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Dominant contribution of the proteasome and metalloproteinases to TAP-independent MHC-I peptide repertoire

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ABSTRACT

Tumors frequently display defects in the MHC-I antigen processing machinery, such as deficiency of the peptide transporter TAP. Interestingly, the residual peptide repertoire contains neo-antigens which are not presented by processing-proficient cells. We termed these immunogenic peptides TEIPP ('T-cell epitopes associated with impaired peptide processing') and were interested to unravel their TAP-independent processing pathways. With an array of chemical inhibitors we assessed the participation of numerous proteases to TAP-independent peptides and found that the previously described catalytic enzymes signal peptidase and furin contributed in a cell-type and MHC-I allele-specific way. In addition, a dominant role for the proteasome and metallopeptidases was observed. These findings raised the question how these proteasome products get access to MHC-I molecules. A novel TEIPP peptide-epitope that represented this intracellular route revealed that the lysosomal peptide transporter ABCB9 ('TAP-like') was dispensable for its presentation. Interestingly, prevention of endolysosomal vesicle acidification by bafilomycin enhanced the surface display of this TEIPP peptide, suggesting that this proteasome-dependent pathway intersects endolysosomes and that these antigens are merely destroyed there. In conclusion, the proteasome has a surprisingly dominant role in shaping the TAP-independent MHC-I peptide repertoire and some of these antigens might be targeted to the endocytic vesicular pathway.
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1. 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 their 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 the TAP1/TAP2 peptide transporter, 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 TAP (Cerundolo and de la Salle, 2006; Glas et al., 1998; Lampen and van Hall, 2011; Lampen et al., 2010; Oliveira and van Hall, 2013; Weinzierl et al., 2008).

We have characterized some TAP-independent peptides from tumors that displayed defects in this peptide transporter and found that these constitute neo-antigens for the $CD8^+$ T cell system (Lampen and van Hall, 2011; Seidel et al., 2012; Van Hall et al., 2006). We called these unique peptide-epitopes TEIPP, for 'T-cell epitopes associated with impaired peptide processing'. Interestingly, TEIPP peptides derive from normal housekeeping proteins, but are not presented on cells with competent function of antigen processing. Our findings implied that the conventional processing pathway, via the proteasome-TAP secretory route prevents the MHC-I loading of the alternative peptide repertoire, including TEIPP peptides (Durgeau et al., 2011; Oliveira et al., 2011). A second consequence is that TEIPP peptides make use of alternative TAP-independent processing pathways, one of which was unraveled by examining the liberation of a C-terminal peptide-epitope derived from the ceramide synthase Trh4 protein (Oliveira et al., 2013; Van Hall et al., 2006). This ER-membrane spanning protein is hydrolyzed by the intramembrane-cleavage activity of the aspartyl protease

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signal peptide peptidase (SPP), independent from proteasomes. Proteolysis within the ER-membrane allowed for TAP-independent loading of this TEIPP peptide.

Tumors with processing defects still present a broad repertoire of peptides in their MHC-I molecules, emphasizing the existence of yet other alternative processing pathways (Oliveira and van Hall, 2013; Oliveira et al., 2010; Van Hall et al., 2006). Studies with TAP-deficient cells and animals have shown that MHC-I ligands can be formed in the secretory and vesicular compartments. Initial peptides identified in cells lacking the TAP transporters were derived from leader sequences (Henderson et al., 1992; Wei and Cresswell, 1992). Leader sequences address fresh translational products to the ER and are removed by signal peptidases (SP) in close proximity of the ER membrane, thereby releasing these leaders. After cleavage by signal peptide peptidase (SPP), certain hydrophobic domains of the signal peptides end up in the ER lumen and, after further trimming, can be loaded unto MHC-I molecules (Henderson et al., 1992; Martoglio and Dobberstein, 1998; Wei and Cresswell, 1992). Other domains of leader sequences fall back in the cytosol, where they need access to the ER via TAP. In addition to this TAP-independent peptide loading pathway, two proprotein convertases have been shown to generate MHC-I ligands in the trans-Golgi network: furin and PC7 (Gil-Torregrosa et al., 1998, 2000; Leonhardt et al., 2010; Tiwari et al., 2007). 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. Several reports indicate that TAP-independent peptides can also be generated in the vesicular pathway, located in endolysosomes, although the responsible proteolytic enzymes largely remain unknown (Del Val et al., 2011; Oliveira and van Hall, 2013; Tey and Khanna, 2012; Tiwari et al., 2007; Weinzierl et al., 2008).

In this report we studied the processing pathway of a new K^b-presented TEIPP epitope recognized by a specific $CD8^+$ T-cell clone capable of killing TAP-deficient tumor cells (Van Hall et al., 2006). Surprisingly, the proteasome was responsible for the generation of this epitope, although TAP-positive cells did not present this peptide by K^b at their cell surface. Furthermore, our data indicate that MHC-I loading of the TEIPP epitope did not require endolysosomal processing, as inhibition of acidification of these compartments did not prevent peptide presentation. The display of this TEIPP peptide was actually enhanced by the proton pump inhibitor bafilomycin, suggesting that endolysosomal processing destroys the peptide. Finally, we found that the proteasome strongly contributed to the general surface display of peptide/MHC-I in TAP-deficient cells and therefore speculate that the majority of these peptides might gain access to MHC-I loading compartments in the endocytic vesicular pathway.

2. Results

2.1. TAP-mediated peptide transport activity in tumor cell panel

The novel category of tumor antigens TEIPP that we previously described is characterized by their selective MHC-I-mediated presentation on TAP-deficient cells. The presentation of TEIPP peptides is surprisingly promoted by the absence of a functional peptide transport across the ER membrane by the TAP1/2 heterodimer (Durgeau et al., 2011; Oliveira et al., 2011; Oliveira and van Hall, 2013; Seidel et al., 2012). In the current study we used a panel of TAP-negative mouse tumor cell lines: the RMA-S lymphoma with an early stop-codon in the *TAP2* gene (Attaya et al., 1992) and the MCA fibrosarcoma from a *TAP1*-knockout mouse (Van Hall et al., 2006). TAP transport activity was measured in these cells and their TAP-proficient counterparts RMA and MCA cells in which the mouse *TAP1* gene was introduced ('MCA.TAP1'). Both the TAP2-deficient RMA-S as well as the TAP1-deficient MCA tumor line completely failed to transport the reporter peptide across the ER membrane, whereas the TAP-competent lines showed efficient translocation (Suppl. Fig. 1). These data indicated that this cell panel represented the two extremes of TAP activity and were useful for our further analyses.

Table 1
Chemical protease inhibitors used in this study.

Inhibitor	Specificity	Concentration
Epoxomicin	Proteasome	400 nM
3,4-Dichloroisocoumarin(DCI)	Signal peptidase	5 μM
Butabindide oxalate	Tripeptidylpeptidase II	400 μM
Leucinethiol	ERAAP	15 μM
1,10-Phenanthroline	Metalloproteases	50 μM
z-vad-fmk	Caspases	25 μM
Calpeptin	Calpains	30 μM
Calpain inhibitor IV	Calpain II	13 μM
PD150606	Calpains	50 μM
Decanoyl-RVKR	Furinconvertase	10 μM
$(z\text{-}LL)_2$-ketone	Signal peptide peptidase	5 μM
Aprotinin	Trypsin, chemotrypsin	10 μM
Bestatin	Metallo-aminopeptidases	200 μM
DAPT	γ-Secretase	10 μM
Captopril	ACE and ACE-like proteases	100 μM
Leupeptin	Trypsin-like and cysteine proteases	200 μM
Bafilomycin-A	Endosomal acidification	200 nM

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

Two processing pathways have been described to deliver TAP-independent peptides. The furin convertase can liberate C-terminal peptides in the Golgi, representing the secretory route and N-terminal leader sequences are liberated in the ER by a signal peptidase- and SPP-dependent pathway (Gil-Torregrosa et al., 1998, 2000; Martoglio and Dobberstein, 1998). We were interested to estimate the contribution of these and several other proteases to the generation of TAP-independent peptides. For that, we measured total D^b and K^b surface molecules in a recovery assay applying an array of protease inhibitors (Table 1). TAP-deficient RMA-S and MCA tumor cells and their TAP-proficient counterparts, were tested and percent inhibition of MHC-I recovery was measured by flow cytometry after 5 h (Fig. 1). The data revealed that the known TAP-independent pathways, based on furin (inhibitor Dec-RVKR), SP (inhibitor DCI) and SPP (inhibitor $z\text{-}LL_2$-ketone) were active and indeed contributed to the overall MHC-I presentation. Interestingly, their contribution was cell-type (lymphoma versus fibrosarcoma) and MHC-I allele (D^b versus K^b) specific. However, their individual contribution was marginal on TAP-deficient cells (Fig. 1). The most dominant inhibitor was epoxomicin, the specific inhibitor for proteasome activity. Approximately 50% recovery of the normal MHC-I levels on TAP-deficient cells could be blocked with epoxomicin, surprisingly comparable to that of the TAP-proficient counterparts (Fig. 1). We concluded that the proteasome-mediated processing pathway dominantly participate in the formation of the TAP-independent peptide repertoire and that the proteasome is more prominent than the previously described furin-, SP- and SPP-mediated pathways.

2.3. Metalloproteases are particularly important for the TAP-independent peptide repertoire

We analyzed if the TAP-independent pathways have different relative contributions in TAP-deficient versus TAP-proficient cells.

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