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INTRODUCTION

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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 (IFNγ), 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 selection. 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 lineage. 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. 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 epitopes. 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 β2-microglobulin (β2m) with help from chaperones such as calreticulin, tapasin and ERp57 of the PLC. 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.
**Importance of TAP-independent processing pathways**

The proteasome-TAP pathway is considered as the conventional processing route \(^8-10\). 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 mice \(^11-13\). 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 TAP2 \(^14\). 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 TCR\(\alpha\beta\)CD\(8^+\) 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 cells \(^15\). These observations argue that the TAP-independent processing pathway sufficiently compensate for the loss of the conventional pathway in order to select a functional CD\(8^+\) T-cell repertoire and, moreover, to control virus infections \(^14\). 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 CD\(8^+\) T-cells \(^11-13\). 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 charge \(^18\). 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. 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 affinity. 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 ER. They described the presentation of TAP-independent peptides from one ER-resident protein, Jaw1, and proteins in the secretory pathway, like ovalbumin and CD23. 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. 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 residues. 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 is any amino acid and “↓” indicates the cleavage position). 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 cells. 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. 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 mechanism.

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-I. 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 mechanism. 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.

I THE CASE OF IMMUNE ESCAPE OF TUMORS

Tumors frequently display processing defects

One hallmark of tumors is their ability to evade immune recognition. Among others, a common way to escape is through loss of antigen presentation by MHC-I molecules. 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 types. 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. 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). 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. 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 β2m 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 subunits.

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 killing. 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 CTL.

**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 cells. 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). Recognition depended on the β2m light chain and residual MHC-I molecules on the tumor cells. 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 proteins, induced recognition of these cells by the novel CTL category. Reactivity of these CTL was clearly T-cell receptor dependent and their phenotype was indistinguishable from that of conventional CD8+ CTL. 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).

**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 TEIPP. 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). The epitope was located at the very C-terminus...
of Trh4, which is an ER membrane spanning fatty acid regulator. Since this protein is ubiquitously expressed, it is logical that every tumor type harboring an APM defect was recognized by this CTL. 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.

**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. 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 CTL. Examples of viral proteins targeting TAP are ICP47 (HSV), US6 (HCMV), BNLF2a (EBV), and

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**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.
UL49.5 (varicella viruses). By introducing UL49.5 via gene transfer in human DCs, TAP activity was successfully inhibited\textsuperscript{55}. 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\textsuperscript{+} T-cell pool as they are readily detectable at the bulk T-cell culture level\textsuperscript{55}. Furthermore, \textit{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 manner\textsuperscript{66}. 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 peptide\textsuperscript{67}. Therefore, we consider this CTL epitope, derived from a primary human tumor, as the first molecular defined human TEIPP.

\textbf{I 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 \textit{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\textsuperscript{+} 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 \textit{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 (1-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 Kb 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.

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Introduction
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