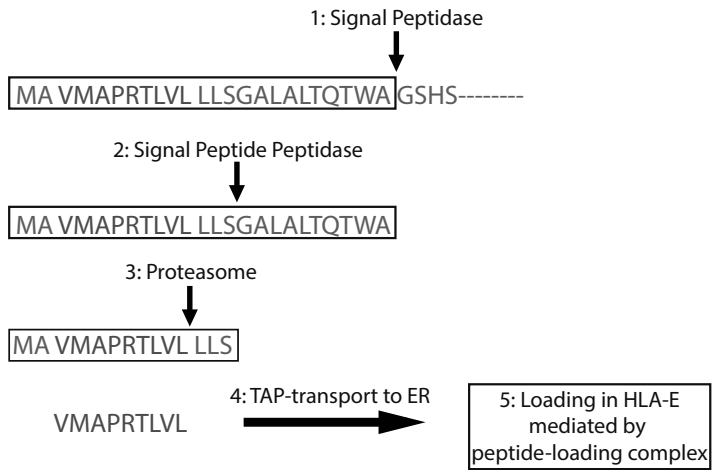


Figure 2. The processing requirements for proper presentation of Qdm peptides by Qa-1/HLA-E. The signal sequence of HLA-A2 molecules is taken as an example in this figure. The liberation of the Qdm peptide from its protein context needs at least three distinct proteolytic enzymes: signal peptidase (1) to remove the leader from HLA-A2 proteins, the signal peptide peptidase (2) to release the membrane associated peptide from the ER membrane and the proteasome (3) to generate the right C-terminus. It is unknown if the two N-terminal amino acids are removed by the proteasome or by aminopeptidases in the ER. The peptide loading complex involves at least tapasin, ERp57 and calreticulin.

infection or transformation, are thus marked for detection by immune cells, due to the lack of Qdm presentation. Intracellular pathogens are, however, able to sabotage this system and escape immune detection. Intriguingly, processing defects can lead to replacement of Qdm-like peptides with an alternative, surprisingly broad repertoire of peptides², supporting earlier indications that the binding motif of Qa-1/HLA-E molecules is much less stringent as generally appreciated²⁷. This novel repertoire is a clear witness of the other face of Qa-1, which will be discussed later.

| PRESENTATION OF Qdm PEPTIDES BY Qa-1/HLA-E IS SENSED BY CD94/NKG2 RECEPTORS

The importance of Qa-1/HLA-E is illustrated by the fact that Qdm-like peptides have been broadly determined across mammalian species, including mouse, rat, dog, cat, cow, gorilla, chimpanzee and human²⁸. The sequences of all these peptides are strikingly conserved and actually all efficiently bind HLA-E. The identification of the cognate receptor for this non-variable Qdm/MHC-Ib complex was a breakthrough and strongly boosted the unraveling of biological functions of this receptor-ligand pair. Tetramerized peptide/HLA-E complexes revealed the heterodimeric CD94/NKG2 surface molecules as the specific and selective receptors for HLA-E when filled with Qdm-like peptide^{29,30}. These receptors are invariant transmembrane molecules that are expressed by approximately half of all natural killer (NK) cells and activated CD8⁺ T cells³¹. The CD94 subunit makes most contact with peptide residues and the upward pointing alpha helices of HLA-E³²⁻³⁴. The NKG2 subunit hardly interacts with peptide residues, but contains the signal transduction domain³²⁻³⁴. NKG2 receptor genes are clustered in the NK-complex locus, and come in different flavors: NKG2A, NKG2C, and NKG2E. These three variants differ from each other only at few amino acids, which determine their affinity for CD94 and whether the specific CD94/NKG2 heterodimer acts as an inhibitory or activating receptor. NKG2C and NKG2E are less frequently expressed on lymphocytes, lack an ITIM motif in their cytoplasmic domains and associate with the activating adaptor molecule DAP12, whereas the inhibitory ITIM-bearing NKG2A recruits phosphatases, like SHP-1 to the signal transducing synapse^{5,31}. The overall structures of the inhibiting and activating receptors are quite similar and their 'footprint' on the peptide/HLA-E complex is also conserved³²⁻³⁴. However, the binding affinity of the activating receptors is considerably lower than that of the inhibitory ones.

Reactivity of NK cells is governed by the balance between incoming activating and inhibiting signals from surface receptors; the CD94/NKG2A receptor behaves like a strong silencer on NK cells. We have to keep in mind that the non-variable Qdm/HLA-E molecules are widely expressed by most nucleated cells of the body, so that NK cells, which constitute approximately 10% of lymphocytes in the blood continuously sense the presence of HLA-E. NK cells have to overcome this input of inhibitory signals by ligation of activating receptors. On the other hand, the absence of this Qdm/HLA-E complex on

target cells increases their susceptibility to killing. Hence, CD94/NKG2A enables NK cells to sense the absence of 'self' MHC on target cells in an indirect way and behaves like a remote sensor for NK cells to select target cells for lysis³¹. The discovery of such HLA-interacting receptors forms the molecular basis of the 'missing self' hypothesis that was postulated by Ljunggren and Kärre in the 1980's³⁵, which formulated that NK cells exhibit preference for tumor cells with low MHC class Ia expression. More recently, the important role of Qa-1 to protect normal cells from NK mediated lysis was illustrated by using genetically modified mice completely lacking Qa-1, or expressing Qa-1 molecules that failed to interact with CD94/NKG2A receptors³⁶. T lymphocytes from mice carrying an aberrant Qa-1 molecule failed to expand *in vivo* and were very sensitive for NK attack. Depletion of NK cells completely restored the clonal expansion upon antigen encounter³⁶. Furthermore, the Qdm peptide could be exploited to protect adoptively transferred cells^{37, 38}. Collectively, these studies indicate that immunosurveillance of NK cells is particularly efficient in the hematopoietic system and that sensing the Qdm presentation by Qa-1/HLA-E plays a key role in the regulating NK reactivity.

During the last decade it has become evident that also activating receptors contribute to the establishment of NK cell reactivity³¹. The activating partners of CD94 (NKG2C and NKG2E) engage the same Qdm/HLA-E complex as the inhibiting receptor NKG2A, although with lower affinity³⁹. It is still puzzling how two receptors recognizing the same ligand can have opposing functions. Nevertheless, the increase of NKG2C positive NK cells after CMV infection⁴⁰ eludes to an adaptation of the NK repertoire favoring protection against infectious agents.

| CD94/NKG2A RECEPTORS ARE ALSO EXPRESSED BY LYMPHOCYTES IN ADAPTIVE IMMUNITY

In addition to being expressed on NK cells, CD94/NKG2A receptors are also expressed on CD8⁺ T cells. Approximately 5% of circulating CD8⁺ T cells express both CD94 and NKG2A. The expression of these NK receptors is found on conventional antigen-specific T cells with classically rearranged TCR $\alpha\beta$ chains, and it has created considerable confusion to designate these cells as a separate subpopulation called NKT cells. The currently emerging picture instead is that this subpopulation of CD8⁺ T cells belongs to the antigen-experienced memory pool, and that the CD94/NKG2A receptors are induced by activation at T cell priming or by the cytokines IL-12, IL-15 or TGF β ³¹. These receptors might even directly promote the formation of memory cells, since CD94/NKG2A positive T cells seem to be less prone to activation-induced cell death⁴¹. Viruses are again likely involved in inducing CD94/NKG2A receptor expression on T cells: examples include the strong increase of this receptor on antigen-specific T cells after polyoma virus infection in a mouse model; the regulating effect of CD94/NKG2A on the cytolytic function of T cells in a Herpes Simplex virus model; and the steep increase of T cell subpopulations carrying this receptor after CMV seroconversion in

transplant patients⁴²⁻⁴⁴. In general, the frequencies of CD94/NKG2A expressing T cells are much higher than those expressing other NK-related receptors, e.g. NK1.1, DX5, Ly49 and KIR. Considering the large pool of T cells that expresses the Qdm/HLA-E-recognizing receptor in a given individual, these receptors might be regarded in fact as 'T cell associated receptors' instead of 'NK receptors'. Anyhow, the prevailing conclusion seems that expression of CD94/NKG2A on activated CD8+ T cells is common, and that QA-1/HLA-E detection by these adaptive lymphocytes might dampen overt cytotoxicity. Pathogens are apparently able to exploit this system to prevent total eradication.

I HCMV BRINGS ALONG ITS OWN Qdm PEPTIDE

Viruses and especially herpes viruses are able to inhibit MHC class I presentation as a strategy to hide from adaptive T cell immunity. For most herpes viruses, which induce live long persistent infections, this strategy is quite successful. The host is not capable of clearing herpes virus family members like CMV, EBV and HSV, and a high percentage of the human population is latently infected. In situations of immune suppression, such as post transplant patients and the elderly, herpes viruses can reactivate from latency into a productive phase and pathology. A multitude of dedicated viral proteins cooperate in this stealth technology⁴⁵. The important bottleneck of the intracellular processing machinery is the peptide transporter TAP: several herpes virus proteins have been identified with a TAP-destabilizing function. TAP deficiency will directly block the presentation of Qdm-like peptides by HLA-E, since its loading is TAP dependent. This poses the question as to why do such infected cells in the body survive NK controlled immunosurveillance, since an effective shutdown of MHC class Ia presentation should sensitize these cells for NK attack. Part of the answer was provided by studying the HLA-E cell surface display on human CMV infected cells. Infection with CMV indeed led to the decrease of Qdm-like peptides in HLA-E, due to the action of immune evasion molecules that target the display of classical HLA. However, HLA-E cell surface presentation was retained by loading a peptide from one of the viral proteins, UL40^{46,47}. The signal peptide from UL40 contains an exact Qdm-like sequence and the supply of this viral peptide is able to substitute for the lack of natural leader peptides from classical MHC class Ia. Of course, loading of the UL40 leader peptide needed to be TAP-independent since this transporter is blocked by US6 in CMV infected cells⁴⁵. Intriguingly, the flanking amino acids of the UL40 signal peptide differs from that of the class Ia and it is exactly this segment that specifies the SPPase cleavage site⁴⁸. The final result is that the processed UL40 leader directly falls back in the lumen of the ER, instead of arriving in the cytosol where it would need to regain access to the ER via TAP. In this manner, CMV bypasses the normal HLA-E loading system by developing a shortcut which is necessary because of its own obstruction of the classical peptide presentation pathway. HCMV is not the only virus that brings along an own Qdm peptide, but this example clearly illustrates that viral pathogens have developed dedicated mechanisms to protect themselves for NK recognition via the HLA-E pathway.

I CELLULAR STRESS REMOVES Qdm PEPTIDES FROM Qa-1/HLA-E MOLECULES

Thus far, we discussed Qa-1/HLA-E molecules carrying signal peptides derived from classical MHC Ia molecules, which are the dominant constituents of the HLA-E binding groove. These Qdm-like peptides bind with relatively high affinity to HLA-E and render this molecule an essentially non-variable structure, in contrast to other MHC class I a/peptide molecules. On this side of the Janus face Qa-1/HLA-E looks and acts like an innate molecule with an important role in NK- and T-cell reactivity via interacting with the conserved receptors CD94/NKG2. However, in situations of cellular stress and induced expression of heat-shock proteins, HLA-E replaces its Qdm-like peptides by alternative peptides such as heat-shock protein-derived peptides^{49,50}. These peptides share some sequence homology with Qdm, but are clearly distinct. The most interesting aspect of this finding was that the CD94/NKG2A receptors failed to recognize HLA-E molecules binding an hsp60 peptide. As a direct consequence, the stressed cells were no longer protected from NK cell lysis⁵⁰. The CD94/NKG2A receptor was indeed shown to directly interact with side residues of the Qdm peptide and changes at certain amino acid positions, especially p5 and p8, results in failure to bind Qdm/MHC complexes^{8, 33, 34}. Thus, this NK receptor is truly Qdm peptide-specific. Interestingly, the crystal structure of a Qdm-specific alloreactive TCR showed an interface with the Qdm/HLA-E ligand that was quite similar to that of CD94/NKG2A⁵¹. In that sense, the heterodimer CD94/NKG2A shares unique characteristics with the peptide-sensing TCRαβ chains of T lymphocytes.

I Qa-1/HLA-E AS THE RECOGNITION ELEMENT FOR T CELL RECEPTORS: SELF PEPTIDES

The other side of the Janus face of Qa-1/HLA-E shows a MHC class I molecule presenting a surprisingly diverse repertoire of peptides. At first glance, the extensive variability of TCRs and the high specificity for their peptide targets do not associate well with the knowledge we have on the nonpolymorphic Qa-1/HLA-E molecules. The fact that TCRs are able to recognize Qdm peptide variants in Qa-1/HLA-E was evidently shown in alloreactive responses, as described above. Furthermore, one of the hsp60 peptides that can replace Qdm is also a target for TCRs^{49,52}. These Qa-1-restricted CD8⁺ T cells cross-react to the *Salmonella* derived GroEl peptide, an example of molecular mimicry, and might explain autoimmune pathology in reactive arthritis⁵². Examples of other 'self' peptides presented by Qa-1/HLA-E are those that arise from TCR chains, multidrug resistance-associated protein and those that are associated with antigen processing defects^{2, 53, 54}. The peptides that are derived from V-segments of TCRs are targeted by a population of CD8⁺ regulatory T cells and mediate alleviation of autoimmune disorders via dampening the pathogenic CD4⁺ T cells⁵⁵.

Recently, we described a diverse repertoire of Qa-1-presented peptides that is associated with antigen processing defects^{2, 56}. As mentioned above, the presentation of Qdm is strictly dependent on the integrity of the antigen processing machinery (figure 2).

In cases where one of the processing components is downregulated or mutated, as found in a high frequency in tumor cells, Qdm does not emerge at the cell surface. Interestingly, the surface expression of Qa-1/HLA-E molecules is relatively preserved^{10, 57, 58}, most likely due to the replacement with an unique set of 'self' peptides. The novel peptides that replace Qdm in TAP-negative cells originated from normal proteins within the cell and constitute epitopes for CD8⁺ T cells, since they represent neo-antigens that are normally not displayed by Qa-1^{2, 56}. This T cell subpopulation was reactive against tumors with processing deficiencies like TAP defects, and our findings strongly suggested that the novel Qa-1 peptides are suitable for vaccination-based therapies, benefiting from the selective activation of the Qa-1-restricted effector CD8⁺ T cells. Interestingly, multiple tumors of different MHC haplotypes were recognized by this T cell subset, due to the conserved nature of Qa-1. Moreover, tumors of different tissue origin were targets, because Qa-1 is expressed by most nucleated cells of the body. In contrast to what is sometimes thought, Qa-1/HLA-E proteins are not restricted to the hematopoietic system, but can be detected ubiquitously. Together with the organization of the Human Protein Atlas (www.proteinatlas.org), we charted all human tissues for HLA-E expression and observed an expression pattern quite similar to that of HLA class Ia molecules.

Importantly, the novel peptide repertoire that we determined in Qa-1 did not fulfill the binding motif as previously described for Qa-1 and HLA-E^{8, 9, 59}. The major anchor binding pockets for the peptides, positions 2 and 9, are optimally accommodating leucine/methionine and aliphatic amino acids, respectively. This binding motif is not unique for Qa-1/HLA-E and is actually similar to the binding motif for the classical HLA-A2 allele^{60, 61}. Our study seems to indicate that binding requirements of Qa-1/HLA-E are not so strict, as suggested before²⁷. Together, these findings portrait Qa-1/HLA-E as a highly flexible MHC class I molecule with an important function to present a broad peptide repertoire to $\alpha\beta$ T cells, completely different from the non-variable Qdm structure described above.

I HLA-E AS THE RECOGNITION ELEMENT FOR T CELL RECEPTORS: PATHOGEN-DERIVED PEPTIDES

The adaptive face of Qa-1/HLA-E is also revealed in studying infectious pathogens. Several pathogens have been reported to encode HLA-E binding peptides: *Salmonella*, *Listeria monocytogenes*, LCMV, HIV, HBV, EBV, HCV and *Mycobacterium tuberculosis* (*Mtb*)^{4, 5}. This long list points at the existence of a subset of CD8⁺ T cells that sense pathogen derived antigens in Qa-1/HLA-E molecules with their TCRs. How large this population of non-classically restricted cells is, remains elusive and in addition to Qa-1/HLA-E, also other non-classical presentation molecules have been associated with T cell recognition. The T cell repertoire in mice that lack classical MHC class Ia molecules suggests that approximately 10% of the CD8⁺ pool is restricted by non-classical MHC class Ib molecules⁶².

First indications that *Mtb* antigens are presented by the conserved HLA-E molecule date back to 2002⁶³. The identity of the peptide-epitopes recognized by the human HLA-E-

restricted CD8⁺ T-cell clones, however, remained elusive. Recently, we studied CD8⁺ T cell recognition of peptides that contained *in silico* predicted HLA-E epitopes for recognition by human T cells¹. Newly identified *Mtb* peptide epitopes were found by measuring proliferation of human CD8⁺ T-cells from PPD-responsive adults and BCG-vaccinated infants. The responding cells had cytotoxic activity, and lysed target cells in a peptide-specific and HLA-E dependent fashion, strongly suggesting peptide/HLA-E engagement by the TCR. Live mycobacterium infected monocytes were killed by these effector T cells, demonstrating proper antigen presentation by infected target cells. Strikingly, in addition to their cytolytic capacity, these *Mtb*-reactive T-cell lines also exhibited strong immunosuppressive properties, as they inhibited proliferation of unrelated responder T-cells. This suppression was dose-dependent, required cell-cell contact and was mediated, at least in part, by membrane bound TGFβ1 (mTGFβ1)¹. T cell lines and clones derived from limiting dilution assays demonstrated that the dual activity could be, but was not necessarily mediated by the same HLA-E/peptide induced T cells. So, both types of HLA-E mediated immune reactivity might play a role during infection. The relative contribution of these two activities to resolution and persistence of the mycobacteria remains to be studied. We need to decipher if HLA-E mediated antigen presentation in tuberculosis preferentially activates T-cells with regulatory properties as compared to T-cells with cytolytic activity. Interesting in this context is the finding that mycobacteria reside in phagosomes of antigen presenting cells, especially macrophages, and that these phagosomes are enriched in HLA-E. This at least suggests a relatively large presentation of mycobacterial peptides on human HLA-E⁶⁴. Recent mouse experiments have indicated that regulatory T cells are “bad guys” in tuberculosis when it comes to control of bacterial growth, especially early during infection⁶⁵⁻⁶⁷. This balance between effector and regulatory immunity in the context of HLA-E might allow partial clearance of pathogen from the host, thus providing sufficient levels of protection while avoiding excessive inflammation and pathology, but at the expense of pathogen persistence and chronic infection.

In addition to its potential contribution to immune suppression via regulatory T cells, HLA-E might offer unique opportunities to embark on vaccination strategies for tuberculosis, due to the low level of variation in HLA-E in human populations. *Mtb* vaccination strategies that focus on HLA-E might be beneficial especially in settings with highly prevalent co-infection with HIV. In Southern Africa alone approximately 70% of TB-patients are also HIV-infected. HIV infection down-regulates expression of classical HLA-A and -B molecules through its Nef proteins, thus decreasing antigen presentation capacities⁶⁸. In contrast, however, HLA-E is resistant to HIV-nef-mediated down-regulation due to a single amino acid substitution in the HLA-E cytoplasmic tail⁶⁹. Thus, while HIV might inhibit antigen presentation by HLA class Ia molecules on monocytes and macrophages⁷⁰, HLA-E dependent antigen presentation is likely less affected by HIV co-infection. Thus, targeting *Mtb*-specific HLA-E-restricted immunity by vaccination may be a novel and advantageous approach.

I SUMMARY AND PROSPECTS

We have reviewed recent studies which demonstrate that Qa1/HLA-E molecules reveal different faces and phases in NK- and T-cell immunity, with important implications for immuno-surveillance and immune regulation: first, Qa1/HLA-E molecules interact in an ‘innate’ immune fashion, through one molecular face, with members of the heterodimeric CD94/NKG2 receptor family. The NKG2 member determines whether ligation leads to inhibiting (NKG2A) or stimulating (NKG2C) signals to NK- and T-cells. The second role of Qa1/HLA-E molecules has only more recently become appreciated, notably their capacity to present a highly variable array of antigenic peptides to T cell receptors on CD8⁺ T cells. Under usual homeostatic conditions the peptide repertoire bound to Qa1/HLA-E is highly restricted, and largely confined to self MHC signal sequences. However, under cellular stress conditions such as malignant cell transformation or intracellular infection with chronic pathogens, a new peptide repertoire is loaded onto Qa1/HLA-E molecules and is presented to CD8⁺ T cells with cytotoxic activity, able to kill tumor cells or infected cells. A brief overview of these different roles is summarized in Table II.

These new findings not only significantly extend the role of Qa1/HLA-E in NK and CD8⁺ T cell surveillance, but due to the extremely limited genetic polymorphism of Qa1/HLA-E also offer interesting perspectives for vaccination with high affinity peptides in cancer and infectious disease. In both cases efforts should similarly evolve around identifying the relevant antigens, which potentially can be useful in therapeutic as well as prophylactic vaccination.

Clearly, more research is needed to dissect the role of Qa1/HLA-E bound peptides in cancer and infection. One question concerns their precise vaccine potential, that is the efficiency by which peptide-specific CD8⁺ T cells are capable of detecting and eradicating MHC-class Ia downregulation on tumor cell escape variants. The potential adverse effects need to be assessed as well, such as induction of auto-immune like phenomena in the case that such peptides are also expressed during “normal” cellular stress during local inflammation.

Another key question is what functional roles Qa1/HLA-E peptide-specific CD8⁺ T cells exactly play: next to CTL, it is clear that also Tregs are induced. What dictates the balance in immune effector vs. regulatory mechanism at this level? Are different CD8⁺ T cell subsets involved with either unique CTL or Treg activity, or is dual functionality the common rule?

Table II. Peptides presented by Qa-1/HLA-E to innate and adaptive immune receptors

Immune receptor	peptide origin	self or non-self
CD94/NKG2	Qdm from self MHC Ia	self
	Qdm from HCMV	non-self
T cell receptor	Qdm from allo MHC Ia	non-self
	hsp60 and GroEl	self and non-self
	Vβ TCR	self
	associated with processing defects	induced self
	M. tuberculosis	non-self

How can we identify and manipulate these functional activities for therapeutic purposes? Clearly, the recent studies we have discussed here shed exciting new light on the potential new role of Qa1/HLA-E mediated antigen presentation in cancer and infection.

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I REFERENCES

- Joosten, S.A. et al. Mycobacterium tuberculosis peptides presented by HLA-E molecules are targets for human CD8 T-cells with cytotoxic as well as regulatory activity. *PLoS Pathog* **6**, e1000782 (2010).
- Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-21 (2010).
- Hermel, E. et al. Polymorphism and conservation of the genes encoding Qa1 molecules. *Immunogenetics* **56**, 639-649 (2004).
- Jensen, P.E., Sullivan, B.A., Reed-Loisel, L.M. & Weber, D.A. Qa-1, a nonclassical class I histocompatibility molecule with roles in innate and adaptive immunity. *Immunol. Res.* **29**, 81-92 (2004).
- Rodgers, J.R. & Cook, R.G. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5**, 459-471 (2005).
- Aldrich, C.J. et al. Identification of a Tap-dependent leader peptide recognized by alloreactive T cells specific for a class Ib antigen. *Cell* **79**, 649-658 (1994).
- DeCloux, A., Woods, A.S., Cotter, R.J., Soloski, M.J. & Forman, J. Dominance of a single peptide bound to the class Ib molecule, Qa-1b. *J. Immunol.* **158**, 2183-2191 (1997).
- Kraft, J.R. et al. Analysis of Qa-1(b) peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J. Exp. Med.* **192**, 613-624 (2000).
- Miller, J.D. et al. Analysis of HLA-E peptide-binding specificity and contact residues in bound peptide required for recognition by CD94/NKG2. *J Immunol* **171**, 1369-1375. (2003).
- Kambayashi, T. et al. The nonclassical MHC class I molecule Qa-1 forms unstable peptide complexes. *J. Immunol.* **172**, 1661-9 (2004).
- Grimsley, C. et al. Definitive high resolution typing of HLA-E allelic polymorphisms: identifying potential error in existing allele data. *Tissue Antigens* **60**, 206-212 (2002).
- Strong, R.K. et al. HLA-E allelic variants. Correlating differential expression, peptide affinities, crystal structures, and thermal stabilities. *J. Biol. Chem.* **278**, 5082-90 (2003).
- O'Callaghan, C.A. et al. Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E. *Mol. Cell* **1**, 531-541 (1998).
- Grimsley, C. & Ober, C. Population genetic studies of HLA-E: evidence for selection. *Hum Immunol* **52**, 33-40 (1997).
- Braud, V.M., Allan, D.S.J., Wilson, D. & McMichael, A.J. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* **8**, 1-10 (1997).
- Lee, N., Goodlett, D.R., Ishitani, A., Marquardt, H. & Geraghty, D.E. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* **160**, 4951-4960 (1998).
- Hunt, J.S. Stranger in a strange land. *Immunol Rev* **213**, 36-47 (2006).
- Pietra, G. et al. HLA-E-restricted recognition of cytomegalovirus-derived peptides by human CD8+ cytolytic T lymphocytes. *Proc Natl Acad Sci U S A* **100**, 10896-901 (2003).
- Salerno-Goncalves, R., Fernandez-Vina, M., Lewinsohn, D.M. & Szein, M.B. Identification of a human HLA-E-restricted CD8+ T cell subset in

- volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J Immunol* **173**, 5852-62 (2004).
20. Mazzarino, P. et al. Identification of effector-memory CMV-specific T lymphocytes that kill CMV-infected target cells in an HLA-E-restricted fashion. *Eur J Immunol* **35**, 3240-7 (2005).
 21. Martoglio, B. & Dobbertein, B. Signal sequences: more than just greasy peptides. *trends in Cell Biol.* **8**, 410-415 (1998).
 22. Cresswell, P., Ackerman, A.L., Giodini, A., Peaper, D.R. & Wearsch, P.A. Mechanisms of MHC class I-restricted antigen processing and cross-presentation. *Immunol Rev* **207**, 145-57 (2005).
 23. Lemberg, M.K., Bland, F.A., Weihofen, A., Braud, V.M. & Martoglio, B. Intramembrane proteolysis of signal peptides: an essential step in the generation of HLA-E epitopes. *J. Immunol.* **167**, 6441-6 (2001).
 24. Bland, F.A., Lemberg, M.K., McMichael, A.J., Martoglio, B. & Braud, V.M. Requirement of the proteasome for the trimming of signal peptide-derived epitopes presented by the nonclassical major histocompatibility complex class I molecule HLA-E. *J. Biol. Chem.* **278**, 33747-52 (2003).
 25. Bai, A., Broen, J. & Forman, J. The pathway for processing leader-derived peptides that regulate the maturation and expression of Qa-1. *Immunity* **9**, 413-421 (1998).
 26. Li, L. et al. Differential requirement for tapasin in the presentation of leader- and insulin-derived peptide antigens to Qa-1b-restricted CTLs. *J. Immunol.* **173**, 3707-15 (2004).
 27. Stevens, J., Joly, E., Trowsdale, J. & Butcher, G.W. Peptide binding characteristics of the non-classical class Ib MHC molecule HLA-E assessed by a recombinant random peptide approach. *BMC Immunol* **2**, 5 (2001).
 28. Kurepa, Z., Hasemann, C.A. & Forman, J. Qa-1(b) binds conserved class I leader peptides derived from several mammalian species. *J. Exp. Med.* **188**, 973-978 (1998).
 29. Braud, V.M. et al. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* **391**, 795-799 (1998).
 30. Vance, R.E., Jamieson, A.M. & Raulet, D.H. Recognition of the class Ib molecule Qa-1b by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J. Exp. Med.* **190**, 1801-1812 (1999).
 31. Lanier, L.L. NK cell recognition. *Annu. Rev. Immunol.* **23**, 225-274 (2005).
 32. Sullivan, L.C. et al. The heterodimeric assembly of the CD94-NKG2 receptor family and implications for human leukocyte antigen-E recognition. *Immunity* **27**, 900-11 (2007).
 33. Kaiser, B.K., Pizarro, J.C., Kerns, J. & Strong, R.K. Structural basis for NKG2A/CD94 recognition of HLA-E. *Proc Natl Acad Sci U S A* **105**, 6696-701 (2008).
 34. Petrie, E.J. et al. CD94-NKG2A recognition of human leukocyte antigen (HLA)-E bound to an HLA class I leader sequence. *J Exp Med* **205**, 725-735 (2008).
 35. Ljunggren, H.G. & Karre, K. In search of the 'missing self': MHC molecules and NK cell recognition. *Immunol. Today* **11**, 237-244 (1990).
 36. Lu, L. et al. Regulation of activated CD4+ T cells by NK cells via the Qa-1-NKG2A inhibitory pathway. *Immunity* **26**, 593-604 (2007).
 37. Colmenero, P. et al. Qa-1(b)-dependent modulation of dendritic cell and NK cell cross-talk in vivo. *J Immunol* **179**, 4608-15 (2007).
 38. Persson, C.M., Assarsson, E., Vahlne, G., Brodin, P. & Chambers, B.J. Critical role of Qa1b in the protection of mature dendritic cells from NK cell-mediated killing. *Scand J Immunol* **67**, 30-6 (2008).
 39. Vales-Gomez, M., Reyburn, H.T., Erskine, R.A., Lopez-Botet, M. & Strominger, J.L. Kinetics and peptide dependency of the binding of the inhibitory NK receptor CD94/NKG2A and the activating receptor CD94/NKG2C to HLA-E. *EMBO J.* **18**, 4250-4260 (1999).
 40. Guma, M. et al. Expansion of CD94/NKG2C+ NK cells in response to human cytomegalovirus-infected fibroblasts. *Blood* **107**, 3624-31 (2006).
 41. Gunturi, A., Berg, R.E. & Forman, J. Preferential survival of CD8 T and NK cells expressing high levels of CD94. *J Immunol* **170**, 1737-45 (2003).
 42. Moser, J.M., Gibbs, J., Jensen, P.E. & Lukacher, A.E. CD94-NKG2A receptors regulate antiviral CD8+ T cell responses. *Nature immunol.* **3**, 189-195 (2002).
 43. Suvas, S., Azkur, A.K. & Rouse, B.T. Qa-1b and CD94-NKG2a interaction regulate cytolytic activity of herpes simplex virus-specific memory CD8+ T cells in the latently infected trigeminal ganglia. *J Immunol* **176**, 1703-11 (2006).
 44. van Stijn, A. et al. Human cytomegalovirus infection induces a rapid and sustained change in the expression of NK cell receptors on CD8+ T cells. *J Immunol* **180**, 4550-60 (2008).
 45. Griffin, B.D., Verweij, M.C. & Wiertz, E.J. Herpesviruses and immunity: the art of evasion. *Vet Microbiol* **143**, 89-100 (2010).
 46. Tomasec, P. et al. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by

- human cytomegalovirus gpUL40. *Science* **287**, 1031 (2000).
47. Wang, E.C. et al. UL40-mediated NK evasion during productive infection with human cytomegalovirus. *Proc Natl Acad Sci U S A* **99**, 7570-5 (2002).
 48. Lemberg, M.K. & Martoglio, B. Requirements for signal peptide peptidase-catalyzed intramembrane proteolysis. *Mol. Cell* **10**, 735-744 (2002).
 49. Davies, A. et al. A peptide from heat shock protein 60 is the dominant peptide bound to Qa-1 in the absence of the MHC class Ia leader sequence peptide Qdm. *J. Immunol.* **170**, 5027-33 (2003).
 50. Michaelsson, J. et al. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J. Exp. Med.* **196**, 1403-14 (2002).
 51. Hoare, H.L. et al. Structural basis for a major histocompatibility complex class Ib-restricted T cell response. *Nat Immunol* **7**, 256-264 (2006).
 52. Lo, W.-F. et al. Molecular mimicry mediated by MHC class Ib molecules after infection with Gram-negative pathogens. *Nat. Medicine* **6**, 215-218 (2000).
 53. Jiang, H. & Chess, L. The specific regulation of immune responses by CD8+ T cells restricted by the MHC class Ib molecule, Qa-1. *Annu. Rev. Immunol.* **18**, 185-216 (2000).
 54. Wooden, S.L., Kalb, S.R., Cotter, R.J. & Soloski, M.J. HLA-E binds a peptide derived from the ATP-binding cassette transporter multidrug resistance-associated protein 7 and inhibits NK cell-mediated lysis. *J. Immunol.* **175**, 1383-1387 (2005).
 55. Lu, L., Werneck, M.B.F. & Cantor, H. The immunoregulatory effects of Qa-1. *Immunol Rev* **212**, 51-59 (2006).
 56. Van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Medicine* **12**, 417-424 (2006).
 57. Furukawa, H. et al. Cell surface expression of HLA-E molecules on PBMC from a TAP1-deficient patient. *Tissue Antigens* **53**, 292-5 (1999).
 58. Palmisano, G.L. et al. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum. Immunol.* **66**, 1-12 (2005).
 59. Braud, V., Jones, E.Y. & McMichael, A. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur J Immunol* **27**, 1164-9 (1997).
 60. Sensi, M. et al. Peptides with dual binding specificity for HLA-A2 and HLA-E are encoded by alternatively spliced isoforms of the antioxidant enzyme peroxiredoxin 5. *Int Immunol* **21**, 257-68 (2009).
 61. Nattermann, J. et al. The HLA-A2 restricted T cell epitope HCV core 35-44 stabilizes HLA-E expression and inhibits cytotoxicity mediated by natural killer cells. *Am J Pathol* **166**, 443-53 (2005).
 62. Perarnau, B. et al. Single H2Kb, H2Db and double H2KbDb knockout mice: peripheral CD8+ T cell repertoire and anti-lymphocyte choriomeningitis virus cytolytic responses. *Eur. J. Immunol.* **29**, 1243-1252 (1999).
 63. Heinzel, A.S. et al. HLA-E-dependent presentation of Mtb-derived antigen to human CD8+ T cells. *J. Exp. Med.* **196**, 1473-1481 (2002).
 64. Grotzke, J.E. et al. The Mycobacterium tuberculosis phagosome is a HLA-I processing competent organelle. *PLoS Pathog* **5**, e1000374 (2009).
 65. Kursar, M. et al. Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis. *J Immunol* **178**, 2661-5 (2007).
 66. Scott-Browne, J.P. et al. Expansion and function of Foxp3-expressing T regulatory cells during tuberculosis. *J Exp Med* **204**, 2159-69 (2007).
 67. Shafiani, S., Tucker-Heard, G., Kariyone, A., Takatsu, K. & Urdahl, K.B. Pathogen-specific regulatory T cells delay the arrival of effector T cells in the lung during early tuberculosis. *J Exp Med* **207**, 1409-20 (2010).
 68. Collins, K.L., Chen, B.K., Kalams, S.A., Walker, B.D. & Baltimore, D. HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* **391**, 397-401 (1998).
 69. Cohen, G.B. et al. The selective downregulation of class I major histocompatibility complex proteins by HIV-1 protects HIV-infected cells from NK cells. *Immunity* **10**, 661-71 (1999).
 70. Noursadeghi, M., Katz, D.R. & Miller, R.F. HIV-1 infection of mononuclear phagocytic cells: the case for bacterial innate immune deficiency in AIDS. *Lancet Infect Dis* **6**, 794-804 (2006).

7

DISCUSSION

| MODEL OF TEIPP-PEPTIDE IMMUNOGENICITY

The study of immunity against TAP-deficient cells led to the discovery of peptides presented by such TAP-deficient cells. Some of these peptides constituted antigens to CTL and these CTL only recognized TAP-deficient cells but not normal cells¹. These peptides were called “T-cell epitopes associated with impaired peptide processing” (TEIPP). Therefore, TEIPP corresponds to immunogenic peptides that are presented only in cases of processing deficiency and not by normal cells. Also, they are derived from normal housekeeping proteins and therefore constitute normal self-peptides (non-mutated, not pathogen- or tumor-specific). The immunogenicity of TEIPP peptides exists because they are not presented by normal cells including the thymus. Therefore, there is no central tolerance against these peptides and the TEIPP-CTL can be released in the periphery. Because of this TEIPP peptides can be regarded as neo-antigens.

Why are TEIPPs not presented by MHC-I of normal cells?

TEIPPs are present in normal cells

Since the peptides recognized by TEIPP-specific CTL are derived from housekeeping proteins, it became interesting to understand why TEIPPs are not presented by processing-intact cells. Convincing data shows that TEIPP peptides are actually produced within processing intact cells, but somehow are not or not sufficiently presented by their surface MHC-I molecules. Previous studies have shown that TAP-positive tumor cells can be rendered sensitive to TEIPP-specific CTL after treatment with proteasome inhibitors (e.g. lactacystin) or by deficiency of other APM components (e.g. tapasin)¹. Furthermore, introduction of viral TAP inhibitors in DCs brings these internal peptides to the cell surface for MHC-I presentation². Taking the Trh4-derived TEIPP peptide as a model, we have analyzed the expression of the *Trh4* gene in several epithelial populations isolated from wild-type and TAP1-ko mice (data presented in chapter 2). This analysis revealed the same level of RNA transcripts between the normal and knockout populations, suggesting that the proteins levels would also be comparable. The liberation of the Trh4 peptide is performed by SPP which is active in TAP-positive as well as TAP-negative targets. Therefore, the generation of the Trh4 peptide is comparable between normal and TAP-deficient cells. But while APC from TAP-ko mice were recognized by Trh4-specific CTL, the wild-type-derived APC were not. This shows that there is difference in the MHC-I presentation of this TEIPP but not in expression. Additionally, enhancement of *Trh4* expression in TAP-positive RMA cells by gene transfer, using vectors with a strong heterologous promoter, resulted in presentation of the Trh4-derived peptide. Therefore, there is strong evidence for the production of Trh4-derived peptide and other TEIPP in normal cells and that the failure to present on normal cells has to be explained by failure to load in MHC-I.

Peptide competition hypothesis

What does prevent TEIPP from being presented in normal cells? One hypothesis is that binding affinity of TEIPP peptides might be lower than other peptides and therefore

loose competition with high affinity peptides that arrive in the ER via the TAP-transport. However, we found that the Trh4-derived TEIPP peptide has a good binding affinity and is capable of forming stable D^b/Trh4 complexes, showing that it is an optimal MHC-I ligand (data presented in chapter 2). Nevertheless, it is still possible that other TEIPPs are indeed poor binders and that explains their absence from normal cells, but so far we did not find evidence for that. To understand the absence of the Trh4-peptide from normal cells we decided to look for the limiting step of Trh4 peptide presentation. We found that increasing the availability of MHC-I molecules in TAP-deficient cells did not augment the presentation of Trh4, indicating that TAP-deficiency *per se* creates the opportunity for all available Trh4 peptides to be loaded, irrespective of MHC-I levels. This suggested that the peptide quantity available for loading into MHC-I was the limiting factor. Indeed, increasing the quantity of the Trh4 peptide in TAP-deficient cells increased the presentation of Trh4 and in normal cells resulted in the presentation of Trh4 whereas it does not normally occur. Interestingly, increasing recognition by the TEIPP-specific CTL clone correlated with fold of overexpression in target cells showing that the D^b/Trh4 complexes increased proportionally, implying that TAP function constitute a strong barrier for TEIPP. A vast excess of TAP-pumped competing peptides is present in the ER and prevents TEIPP loading to MHC-I. These findings argue that TEIPP peptides, such as Trh4, are underrepresented in the ER and under normal conditions fail to find peptide-receptive MHC-I molecules in the ER (Figure 1). We have confirmed that the C-terminal Trh4 peptide is liberated in the ER, by the activity of SPP, as described in chapter 3. The study of a human TEIPP antigen corroborates these findings. This antigenic peptide is encoded by the human *CALCA* gene and derives from the signal sequence of preprocalcitonin (ppCT) protein. This peptide is liberated in the ER lumen by sequential cleavage with SP and SPP, independently from proteasomes and TAP³. The presentation of the ppCT peptide to specific-CTL was found in human lung and medullary thyroid carcinomas (MTCs) that had very low expression of TAP. Presentation of the ppCT peptide occurred also in normal non-transformed cells, such as DCs, after knockdown of TAP. Overexpression of the *CALCA* gene in DCs and TAP-positive tumor cells resulted in recognition by the specific CTL clone⁴. Thus, these facts support the model of peptide competition in the ER as a factor that prevents presentation of TEIPP antigens in normal cells.

Mechanism of peptide competition

We can speculate how such a peptide competition mechanism actually works. TAP is one of the components of the PLC and links peptide transport into the ER with peptide loading. These two processes physically linked by TAP enhance the efficiency of peptide presentation. TAP-transported peptides readily get in contact with trimming enzymes and chaperones needed for optimal peptide binding to MHC-I that are also part of the PLC. The MHC-I molecules within the PLC have direct contact with the peptides that come from the cytosol, a big pool that contains peptides with optimal binding properties. Therefore these are favored over peptides situated in the ER lumen separate from the

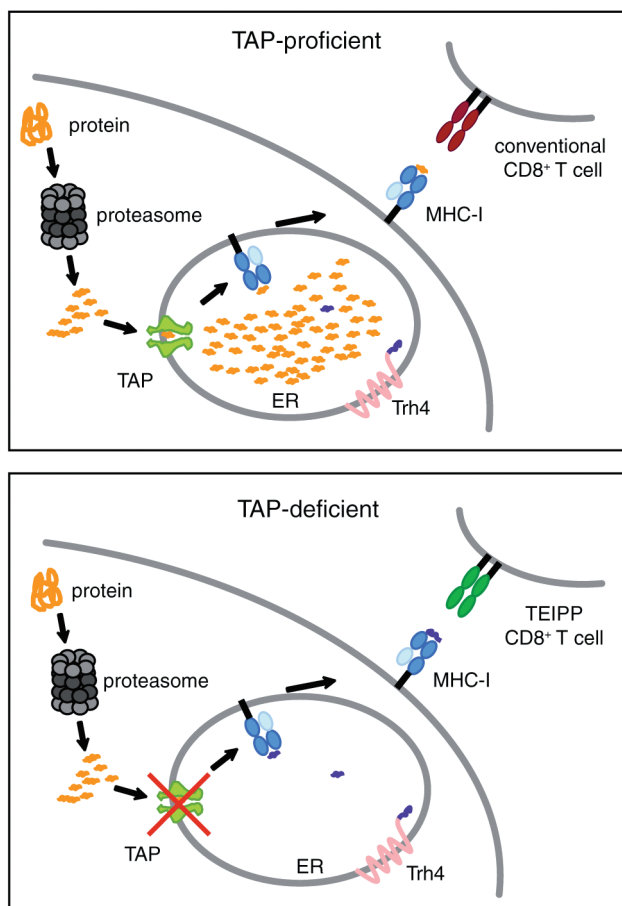


Figure 1. Schematic illustration to explain why Trh4 peptides fail to be presented by processing-proficient cells. We hypothesize that Trh4 peptides are underrepresented in the ER and lose competition with the vast excess of TAP-pumped peptides under normal conditions. Under TAP-deficiency, those peptides are not transported to the ER and Trh4 peptides get the chance to be presented instead. Binding affinity and stability of the Trh4 epitope with its MHC-I molecule seem sufficient for presentation. Adapted from: Seidel UJ. *et al.* A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens. *Cancer Immunology, Immunotherapy* 2012, 61(1): 119-125.

transport complex. As discussed above, peptides such as the TEIPP Trh4-derived peptide are liberated in the ER in normal cells but are not loaded into MHC-I. The quantity of these peptides seems to be too low to overcome the competition of the TAP-transported peptides. In cases where the PLC does not have a functional TAP there is no direct link between the entering peptides and the specialized loading complex. However, the dynamical process of MHC-I presentation continues even without TAP. The available peptides, probably situated in the vicinity of the PLC have the opportunity to be loaded into MHC-I. Additionally, peptides liberated in the subsequent compartments of the

secretory route might get in contact with peptide-receptive MHC-I molecules on their way to the cell surface, which might exchange their ligands. Indeed, a higher number of intracellular peptide-receptive MHC-I molecules is found in TAP-deficient cells⁵.

Alternative ways to be loaded into MHC-I: find peptide-receptive MHC-I in cells

Peptides located in the N- and C- terminus

The mechanism of peptide competition discussed here is a model that explains the immunogenicity of TEIPP peptides, such as the prototype example Trh4-derived peptide. This mechanism might be the base of the immunogenicity of other TEIPP that are liberated in the ER similarly to the Trh4 and ppCT peptides. These are examples of peptides situated in the N- and C-terminal regions of the proteins and can therefore be “easily” liberated by mechanisms such as the processing of leaders and the action of SPP on C-terminal regions of ER-resident proteins. The ER is equipped with sophisticated machinery for optimizing ligand length and quality, and for facilitating peptide loading onto nascent MHC-I molecules. This includes aminopeptidases as well as folding chaperones and editing molecules in the peptide loading complex. In the absence of TAP transporters the loading of peptides into MHC-I occurs without a fully functional peptide loading complex. The details of this mechanism are not yet understood. It has been shown that the loading of peptides in MHC-I can occur with the minimal components of tapasin-ERp57-MHC-I complexes⁶. TEIPP peptides generated inside the ER may have readily access to peptide-receptive MHC-I molecules associated with tapasin-ERp57 or might be actively chaperoned towards these open grooves. Chaperones with high peptide binding capacity in the ER are heat shock proteins (e.g. Hsp96) and PDI, an isomerase that efficiently binds free peptide⁷. It is possible that TAP-independent peptides are captured by these molecules and chaperoned to MHC-I.

Peptides located within proteins

A significant fraction of TAP-independent peptides identified thus far are derived from internal regions of cytosolic proteins^{8,9}. These peptides, most likely, need the degradation of the entire protein for being liberated in more complex degradation steps than the liberation of N- or C-terminal ends. This normally occurs by the action of the proteasome, alone or in combination with other proteases. We studied a TEIPP peptide antigen that was processed by the proteasome and presented independently from TAP (chapter 3 of this thesis). In contrast to Trh4, it is likely that in this case the peptide is generated outside the ER, via one or more proteolytic steps. Novel transport mechanisms from the cytosol into vesicular compartments that enable loading into MHC-class I molecules have been proposed. Apparently such cytosol-derived peptides can have access to MHC-I loading compartments, such as the ER, without the action of TAP. Several epitopes from the LMP2 protein of the Epstein-Barr virus (EBV) are produced by the proteasome and presented without TAP¹⁰. It was suggested that they cross the ER membrane by diffusion due to their hydrophobicity, because all of these were highly hydrophobic. Maybe there are TEIPP peptides with such characteristics of being

generated in the cytosol by the action of the proteasome and then traverse membranes due to their hydrophobic characteristics and do not need TAP.

Peptides generated in post-ER compartments possibly might travel back to the ER for loading in MHC-I molecules. Peptides generated along the secretory route, including Golgi, may use retrograde vesicular transport to get from distant organelles to the ER¹¹. Secondly, MHC-I molecules with sub-optimally bound peptides may exchange them at a second quality control check point in the Golgi¹²⁻¹⁴. Peptidic ligands generated in these secretory compartments may bind to MHC class I molecules in these compartments when the exchange occurs. It is likely that TAP-deficiency promotes the availability of sub-optimally loaded MHC-I molecules and this increases the efficiency of presentation of vesicular and secretory MHC-I ligands.

Autophagy as a way to load MHC-I?

Autophagy could provide a pathway to facilitate the loading of potential MHC-I ligands in endovacuolar compartments. During autophagy, large portions of cytoplasmic content are encapsulated in vesicles, the autophagosomes, and then fused with other vesicles such as lysosomes. The contents of cellular organelles are degraded in these vacuolar compartments in this conserved cellular process of self-eating. Antigens could be liberated by proteases in a lytic vacuole after autophagy and encounter MHC-I complexes that were recycled from the cell surface. It is known that MHC-I molecules constantly recycle from the cell surface to early endosomes as part of the normal MHC-I trafficking. Upon entering gradually acidic environments they might exchange their ligands. This vacuolar pathway of antigen presentation might be of particular importance in TAP-deficient cells or cells infected with viruses that can interfere with TAP or MHC-I processing and transport. In a recent study it has been shown that autophagy promotes the processing of viral antigens for TAP-independent MHC-I presentation. The MHC-I presentation of an endogenous human cytomegalovirus (HCMV) latency associated protein, pUL138, was followed the vacuolar pathway dependent on autophagy¹⁵. Peptide loading occurred within autophagic vesicles associated with lysosomes (autophagolysosomes). Curiously, this pathway generated the same peptide epitope as that generated from conventional processing. Thus, it is likely that this autophagy mediated pathway supplements the conventional pathway and contributes to viral clearance.

The autophagosomal membrane has been proposed to originate from the ER^{16, 17}. If the loading occurs in these compartments, the advantage created by TAP could be the higher number of peptide-receptive MHC-I heavy chains that could enter other loading compartments such as the autophagosome vesicles. However, more research is needed to get further insight on the role of autophagy for MHC-I antigen presentation.

Conclusion

In summary, there are many new interesting pathways ready to be elucidated. And many TEIPP antigens generated and presented via these pathways occur and stimulate the immune system. Indeed the immunogenicity of TEIPP is an important topic to elucidate

since it can be exploited in the combat of many diseases. The study of alternative pathways could be exploited to develop new therapies to combat viral infections and cancer where TAP is frequently inhibited. As we have shown, following TAP-inhibition there is a generalized decrease in MHC-I presentation and the emergence of an alternative peptide repertoire¹⁸. TEIPP peptides are among these TAP-independent peptides and might constitute an important line of host defense for exploitation in therapeutic strategies.

I EXPLOITATION OF TEIPP ANTIGENS IN IMMUNOTHERAPY

Many tumors resist to consecutive conventional treatments such as chemotherapy. Frequently it is observed the outgrowth of tumor escaped variants. These variants frequently display defects in the APM machinery, for example TAP-deficiency, and sometimes complete loss of antigenic presentation or MHC-I or MHC-II alleles¹⁹⁻²¹. These observations are common in advanced stages of the disease such as the metastatic state^{22, 23}. TAP-deficiency causes MHC-I downregulation and hampers the presentation of many TAP-dependent tumor antigens that could be already the target of T-cell therapies. TEIPP antigens are presented in TAP-deficiency or other APM-deficiencies and therefore provide a way to target this escaped variants.

Therapeutic peptide vaccines

The applicability of TEIPP antigens for immunotherapy of cancer has been tested in preliminary studies using mice. The 9-mer TEIPP peptide Trh4 was used as a CD8⁺ T-cell vaccine, combined with a T helper peptide to also stimulate CD4⁺ T-cells. This vaccination scheme prevented the outgrowth of a TAP-deficient lymphoma¹. These results showed that TEIPP antigens can be used in combination with other antigens to be used as a vaccine that can tackle tumor immune escaped variants. In the tumor vaccination field, several immune intervention strategies have been designed and tested to stimulate the power and specificity of the immune system to treat cancer. However, until recently, most of the immune-therapies developed have provided poor tumor regression in humans. Some of these were based on vaccination strategies to induce CD8⁺ T-cell responses against tumor-associated antigens using single CTL epitopes comprising the exact HLA-I binding peptide. Peptide epitopes used in clinical trials to treat metastatic cancer included melanoma-differentiation antigens such as MART-1, gp100, tyrosinase or TRP-2 and cancer-testes antigens such as NY-ESO-1, MAGE-12 or Her2/neu²⁴. The low efficacy of vaccines using minimal CTL epitopes may be caused by inefficient T-cell activation that precludes the ability of the T-cells to infiltrate the solid tumors and become activated. In preclinical mouse studies it was shown that synthetic peptides that were much longer and could reach up to 27 amino acids in length were more effective²⁵⁻²⁹. These longer peptides may be retained in the draining lymph nodes where they could be processed and presented by professional APCs to prime CD8⁺ T-cells. Additionally, some of the synthetic peptides also contained CD4⁺ T-cell helper epitopes. These two factors, processing and presentation of peptides by APCs and the induction of CD4⁺ T-cell help are important factors that enhanced efficacy of these studies in mice but lacking in most

of the clinical trials in humans. This realization provided the development of even more complex peptide formulations, for instance the synthetic long peptides (SLP). These contain overlapping long peptides that span the whole protein and contain multiple CD8⁺ and CD4⁺ T-cell epitopes. A clinical trial was conducted in twenty women carrying pre-malignant vulvar lesions associated with HPV16 infection using synthetic long peptides (SLP)³⁰. This vaccine formulation contained multiple MHC-I and MHC-II overlapping peptides (27-34 amino-acids) spanning the sequence of HPV16 E6 and E7 proteins. Five women had complete regression of the lesion and this was associated with the induction of interferon- γ -associated CD4⁺ and CD8⁺ T-cell responses. Therefore, it might be worthwhile to test if we can formulate long-TEIPP antigens to use as vaccines. One advantage of TEIPP antigens relative to tumor associated antigens, such as differentiation antigens, is that they behave like foreign antigens because they are not presented in the thymus in normal conditions. This implies that the T-cells are not affected by tolerance which might contribute to the development of a robust anti-tumor response and enhance the efficacy of tumor-specific immunotherapy.

Tumor-cell based vaccines

Others demonstrated that tumor-cell vaccines consisting of TAP-impaired tumor cell lines that present TEIPP peptides might also induce immune protection, provided that enough co-stimulation on the tumor cell vaccine is at hand^{31, 32}. The applicability of this variety of therapeutic immune interventions shows that TEIPPs can be exploited alike conventional tumor antigens, despite their unconventional character (Table 1). Altogether, the understanding of the mechanisms underlying the generation of tumor immunity and tumor escape is needed to provide a framework for developing novel immunotherapies.

Dendritic cell-based vaccination

As mentioned above, the processing of tumor antigens by APCs is important in immunotherapies. DCs are potent antigen-presenting cells of the immune system and can promote protective immunity. Upon acquisition of antigens, DCs can instruct the type of immune response to be induced. Immunization with TAP-deficient DCs mediated protection to TAP-deficient tumor challenge². This DC-based cellular vaccine is characterized by

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

Adapted from: Lampen M.H. et al, Current Opinion in Immunology 2011, 23(2):293-8

presentation of a variety of endogenous TEIPP antigens by MHC-I molecules. Importantly, in this vaccination strategy TEIPP antigens do not require to be molecularly identified. Clinical trials have shown that DC-based vaccines can directly induce specific antitumor immune responses. A prostate cancer vaccine that includes DCs pulsed with prostate cancer-associated antigens has been approved for cell-based immune-therapy. Patients given this formulation, called Provenge (Dendreon), showed a small improvement in overall survival in a clinical trial, highlighting the need to improve current DC-based vaccination strategies^{33,34}.

The TAP-deficient DCs can also be used to treat viral infections. Many viruses establish persistent infections in their host. Herpesviruses, for instance, employ several immune evasion strategies to accomplish this. They code for specific molecules that greatly interfere with presentation of viral antigens and therefore “hide” from the immune system³⁵. These molecules interfere with many steps of the MHC-I antigen presentation route³⁵⁻³⁷. A frequent target within this pathway is the TAP transporter. Herpes simplex virus (HSV)-1 and 2 code for ICP47, a cytosolic protein that inhibits TAP function by interfering with peptide binding to the transporter. Others, such as the transmembrane glycoprotein US6 of human cytomegalovirus (HCMV) inhibit ATP binding to TAP and impair peptide transport. However, cells that have these TAP-inhibitors are still capable of presenting TEIPP peptides. Introduction of viral TAP-inhibiting molecules into dendritic cells efficiently induces the display of TEIPP peptides, resulting in recognition by TEIPP-specific CTL. Human DCs electroporated with RNA encoding the bovine herpes virus (BHV)-1 gene *UL49.5* were used for stimulations of autologous peripheral-blood mononuclear cells (PBMCs)³⁸. The *UL49.5* protein from this herpes-virus efficiently inhibits the human peptide transporter TAP². The CD8⁺ T-cell clones isolated from these cultures exhibited different specificities, by virtue of different TCR usage, and displayed different HLA-I restrictions. These CTL clones were capable of lysing other TAP-inhibited targets and did not react against TAP-positive cells, showing that these CTL clones exhibited TEIPP-specificity³⁸. Moreover, these target cells displayed other viral TAP-inhibitors, showing that the T-cell clones raised strategy can be used to fight different viral infections. Together, these findings show that it is possible to raise CD8⁺ TEIPP-T-cell responses against viral infected cells by using professional antigen presenting cells, such as dendritic cells, rendered TAP-inhibited. These DCs are able to display TEIPP peptides as the infected cells and can prime naïve T-cells into effector cells. Partial inhibition of TAP-function in the DCs is sufficient to exhibit these responses^{2,38}. The technique of RNA electroporation, that could be used to introduce the viral TAP-inhibitors, is feasible in clinical settings. Also, it has the advantage that the nucleotides are not integrated in the host genome, which happens with the use of viral vectors.

Therefore, TEIPP might also be exploited in several ways to combat infections of viruses that hide from T cell immunity by down modulation of conventional antigen processing. The study of alternative presentation pathways will most likely provide further fundamental insight in TEIPP peptide presentation and facilitate the future application of this knowledge for immunotherapeutic intervention of cancer and infectious diseases.

Adoptive T-cell transfer

Cell-based vaccinations using adoptively transferred TEIPP-specific CTL were able to eradicate TAP-deficient tumor cells *in vivo* and control the outgrowth of a lethal dose¹. In adoptive cell transfer (ACT) techniques tumor-specific T-cells are expanded *in vitro* and then reintroduced in large numbers into the patients. During this *in vitro* growth phase, the properties of these cells may be monitored such as their recognition potential. It has been shown that T-cell characteristics such as young and rapidly growing cells were beneficial³⁹⁻⁴². Immune cells generated and activated *ex vivo* can be infused in a highly activated state, already displaying the necessary lytic and cytokine secreting activities required to mediate the destruction of even large solid tumor masses. Antitumor T-cells used for cell transfer can be generated *in vitro* from tumor infiltrating lymphocytes (TILs) or from peripheral blood lymphocytes. Also, during *in vitro* procedures these cells may be modified through genetic engineering, such as T-cell receptor (TCR) gene transfer strategies⁴³. In this strategy, TCR of desired specificity are introduced by gene transfer into autologous or donor-derived T-cell populations in short-term *ex vivo* cultures, and then infused into the patients to provide T-cell reactivity against defined antigens⁴³⁻⁴⁶. TCR gene transfer experiments with TEIPP specific TCRs has shown that TEIPP reactivity can be obtained via this strategy. Therefore, ACT and gene transfer techniques can be applied for TEIPP.

Advantages of HLA-E-presented TEIPP in immunotherapy

TEIPP-specific CTL restricted by the non-classical MHC-I molecule Qa-1 are capable of targeting TAP-deficient tumor cells such as other TEIPP-specific CTL restricted by classical MHC-I molecules^{1, 2, 9}. The particular characteristics of Qa-1 turn this molecule into an attractive target for TEIPP studies. The Qa-1 binding groove shares a conserved structure with the human homologue HLA-E⁴⁷. Therefore the peptides presented by these two MHC-I molecules would be very similar, implying that the discovery of Qa-1 presented peptides could directly provide TEIPP epitopes to be used in human experiments. In fact, it was observed that human cells transfected with Qa-1 were recognized by our mouse Qa-1-TEIPP CTL clones (unpublished data). We found a surprisingly diverse peptide repertoire of more than 100 different peptides in Qa-1 on TAP-deficient cells, described on chapter 5. The peptides originated from housekeeping proteins within the cell and some of them constituted neo-antigens according to the TEIPP concept with the ability to give rise to CD8⁺ T cell responses. Therefore a high number of antigens can be targeted in tumors by Qa-1-specific CTL.

Qa-1 and HLA-E exhibit low polymorphism, consequently the presented peptides are widely distributed within the population⁴⁷⁻⁴⁹. The independency of MHC typing on the application of treatment strategies based on Qa-1/HLA-E-binding peptides contrasts with most of the other MHC-I molecules. Interestingly, Qa-1-TEIPP CTL clones recognized TAP-deficient tumors from different mouse MHC haplotype backgrounds by virtue of the conserved nature of Qa-1. Therefore, Qa-1/HLA-E-presented TEIPP antigens fulfill some requirements of ideal tumor antigens. As such, the potential use of TEIPP peptides as vaccines is boosted.

Moreover, Qa-1 shows a wide tissue distribution which allows the targeting of different histological tumor types. One Qa-1-TEIPP CTL clone was capable of recognizing colon tumors, melanoma and lymphoma (described on chapter 5). Qa-1 shows a relative maintenance of surface levels on TAP-negative conditions even in cells that lost expression of classical MHC-I heavy chains^{47, 48, 50}. This implies that tumors with processing defects as well as tumors with MHC-I allele loss can be targeted by these Qa-1-TEIPP CTL. In ovarian and cervical cancers, HLA-E expression was found in equal or even higher levels than normal tissues. This was strongly associated with APM deficiencies such as TAP-deficiency, HLA-I and HLA-II loss⁵¹.

Conclusion

For more than 60 years it has been shown that the host's immune system is capable of fighting against cancer. One challenge of immune-therapy against cancer is the start of a robust and specific CD8⁺ and CD4⁺ T-cell response that is capable of fighting against an established disease. Tumors normally harbor immune suppressive environments and it might be useful to relief the local suppressive milieu in combination with T-cell vaccination strategies. For instance, tumors can be infiltrated with regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs)⁵²⁻⁵⁵. Therapeutic strategies that aim to break the tolerogenic environment and induce inflammation might greatly enhance attraction of T-cells to the tumor area and promote T-cell infiltration⁵⁶. Therefore, the use of chemotherapy or radiotherapy or other means that promotes the release of proinflammatory signals in combination with immune-therapy might be beneficial and are now focus of research⁵⁷. For instance, TLR ligands might increase the magnitude of T-cell response by strongly activate DCs and polarize it into an inflammatory type of immune response (T-helper 1). Covalent linking of TLR ligands to peptide vaccines is one strategy currently under study. Since the vaccine might also activate pre-existing antigen specific T-cells it may be useful to deplete T-cells prior to the vaccination, for instance by administering cyclophosphamide to the patients. These and other techniques might help to improve the T-cell responses stimulated by therapeutic vaccines. TEIPP antigens can be exploited to tackle T cell defense against processing deficient tumors and thus be complementary to conventional tumor antigen vaccination.

REFERENCES

1. Van Hall, T. et al. Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat. Medicine* **12**, 417-424 (2006).
2. Chambers, B. et al. Induction of protective CTL immunity against peptide transporter TAP-deficient tumors through dendritic cell vaccination. *Cancer Res* **67**, 8450-5 (2007).
3. El Hage, F. et al. Preprocalcitonin signal peptide generates a cytotoxic T lymphocyte-defined tumor epitope processed by a proteasome-independent pathway. *Proc Natl Acad Sci U S A* **105**, 10119-24 (2008).
4. Durgeau, A. et al. Different expression levels of the TAP peptide transporter lead to recognition of different antigenic peptides by tumor-specific CTL. *J Immunol* **187**, 5532-9 (2011).
5. Day, P.M., Esquivel, F., Lukszo, J., Bennink, J.R. & Yewdell, J.W. Effect of TAP on the generation and intracellular trafficking of peptide-receptive major histocompatibility complex class I molecules. *Immunity* **2**, 137-47 (1995).
6. Wearsch, P.A. & Cresswell, P. Selective loading of high-affinity peptides onto major histocompatibility complex class I molecules by

- the tapasin-ERp57 heterodimer. *Nat Immunol* **8**, 873-81 (2007).
7. Spee, P. & Neefjes, J. TAP-translocated peptides specifically bind proteins in the endoplasmic reticulum, including gp96, protein disulfide isomerase and calreticulin. *Eur J Immunol* **27**, 2441-9 (1997).
 8. Del Val, M., Iborra, S., Ramos, M. & Lazaro, S. Generation of MHC class I ligands in the secretory and vesicular pathways. *Cell Mol Life Sci* **68**, 1543-52 (2011).
 9. Oliveira, C.C. et al. The nonpolymorphic MHC Qa-1b mediates CD8⁺ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-21 (2010).
 10. Lautscham, G. et al. Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell recognition reveals a proteasome-dependent, transporter associated with antigen processing-independent pathway. *J Exp Med* **194**, 1053-68 (2001).
 11. Ackerman, A.L., Kyritsis, C., Tampe, R. & Cresswell, P. Access of soluble antigens to the endoplasmic reticulum can explain cross-presentation by dendritic cells. *Nat Immunol* **6**, 107-13 (2005).
 12. Garstka, M. et al. Peptide-receptive major histocompatibility complex class I molecules cycle between endoplasmic reticulum and cis-Golgi in wild-type lymphocytes. *J Biol Chem* **282**, 30680-90 (2007).
 13. Ghanem, E. et al. The transporter associated with antigen processing (TAP) is active in a post-ER compartment. *J Cell Sci* **123**, 4271-9 (2010).
 14. Howe, C. et al. Calreticulin-dependent recycling in the early secretory pathway mediates optimal peptide loading of MHC class I molecules. *EMBO J* **28**, 3730-44 (2009).
 15. Tey, S.K. & Khanna, R. Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway. *Blood* **120**, 994-1004 (2012).
 16. Mijaljica, D., Prescott, M. & Devenish, R.J. Endoplasmic reticulum and Golgi complex: Contributions to, and turnover by, autophagy. *Traffic* **7**, 1590-5 (2006).
 17. Yorimitsu, T. & Klionsky, D.J. Eating the endoplasmic reticulum: quality control by autophagy. *Trends Cell Biol* **17**, 279-85 (2007).
 18. Oliveira, C.C. et al. Peptide transporter TAP mediates between competing antigen sources generating distinct surface MHC class I peptide repertoires. *Eur J Immunol* **41**, 3114-24 (2011).
 19. Ferris, R.L., Whiteside, T.L. & Ferrone, S. Immune escape associated with functional defects in antigen-processing machinery in head and neck cancer. *Clin Cancer Res* **12**, 3890-5 (2006).
 20. Seliger, B. Molecular mechanisms of MHC class I abnormalities and APM components in human tumors. *Cancer Immunol Immunother* **57**, 1719-26 (2008).
 21. Seliger, B., Maeurer, M.J. & Ferrone, S. Antigen-processing machinery breakdown and tumor growth. *Immunology Today* **21**, 455-464 (2000).
 22. Cabrera, T. et al. HLA class I expression in metastatic melanoma correlates with tumor development during autologous vaccination. *Cancer Immunol Immunother* **56**, 709-17 (2007).
 23. Vitale, M. et al. HLA class I antigen down-regulation in primary ovary carcinoma lesions: association with disease stage. *Clin Cancer Res* **11**, 67-72 (2005).
 24. Rosenberg, S.A., Yang, J.C. & Restifo, N.P. Cancer immunotherapy: moving beyond current vaccines. *Nat Med* **10**, 909-15 (2004).
 25. Aichele, P., Hengartner, H., Zinkernagel, R.M. & Schulz, M. Antiviral cytotoxic T cell response induced by in vivo priming with a free synthetic peptide. *J Exp Med* **171**, 1815-20 (1990).
 26. Gao, X.M., Zheng, B., Liew, F.Y., Brett, S. & Tite, J. Priming of influenza virus-specific cytotoxic T lymphocytes vivo by short synthetic peptides. *J Immunol* **147**, 3268-73 (1991).
 27. Kast, W.M. et al. Protection against lethal Sendai virus infection by in vivo priming of virus-specific cytotoxic T lymphocytes with a free synthetic peptide. *Proc Natl Acad Sci U S A* **88**, 2283-7 (1991).
 28. Schulz, M., Zinkernagel, R.M. & Hengartner, H. Peptide-induced antiviral protection by cytotoxic T cells. *Proc Natl Acad Sci U S A* **88**, 991-3 (1991).
 29. Mineev, B.R., McFarland, B.J., Spiess, P.J., Rosenberg, S.A. & Restifo, N.P. Insertion signal sequence fused to minimal peptides elicits specific CD8⁺ T-cell responses and prolongs survival of thymoma-bearing mice. *Cancer Res* **54**, 4155-61 (1994).
 30. Kenter, G.G. et al. Vaccination against HPV-16 oncoproteins for vulvar intraepithelial neoplasia. *N Engl J Med* **361**, 1838-47 (2009).
 31. Li, X.L. et al. Effect of B7.1 costimulation on T-cell based immunity against TAP-negative cancer can be facilitated by TAP1 expression. *PLoS One* **4**, e6385 (2009).
 32. Li, X.L. et al. Priming of immune responses against transporter associated with antigen processing (TAP)-deficient tumours: tumour direct priming. *Immunology* **128**, 420-8 (2009).

33. Higano, C.S. et al. Sipuleucel-T. *Nat Rev Drug Discov* **9**, 513-4 (2010).
34. Kantoff, P.W. et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. *N Engl J Med* **363**, 411-22 (2010).
35. Abele, R. & Tampe, R. The TAP translocation machinery in adaptive immunity and viral escape mechanisms. *Essays Biochem* **50**, 249-64 (2011).
36. Kim, S. et al. Human cytomegalovirus microRNA miR-US4-1 inhibits CD8(+) T cell responses by targeting the aminopeptidase ERAP1. *Nat Immunol* **12**, 984-91 (2011).
37. Verweij, M.C. et al. Structural and functional analysis of the TAP-inhibiting UL49.5 proteins of varicelloviruses. *Mol Immunol* **48**, 2038-51 (2011).
38. Lampen, M.H. et al. CD8+ T cell responses against TAP-inhibited cells are readily detected in the human population. *J Immunol* **185**, 6508-17 (2010).
39. Gattinoni, L. et al. A human memory T cell subset with stem cell-like properties. *Nat Med* **17**, 1290-7 (2011).
40. Hinrichs, C.S. et al. Adoptively transferred effector cells derived from naive rather than central memory CD8+ T cells mediate superior antitumor immunity. *Proc Natl Acad Sci U S A* **106**, 17469-74 (2009).
41. Huang, J. et al. Modulation by IL-2 of CD70 and CD27 expression on CD8+ T cells: importance for the therapeutic effectiveness of cell transfer immunotherapy. *J Immunol* **176**, 7726-35 (2006).
42. Zhou, J. et al. Telomere length of transferred lymphocytes correlates with in vivo persistence and tumor regression in melanoma patients receiving cell transfer therapy. *J Immunol* **175**, 7046-52 (2005).
43. Schumacher, T.N. T-cell-receptor gene therapy. *Nat Rev Immunol* **2**, 512-9 (2002).
44. Hawkins, R.E. et al. Development of adoptive cell therapy for cancer: a clinical perspective. *Hum Gene Ther* **21**, 665-72 (2010).
45. Kessels, H.W., Wolkers, M.C. & Schumacher, T.N. Adoptive transfer of T-cell immunity. *Trends Immunol* **23**, 264-9 (2002).
46. Slansky, J.E. & Jordan, K.R. The Goldilocks model for TCR-too much attraction might not be best for vaccine design. *PLoS Biol* **8** (2010).
47. Rodgers, J.R. & Cook, R.G. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5**, 459-471 (2005).
48. Davies, A. et al. A peptide from heat shock protein 60 is the dominant peptide bound to Qa-1 in the absence of the MHC class Ia leader sequence peptide Qdm. *J Immunol* **170**, 5027-33 (2003).
49. Kambayashi, T. et al. The nonclassical MHC class I molecule Qa-1 forms unstable peptide complexes. *J Immunol* **172**, 1661-9 (2004).
50. Kraft, J.R. et al. Analysis of Qa-1(b) peptide binding specificity and the capacity of CD94/NKG2A to discriminate between Qa-1-peptide complexes. *J Exp Med* **192**, 613-624 (2000).
51. Gooden, M. et al. HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8(+) T lymphocytes. *Proc Natl Acad Sci U S A* **108**, 10656-61 (2011).
52. Arens, R. Rational design of vaccines: learning from immune evasion mechanisms of persistent viruses and tumors. *Adv Immunol* **114**, 217-43 (2012).
53. Curiel, T.J. et al. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* **10**, 942-9 (2004).
54. Drake, C.G., Jaffee, E. & Pardoll, D.M. Mechanisms of immune evasion by tumors. *Adv Immunol* **90**, 51-81 (2006).
55. Nagaraj, S. & Gabrilovich, D.I. Tumor escape mechanism governed by myeloid-derived suppressor cells. *Cancer Res* **68**, 2561-3 (2008).
56. Flavell, R.A., Sanjabi, S., Wrzesinski, S.H. & Licona-Limon, P. The polarization of immune cells in the tumour environment by TGFbeta. *Nat Rev Immunol* **10**, 554-67 (2010).
57. Arens, R., van Hall, T., van der Burg, S.H., Ossendorp, F. & Melief, C.J. Prospects of combinatorial synthetic peptide vaccine-based immunotherapy against cancer. *Semin Immunol* (2013).

| NEDERLANDSE SAMENVATTING

Het immuunsysteem speelt een essentiële rol in de bescherming van ons lichaam tegen bedreigingen zoals bacteriën, virussen en andere ziekteverwekkers, maar ook tumorcellen. Het immuunsysteem bestaat uit verschillende groepen van cellen, waaronder de Cytotoxische T-Lymfocyten (CTL). Deze cellen zijn gespecialiseerd in het aanvallen van door ziekteverwekkers geïnfecteerde cellen en tumorcellen. Zij ruimen deze cellen op door moleculen uit te scheiden waardoor de doelwit cel “zelfmoord” pleegt en vervolgens uit het lichaam verwijderd wordt. In enkele nieuwe behandelingsvormen van kanker wordt gebruik gemaakt van deze CTL, zoals bijvoorbeeld therapeutische vaccins die anti-tumor CTL activeren. In ons lichaam gaan ze naar de plek van de tumor om deze daar te bestrijden. De lymfocyten kunnen ook gekweekt, geactiveerd en opgegroeid worden in laboratoria om daarna terug gespoten te worden in de patiënt. Dergelijke experimentele therapieën worden in verschillende klinische studies gebruikt voor de behandeling van verschillende types kanker, zoals bijvoorbeeld baarmoederhalskanker en huidkanker.

De activatie van CTL is belangrijk voor hun functioneren en is afhankelijk van bepaalde factoren. De doelwit cellen worden gezien via een moleculaire receptor die op de oppervlakte van de CTL zit, genaamd de T-cel receptor (TCR). Elke CTL heeft een unieke TCR die ervoor zorgt dat heel selectief een afwijkend stukje eiwit wordt herkend, genaamd het antigeen, op de doelwit cel. Deze kleine stukjes eiwit zijn afkomstig vanuit de cel zelf en worden getransporteerd naar de oppervlakte van de doelwit cel waar ze zichtbaar zijn voor de CTL. Deze peptiden worden alleen herkend door de CTL als ze gebonden zijn aan grotere moleculen, de Major Histocompatibility Complex moleculen (MHC).

Vaak kunnen tumor cellen ontsnappen aan de CTL-gemedieerde verwijdering door te voorkomen dat ze herkend worden door de CTL. In zulke tumor cellen werkt de presentatie van antigenen niet correct en kan drastisch gereduceerd worden. Deze vermindering in het zichtbaar maken van antigenen wordt veroorzaakt door een hapering van een of verschillende moleculen geassocieerd met deze transport route, bijvoorbeeld de proteasoom, tapasin en TAP. Tumor cellen met verminderde oppervlakte presentatie van tumor-geassocieerde antigenen zijn een grote barrière voor de doeltreffendheid van CTL-gebaseerde therapieën die momenteel in ontwikkeling zijn voor kanker.

Niettemin zijn er de laatste tijd vindingen die nieuw licht op dit onderwerp schijnen. Er is een nieuwe categorie van CTL ontdekt die dergelijk ontsnapte tumor cellen toch kan opruimen. De defecten in MHC-I antigeen verwerking zorgt er namelijk voor dat tumor cellen andere peptiden gaat presenteren. De hapering in de normale antigeen presentatie route resulteert in de uitschakeling van de normale peptiden, maar tegelijkertijd in de presentatie van nieuwe tumor antigenen. Deze nieuwe peptiden worden niet gepresenteerd door normale cellen. Er is nu veel onderzoek naar deze CTL en hun gebruik in tumor beschermingsexperimenten. Tumor cellen met een slecht functionerend transportsysteem door TAP of het proteasoom worden herkend en doodgemaakt door deze CTL terwijl de normale cellen hier geen last van hebben. Deze categorie van peptiden worden TEIPP genoemd, dat staat voor 'T cell epitopes associated

with impaired peptide processing'. Hoewel ze niet gepresenteerd worden door normale cellen, komen deze peptiden van eiwitten die niet gemuteerd zijn en die normaal tot expressie komen in alle cellen, zogenaamde huishoud genen. CTL die TEIPP peptiden herkennen kunnen veel verschillende tumor types doodmaken, zoals darm kanker, huidkanker, lymfoom en sarcoom. De eerste onderzoeken naar TEIPP zijn ontwikkeld in muis modellen, maar recent werk laat zien dat TEIPP-CTL ook in mensen voorkomen en ook verschillende kanker types herkennen terwijl normale cellen intact blijven.

De studies naar TEIPP antigenen hebben tot zover laten zien dat deze antigenen veelbelovende kandidaten zijn voor het bestrijden van tumoren die aan een aanval van het immuunsysteem ontsnapt zijn. Echter, verschillende aspecten van TEIPP worden nog slecht begrepen. De studies die in dit proefschrift worden gepresenteerd zijn ontwikkeld om de TEIPP peptiden beter te begrijpen, en dus ook hoe de peptiden gebruikt kunnen worden in de toekomst om kanker te bestrijden. In hoofdstuk 2 komt ter sprake waarom TEIPP peptiden immunogeen zijn. Het muis antigeen van het Trh4 eiwit is gebruikt als model. Wij verhoogde de hoeveelheid van Trh4 eiwit en MHC-I moleculen in cellen met normale TAP functie, om de beperkende stap van Trh4 peptide presentatie te identificeren. De verhoogde expressie van Trh4 was het enige mechanisme dat resulteerde in peptide presentatie in deze cellen. Wij concluderen dat de normale TAP activiteit de presentatie van de Trh4 peptiden onderdrukt en wanneer de TAP functie lager is, dan hebben de Trh4 peptiden en andere gerelateerde peptiden een kans om gepresenteerd te worden aan het cel oppervlakte. Omdat deze peptiden onder normale omstandigheden niet gepresenteerd worden, zien de immuun cellen ze als een "gevaarlijke" situatie en vallen de cellen aan die deze peptiden laat zien. Dit is een model om uit te leggen dat de TEIPP peptiden immunogeen zijn.

In hoofdstuk 3 laten wij zien hoe het Trh4-afgeleide peptide geknipt wordt uit het Trh4 eiwit binnenin de cel. We hebben gevonden dat het enzym 'signal peptide peptidase' (SPP), dat tot een familie van I-CLiPs hoort (intra-membraan knippende aspartyl proteases), de 9-meer Trh4-eiwit vrijmaakt in het endoplasmatisch reticulum (ER). In hoofdstuk 4 bekijken wij de verwerkingsroute van een ander TEIPP antigeen dat aan CTL wordt gepresenteerd. Verassend genoeg ontdekten wij dat deze presentatie zeer afhankelijk was van de functie van proteasomen. Dit betekent dat het peptide vrij gemaakt wordt door het proteasome, maar via een TAP-onafhankelijke route naar het cel oppervlak gaat. Onze data suggereren een rol voor 'autophagy' in de presentatie van dit TAP-onafhankelijke en proteasoom-afhankelijke antigeen. Autophagy is een cellulair mechanisme waarbij organellen in stukjes uiteenvallen en worden "gerecycled".

In hoofdstuk 5, bekijken wij de TEIPP-specifieke CTL die antigenen herkennen via het MHC-I molecuul Qa-1. Qa-1 presenteert normaal peptiden die strikt afhankelijk zijn van TAP activiteit. Echter, eerste onderzoeken laten zien dat TEIPP-specifieke CTL worden geactiveerd wanneer ze gebonden zijn aan Qa-1 moleculen in TAP-negatieve cellen maar niet in TAP-normale cellen. Meer dan 80 peptide sequenties werden gevonden in Qa-1 moleculen op TAP-negatieve cellen. Verschillende van deze peptiden zijn zeer immunogeen, wat laat zien dat ze veelbelovend zijn in het gevecht tegen ontsnapte

tumor cellen. In hoofdstuk 6 is er een overzicht van de verschillende rollen van Qa-1 en de humane variant, HLA-E, in immuniteit. Hoofdstuk 7 rondt dit proefschrift af met een overzicht van de vindingen in dit boekje waarbij onze resultaten bediscussieerd worden.

| CURRICULUM VITAE

Claudia da Cunha Oliveira was born on 15th of February 1985 in Braga, Portugal. She obtained her high school degree in Natural Sciences at Escola Secundária de Amares in 2003. In the same year, she was admitted at University of Minho, Braga, and obtained her diploma in “Biologia Aplicada” (Applied Biology) in 2007. Within this period, she received an Erasmus fellowship and developed her final project at the department of Clinical Oncology, Leiden University Medical Center, under the supervision of Dr. Thorbald van Hall and Prof. Dr. Sjoerd H. van der Burg. In 2008 she obtained a research fellowship to work at Instituto Gulbenkian de Ciência (IGC), Oeiras, Portugal, in the group of Salvatore Valitutti, M.D. She was admitted to the PhD program “Graduate Program in Areas of Basic and Applied Biology” (GABBA) from University of Porto, Porto, Portugal. She started the PhD program in 2009 and during the first 6 months she attended theoretical and practical modules from diverse biological subjects in Porto. In September 2009 she moved to Leiden where she started her PhD thesis work in the department of Clinical Oncology, Leiden University Medical Center, under the supervision of Dr. Thorbald van Hall and Prof. Dr. Sjoerd H. van der Burg.

| LIST OF PUBLICATIONS

Oliveira CC, Querido B, Sluijter M, de Groot AF, van der Zee R, Rabelink MJWE, Hoebe RC, Ossendorp F, van der Burg SH, van Hall T. New role of signal peptide peptidase to liberate C-terminal peptides for MHC class-I presentation. *The Journal of Immunology*, 2013, *in press*.

Oliveira CC, van Hall T. Importance of TAP-independent processing pathways. *Molecular Immunology* 2013, 55(2): 113-116.

Seidel UJ, Oliveira CC, Lampen MH, Hall T. A novel category of antigens enabling CTL immunity to tumor escape variants: Cinderella antigens. *Cancer Immunology, Immunotherapy* 2012, 61(1): 119-125.

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

van Hall T, Oliveira CC, Joosten SA, Ottenhoff TH. The other Janus face of Qa-1 and HLA-E: diverse peptide repertoires in times of stress. *Microbes and Infection* 2010, 12(12-13): 910-918.

Oliveira CC, van Veelen PA, Querido B, de Ru A, Sluijter M, Laban S, van der Burg SH, Offringa R and van Hall T. The nonpolymorphic MHC Qa-1^b mediates CD8⁺ T cell surveillance of antigen-processing defects. *The Journal of Experimental Medicine* 2010, 207(1): 207-221.

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