Research Article

ER-aminopeptidase 1 determines the processing and presentation of an immunotherapy-relevant melanoma epitope

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Dissecting the different steps of the processing and presentation of tumor-associated antigens is a key aspect of immunotherapies enabling to tackle the immune response evasion attempts of cancer cells. The immunodominant glycoprotein gp100209-217 epitope, which is liberated from the melanoma differentiation antigen gp100PMEL17, is part of immunotherapy trials. By analyzing different human melanoma cell lines, we here demonstrate that a pool of N-terminal extended peptides sharing the common minimal epitope is generated by melanoma proteasome subtypes. In vitro and in cellulo experiments indicate that ER-resident aminopeptidase 1 (ERAP1)—but not ERAP2—defines the processing of this peptide pool thereby modulating the T-cell recognition of melanoma cells. By combining the outcomes of our studies and others, we can sketch the
complex processing and endogenous presentation pathway of the gp100209-217- containing epitope/peptides, which are produced by proteasomes and are translocated to the vesicular compartment through different pathways, where the precursor peptides that reach the endoplasmic reticulum are further processed by ERAP1. The latter step enhances the activation of epitope-specific T lymphocytes, which might be a target to improve the efficiency of anti-melanoma immunotherapy.

**Keywords:** CD8$^+$ T cells · proteasome · ER-aminopeptidase · melanoma · gp100

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Introduction**

Recognition and destruction of malignant cells by CD8$^+$ cytotoxic T lymphocytes (CTLs) are the underlying mechanisms of the three leading approaches in cancer immunotherapy. At first, injection of antibodies blocking the immune checkpoints CTLA-4 or PD-1, which causes activation of patient’s T cells, has emerged as a breakthrough in antitumor therapy. Secondly and thirdly, adoptive transfer of tumor-specific CTLs and therapeutic vaccination are under preclinical as well as clinical investigation. A key factor in these latter immunotherapies is the selection of optimal epitopes that are presented by major histocompatibility complex (MHC) class I molecules to CTLs [1–3].

Tumor-expressed T cell epitopes are generated, transported, and presented at the cell surface by MHC class I molecules via the antigen processing and presentation pathway (APP). The majority of these epitopes are produced by proteasome complexes, which are the central proteolytic unities of the ubiquitin-proteasome system. Proteasomes generate MHC class I-restricted epitopes by canonical peptide-bond hydrolysis as well as by post- translational peptide splicing [4]. The proteasome’s 20S catalytic core particle is arranged as four staggered rings, each containing seven non- identical subunits. The outer rings contain the α subunits (α1–α7), which form the ‘gates’ through which substrates enter and products are released. Each of the two inner rings contains the β subunits (β1–β7), three of which (β1, β2, and β5) harbor the six active sites. In some hematopoietic cell types and in the presence of type I and/or type II interferons, the β1, β2, and β5 standard subunits are replaced by their alternative variants, the immunoproteasome subunits β1i/LMP2 (low molecular weight protein), β2i/MECL-1 (multicatalytic endopeptidase complex like 1), and β5i/LMP7, thereby forming de-novo synthesized immunoproteasomes [5, 6]. Besides standard- and immunoproteasome complexes, expression of intermediate- or mixed-type proteasomes containing both standard- and immunoproteasome subunits have been detected in various tumor cells as well as in healthy tissue [7–9]. These proteasome isoforms quantitatively differ in their capability to produce peptides although the existence of proteasome-specific substrate cleavage sites and peptide products is still a matter of discussion [7, 8, 10–15].

Following generation by proteasomes, the epitopes and their precursor-peptides can be destroyed or further processed in the cytosol by aminopeptidases such as tripeptidyl peptidase 2 (TPP2) [16]. Peptides that survive this APP step are often translocated into the ER lumen via TAP, where peptides can be further trimmed by the IFN-γ inducible ER-resident aminopeptidases ERAP1 and/or ERAP2 [17, 18]. In addition, ERAP1/2 has been shown to be constitutively expressed in different tissues and tumor cells, where the ERAP1 level correlated with MHC class I expression [19–21]. Whereas the generation of various, mainly viral, epitopes is enhanced by ERAP1 [22], some tumor epitopes are destroyed by ERAP1 activity [23, 24], thereby suggesting that N-terminal trimming in the ER can be a key step of MHC class I antigen presentation and recognition of both infected and cancer cells [25]. So far, no precise role of ERAP2 in MHC class I epitope generation could be defined, although its specificity has been characterized to be distinct from ERAP1 activity [18, 26]. In general, the specific mechanism of ERAP1 and/or ERAP2 function could not be clarified so far. Although ERAP1 and ERAP2 have been shown to trim peptides in solution separately and also completing each other’s activity, ERAP1/2 heterodimer formation has been proposed to exist in cells. However, the exact nature of the in vivo heterodimer could not be demonstrated so far. An artificially created ERAP1-ERAP2 heterodimer was shown to change the enzymatic parameters of ERAP1 leading to increased peptide trimming efficacy of the ERAP1/2 complex in vitro [27]. Furthermore, ERAP1-ERAP2 heterodimer constructs have been shown to trim precursor peptides while they are bound to MHC class I molecules, albeit no direct ERAP/MHC interaction could be detected [28]. Peptides are further handled by the peptide loading complex, which inserts the peptide into the binding groove of the MHC class I molecules according to their sequence affinity [29, 30].

Initially identified by the Rosenberg lab, the melanoma-associated glycoprotein 100 (gp100)209-217 epitope has been frequently targeted in clinical anti-melanoma trials, mainly in its anchor-modified form (gp100209-217/T210M) to enhance HLA-A*02:01 binding affinity [31–34]. The T210M substitution not only improves the binding of the gp100209-217/T210M epitope to HLA-A*02:01 but also alters significantly the proteasomal processing of that antigenic sequence [35]. The gp100209-217 epitope
is derived from the melanocyte differentiation protein gp100 (also called PMEL or Pmel17), which belongs to the group of differentiation antigens expressed by tumor cells and healthy tissue of origin. PMEL can be frequently detected in metastatic melanomas making it a suitable target for cancer immunotherapy [36].

The gp100\textsubscript{209-217}/T210M epitope can be recognized by CTLs not only in its minimal version but also in its N-terminal extended versions gp100\textsubscript{207-217}/T210M and gp100\textsubscript{208-217}/T210M. The latter peptides bind the HLA-A\textsuperscript{a}02:01 complex with lower affinity than the gp100\textsubscript{209-217}/T210M epitope. Any N-extended version of gp100\textsubscript{209-217} (from gp100\textsubscript{205-217} to gp100\textsubscript{208-217}) produced by proteasomes can be trimmed in vitro by ERAP1 to the length of the minimal gp100\textsubscript{209-217} epitope, whereas further degradation seems to be prevented [35]. According to these results, ERAP-mediated trimming of this pool of peptides in melanoma cells should determine the CTL response and therefore affect the efficacy of immunotherapies targeting this epitope.

To test this hypothesis, we investigated in vitro and in cellulo how ERAP1 and ERAP2 control the activation of the gp100\textsubscript{209-217}/T210M-specific CTL clone, which recognizes both WT gp100\textsubscript{209-217} and mutant gp100\textsubscript{209-217}/T210M epitopes. The experimental outcomes provided the missing information to construct the APP model of this critical melanoma antigen.

Results

Cleavage preferences of proteasome subtypes expressed in human melanoma cell lines

Since the generation and presentation of the pool of gp100\textsubscript{209-217}-containing peptides is influenced by the proteasome isoform content of the cell [10, 12, 13, 35], we investigated the proteasome catalytic-subunit composition in two human melanoma cell lines, i.e. the gp100-expressing Ma-Mel-63a cells and the gp100-negative Ma-Mel-86a cells (Supporting Information Fig. 1A). Both melanoma cell lines expressed a mixed population of proteasome isoforms including what we inferred being the intermediate-type proteasome carrying the \(\beta1/\delta\), \(\beta5i/LMP7\), and \(\beta2/\Omega\) or the \(\beta1i/LMP2\), \(\beta5i/LMP7\), and \(\beta2i/\Omega\) subunits (Fig. 1A). Accordingly, analysis of purified proteasomes by two-dimensional gel electrophoresis revealed the assembly of intermediate-type proteasomes containing the two immunosubunits \(\beta1i/LMP2\) and \(\beta5i/LMP7\) in the absence of \(\beta2i/\gamma\text{MECL-1}\) (Supporting Information Fig. 1B). However, compared to isolated human spleen, \(\beta1i\) and \(\beta5i\) expression was considerably less pronounced in both melanoma cell lines. Based on these observations, we conclude that the melanoma cell lines Ma-Mel-63a and Ma-Mel-86a express intermediate-type proteasomes containing \(\beta1i/LMP2\), \(\beta5i/LMP7\) and \(\beta2/\Omega\).

Exposure of melanoma cell lines to INF-\(\gamma\), which could be a frequent situation in the tumor and microenvironment, resulted in the up-regulation of all three immunosubunits including the \(\beta2i/\gamma\text{MECL-1}\) subunit (Fig. 1B), thereby suggesting that the modification of the proteasome isoform content is modifiable in these cell lines.

To investigate the specific proteolytic activity of melanoma-derived 20S proteasomes we performed in vitro processing experiments of the synthetic peptide gp100\textsubscript{201-230} using 20S proteasomes purified from the two melanoma cell lines. The substrate and peptide products were measured by mass spectrometry (MS) and the MS outcomes were analyzed by the quantification with minimal effort (QME) method, which allowed to quantify the amount of each peptide product and to compute the substrate cleavage-site usage, i.e. the substrate site-specific cleavage strength (SCS) [37]. Correlation between in vitro experiments carried out with purified 20S proteasomes and in cellulo and in vivo experiments has been demonstrated in various studies [7, 8, 12, 13, 38–47]. Proteasomes were purified from cell lines grown in the absence of inflammatory stimuli to analyze their baseline activity. Consistently with previous studies [10, 12, 13, 35], the minimal gp100\textsubscript{209-217} epitope was not detectable in melanoma proteasome digestions, whereas its N-terminal extended versions could be quantified (Fig. 2A). In terms of substrate degradation and preferences of cleavage within the synthetic gp100\textsubscript{201-230} peptide, the proteasomes purified from the two melanoma cell lines showed a similar degradation rate and a similar cleavage pattern, which differed from that

Figure 1. Proteasome content in human melanoma cell lines. (A) Subunit composition of proteasome complexes purified from the cell lines Ma-Mel-63a and Ma-Mel-86a analyzed by immunoblotting using antibodies against the indicated proteasome subunits (two technical replicates, single representative experiment). T2 cells (deficient for \(\gamma\text{MECL-1}\) and T2.27 cells—expressing murine \(\beta1i/LMP2\), \(\beta5i/LMP7\), as well as human \(\beta2/\Omega\)—were used as control and \(\gamma\) served as loading control. (B) Induction of immunoproteasome subunits in Ma-Mel-63a and Ma-Mel-86a cells upon INF-\(\gamma\) treatment analyzed by immunoblotting using antibodies, as indicated. Melanoma cells were exposed to 200 U/ml INF-\(\gamma\) for 48 h and compared to HeLa cells (two biological replicates, one out of two independent experiments is shown). GAPDH served as loading control.
Figure 2. Gp100-related activity of 20S proteasomes isolated from different human cell lines and tissues. (A) Absolute amount of the synthetic substrate gp100201-230 and of the gp100209-217-containing peptides in the in vitro kinetic assays with 20S proteasome purified from Ma-Mel-63a and Ma-Mel-86a cell lines. The absolute amount of the peptide products was computed by quantification with minimal effort (QME). Values represent the mean and lines the SD of two independent experiments and two to three technical replicates. (B) Relative frequency of cleavage (SCS) after each substrate residue of the synthetic substrate gp100201-230 by 20S proteasomes purified from Ma-Mel-63a and Ma-Mel-86a cell lines as well as from human erythrocytes and spleen. SCS was computed by QME from the in vitro kinetic assays measured by MS. Values represent the mean and lines the SD of two independent experiments and two or three technical replicates for the melanoma proteasome and two technical replicates for the human erythrocytes and spleen. (C) Scheme of the applied substrates and detected peptides throughout the study.

of proteasomes purified from human erythrocytes (standard proteasome) and spleen (immunoproteasome-enriched) (Fig. 2A and B). 20S melanoma proteasomes produced the gp100205-217 peptide in considerably larger amounts than the shorter N-extended versions of the gp100209-217 epitope (Fig. 2A and C). This was mediated by a strong cut between S204 and S205 at the gp100205-217 N-terminus (Fig. 2B). All substrate cleavage sites used by erythrocyte and spleen proteasomes were used also by melanoma proteasomes, thereby confirming the hypothesis that proteasome isoforms largely cleave after the same residues although with significant quantitative differences [10, 37]. The SCSs of erythrocyte and spleen proteasomes were similar to those of a previous study carried out with 20S proteasome purified from these specimens [10], thereby confirming the robustness of this type of assay and analysis.

ERAP1 provokes degradation of the gp100205-217 substrate as well as gp100209-217 epitope generation

As already anticipated, 20S proteasomes obtained from melanoma cell lines did not produce the minimal gp100209-217 epitope in detectable amounts, confirming the result of previous studies carried out with various proteasomes and conditions [10, 12, 13, 35].
Two N-extended versions — i.e. gp100208-217 and gp100207-217 — have been shown to induce CTL activation in vitro [35]. They are produced in significant smaller amounts than the gp100205-217 peptide by 20S proteasome purified from melanoma cell lines (Fig. 2A) and other cell types [35], though. These quantitative observations suggest that the trimming of the N-extended versions of the minimal gp100209-217 epitope could play a significant role in the presentation of the gp100209-217 epitope by melanoma cells to CTLs. Therefore, we computed the in vitro efficiency in trimming N-extended versions of the minimal gp100209-217 epitope by recombinant ERAP1. To this end we made use of a mathematical model of ERAP1 activity and inferred the kinetic parameters based on the quantitative substrate degradation measurements of the digestions of the gp100205-217, gp100206-217, gp100207-217, gp100208-217, and gp100209-217 synthetic peptides previously published [35] (Supporting Information Fig. 2A). The degradation kinetics of the substrates recapitulated a Michaelis–Menten-like reaction. Thus, we set out to estimate the Michaelis–Menten parameters kcat, KM, and kcat/KM (Supporting Information Fig. 2B), and reported the kcat/KM values (Table 1), which gave the best confidence in our model. The estimated kcat/KM value of gp100209-217 peptide was significantly lower compared to the parameters of the N-extended versions, thereby reflecting the fact that the epitope was not trimmed by ERAP1 in those conditions. The ERAP1-mediated trimming efficiency for the substrates gp100208-217, gp100207-217, and gp100205-217 was comparable. On the contrary, the substrate gp100206-217 was processed with highest efficiency, most likely due to a higher kcat (Table 1 and Supporting Information Fig. 2B).

Since the gp100205-217 peptide is generated in high amounts by the proteasome of the melanoma cell lines (Fig. 2A), we further investigated the ERAP1/2 dynamics of gp100205-217 processing. When this peptide is trimmed by ERAP1, the gp100206-217, gp100207-217, gp100208-217, and gp100209-217 peptides/epitope are generated and no shorter peptide fragments could be detected (Fig. 3A and B). When we used very high ERAP1 enzyme concentrations (2 μg/ml) a barely detectable gp100210-217 peptide product was measured (Supporting Information Fig. 3A).

Normal concentration of ERAP2 could generate only a small amount of the gp100207-217 epitope (Fig. 3A and C), which could be enhanced by using high ERAP2 enzyme concentrations (Supporting Information Fig. 3B and C). No smaller peptide products could be clearly identified when we used the synthetic peptides gp100207-217, gp100208-217, and gp100209-217 as substrates (Supporting Information Fig. 3B and C and data not shown). This limited trimming activity of ERAP2 is substrate specific since the cleavage of the HIV-gp160313-327 precursor peptide was efficiently carried out as expected (Supporting Information Fig. 4A and B) [17]. In ERAP1/2 digestions, the gp100205-217 substrate degradation rate directly correlated with the ERAP1:ERAP2 relative ratio. Accordingly, more products were detectable by increasing the ERAP1:ERAP2 ratio, although in those assays where ERAP2 was present, the gp100206-217 peptide product could not be identified (Fig. 3A–F).

To further test our hypothesis that ERAP1 promotes the generation of immunogenic gp100205-217-containing epitopes, we performed in vitro and in cellulo experiments by using the metalloprotease inhibitor leucinethiol, which targets ERAP1. Consistently, addition of leucinethiol to the in vitro digestion resulted in a blockade of gp100205-217 degradation and stabilization of the precursor peptide (Fig. 4A and B). Furthermore, activation of a gp100209-217-specific CTL clone was significantly reduced by ERAP1 inhibition in Ma-Mel-63a (Fig. 4C) and UKRV-Mel-15a melanoma cells (Fig. 4D), which both express the gp100 antigen and HLA-A*02:01. As activity control, the ability of leucinethiol treated cell lysates to trim the H-Leu-AMC or the H-Arg-AMC substrate was analyzed. As expected, the turnover of the H-Leu-AMC — representing ERAP1 activity — was significantly reduced in the presence of leucinethiol, whereas the H-Arg-AMC substrate — representing ERAP2 activity — was not affected (Fig. 4E).

**Table 1.** Kcat/KM of the trimming of N-extended versions of the minimal gp100205-217 epitope by recombinant ERAP1. The Michaelis–Menten constants were computed from the in vitro digestions of the 50 μM synthetic peptides gp100205-217, gp100206-217, gp100207-217, gp100208-217, gp100209-217 by 2.5 ng recombinant ERAP1 over time. The MS-measured results of the digestions were published previously [35]. Means and standard deviations of two independent experiments are shown. Kinetic parameters were estimated in a Bayesian framework resulting in parameter distributions and therefore providing confidence estimates (see also Supporting Information Fig. 2).

| Substrate       | Kcat/KM [mM⁻¹ min⁻¹] |
|-----------------|----------------------|
| gp100209-217    | 1.50 ± 1.51          |
| gp100208-217    | 17.68 ± 1.99         |
| gp100207-217    | 14.12 ± 2.11         |
| gp100206-217    | 37.72 ± 5.15         |
| gp100205-217    | 17.37 ± 2.91         |

ERAP1, but not ERAP2, promotes activation of gp100209-217-specific CTL clones by melanoma cell lines

These results suggested a beneficial impact of ERAP1 rather than ERAP2 in defining the presentation of the gp100209-217-containing peptides/epitope by melanoma cells to CTLs. We tested this hypothesis and the ERAP1/2 content in human melanoma cell lines Ma-Mel-63a, Ma-Mel-86a, and UKRV-Mel-15a. They all expressed both ERAP1 and ERAP2, although ERAP2 expression was lower than ERAP1 with different ERAP1:ERAP2 ratio among the melanoma cell lines (Fig. 5A–C). Upon exposure to IFN-γ, ERAP1 expression was enhanced in both Ma-Mel-86a cells and Ma-Mel-63a cells (Fig. 5D) and also in UKRV-Mel-15a cells as shown before [24]. Since ERAP activity could be influenced by the various polymorphisms that these enzymes have, we analyzed the ERAP1 alleles present in the investigated cell lines. Amongst others, we observed two described polymorphisms associated with the risk of cancer development [48, 49]. The R127P polymorphism in Hela, UKRV-Mel-15a and Ma-Mel-63a and the Q730E mutation in Ma-Mel-86a cells suggest an altered peptide trimming activity in the latter [50] (Fig. 5E).

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Figure 3. ERAP1 and ERAP2 trimming activity of the synthetic substrate gp100205-217. (A–F) The epitope precursor peptide gp100205-217 was digested in vitro for 4 h by ERAP1 and/or ERAP2 and the generated peptide fragments were measured by LC–MS. The HPLC-profiles are zoomed from 35 to 50 min. Addition of enzymes as follows: (A) substrate gp100205-217 control, (B) ERAP1 (3 ng), (C) ERAP2 (3 ng), (D) ERAP1/2 ratio I:II (1 ng ERAP1/2 ng ERAP2), (E) ERAP1/2 ratio I:II (1.5 ng ERAP1/1.5 ng ERAP2), and (F) ERAP1/2 ratio II:I (2 ng ERAP1/1 ng ERAP2). The relative MS ion signal of the peptides gp100209-217 (9mer epitope), gp100208-217 (10mer), gp100207-217 (11mer), and gp100206-217 (12mer), which were identified by LC–MS/MS, is indicated. One out of two independent experiments is displayed.

To test whether ERAP1 and ERAP2 could impinge upon the presentation of the gp100209-217-containing peptides/epitope by melanoma cell lines to CTLs, we performed siRNA knock down of either ERAP1 or ERAP2 in Ma-Mel-63a cells. In agreement with our in vitro results, knocking down ERAP1 significantly reduced the gp100209-217-specific CTL activation, whereas silencing ERAP2 displayed no significant alterations of the amount of IFN-γ released by the CTL clone (Fig. 6A–C). Similar results were obtained when we used UKRV-Mel-15a (Supporting Information Fig. 5A and B) and gp100-transfected HeLa cells expressing HLA-A*02:01 (HeLa cells expressing HLA-A*02:01) cell line clones (Fig. 6D–F). This hints toward the hypothesis that ERAP1, rather than ERAP2, promotes the activation of gp100209-217-specific CTLs by increasing the presentation of gp100209-217 epitopes by melanoma cell lines as well as by other cancer cell lines.

Discussion

The modified gp100209-217/(T210M) melanoma epitope has been part of successful phase 2/3 clinical trials combining the gp100209-217/(T210M) peptide with interleukin-2 application [34]. However, combined administration of gp100 peptides together with the CTLA-4-antagonist ipilimumab in patients with metastatic melanoma displayed no improvement in disease progression compared to patients treated with ipilimumab alone, thereby suggesting an insufficient presentation of the gp100209-217 epitope at least in parts of the melanoma [51, 52].

Our results provide further information about the APP of the gp100209-217-containing epitopes and specifically the role that ERAP1 rather than ERAP2 plays in promoting epitope presentation. Both in vitro kinetic analyses and in cellulo inhibitor and siRNA experiments demonstrated that ERAP1 generates and stabilizes the minimal gp100209-217 epitope. This is in contrast to the MART-126-35 (melanoma antigen recognized by T cells) epitope being destroyed by ERAP1 [24]. ERAP1 has been shown to be constitutively expressed in tumor cells, even in the absence of IFN-γ [19, 24]. Analyzing three different melanoma cell lines, we found differences in ERAP1 expression (Fig. 5). We could detect two ERAP1 polymorphisms (R127P and Q730E), which are correlated with an increased risk of cancer development [49, 53]. However, only the Q730E mutation has been shown to display reduced peptide trimming activity suggesting an impaired gp100209-217 epitope generation in melanoma cells expressing the Q730E variant [50]. To note, we did not observe any effect of ERAP2 on gp100209-217 epitope processing, neither in vitro digestions—using ERAP2 alone or in combination with ERAP1—nor in cellular antigen presentation assays analyzing ERAP1/2 activity based on ERAP natural expression in melanoma cells.

By combining the outcome of this and other studies [12, 13, 35, 54], we can sketch how gp100209-217-containing epitopes are presented to CTLs: in the cytosol, proteasomes produce two potential epitopes, i.e. gp100207-217/T210M and gp100208-217/T210M
Figure 4. Endogenous gp100<sub>209-217</sub> epitope generation is inhibited by leucinethiol. (A) LC-MS profiles of in vitro substrate degradation of the 13mer gp100<sub>205-217</sub> precursor peptide by ERAP1 without and with inhibitor leucinethiol after 180 min incubation and (B) corresponding relative substrate degradation. SD (of mean) of duplicate determinations is shown as representative data from two independent experiments. (C) Ma-Mel-63a and (D) UKRV-Mel-15a cells were exposed to 30 µM leucinethiol for 16 h or to DTT as solvent control and were co-incubated with gp100<sub>209-217</sub>-specific T lymphocytes for 16 h. IFN-γ release by gp100-specific CTLs was measured by IFN-γ ELISA. T2 cells without and with gp100<sub>209-217</sub> peptide were used as control for T cell specificity. Data are displayed as mean + SD of three technical replicates. One independent experiment out of two is shown (Student's t-test; *p < 0.05; **p < 0.01). (E) Specific activity of ERAPs in cell lysates of leucinethiol treated Ma-Mel-63a and UKRV-Mel-15a cells. Specific activity was measured through incubation of the cell lysates with the H-Leu-AMC- or the H-Arg-AMC-substrate, which display ERAP1 and ERAP2 activity, respectively. DTT-treated cells served as control. Data are displayed as mean and SD of two technical replicates (one out of two independent experiments).

that share the gp100<sub>209-217</sub>/T210M minimal sequence IT/MDQVPFSV. The WT gp100<sub>207-217</sub> and gp100<sub>208-217</sub> peptides bind with low affinity to the HLA-A*02:01 complex, therefore their in vivo immunogenicity is disputable, and these are most likely only sources for the gp100<sub>209-217</sub> generation in the ER. In contrast, the T210M gp100<sub>207-217</sub> and gp100<sub>208-217</sub> peptides bind to the HLA-A*02:01 complex with higher affinity and trigger a stronger CTL response in vitro than the WT gp100<sub>209-217</sub> epitope [35]. Furthermore, melanoma proteasomes, which contain intermediate-type proteasomes, as shown for both Ma-Mel-63a and Ma-Mel-86a cells here as well as in previously published literature [7, 13], abundantly generate the N-extended version of the gp100<sub>209-217</sub>-epitope, i.e. the gp100<sub>205-217</sub> precursor. In the cytosol, the epitopes (and likely also their N-extended precursors) can be destroyed by TPP2 [54]. The peptides that survive this step can be translocated into the ER lumen via TAPs and to the endosomal compartment by another unknown carrier. In the ER, the peptides gp100<sub>205-217</sub>, gp100<sub>206-217</sub>, gp100<sub>207-217</sub>, and gp100<sub>208-217</sub> can be trimmed by ERAP1—but not by ERAP2—thereby augmenting the amount of the gp100<sub>209-217</sub>-containing epitopes, as shown in the present study and in ref. [35]. For those gp100<sub>209-217</sub>/T210M-containing epitopes that followed the canonical TAP-dependent pathway, the allocation into the HLA-A*02:01 binding groove is likely mediated by the peptide loading complex. For the portion of the gp100<sub>209-217</sub> epitope that is translocated into the endosomal
pathway via an alternative route, the function of tapasin is not mandatory to efficiently present the gp100_{209-217} epitope at the cell surface [54] (Fig. 7).

The APP that triggers a response of gp100_{209-217}/T210M-specific CTLs is therefore branched and can be carried out with success independently of several proteins/enzymes. This supports the use of this pool of epitopes/peptides in immunotherapy because a diversified APP can be a potent tool to tackle the immune-response escape attempts implemented by cancer. For example, we can speculate that even in case that cancer compromises either TAP or tapasin, the presentation of the gp100_{209-217}/T210M-containing epitopes could be enhanced in melanoma by inhibiting TPP2 and/or stimulating ERAP1.

Materials and methods

Cell culture

Melanoma cell lines have been described previously [55]. Melanoma cells, HeLa cells, the TAP-deficient T2 cell line, and T2.27 cells expressing murine β1i/LMP2 and β5i/LMP7 [56] were grown in RPMI1640 (Biochrom) containing 10% FCS, 2 mM L-glutamine, and 100 U/mL penicillin/streptomycin (additives from PAA Laboratories). HeLa transfectants, expressing HLA-A*02:01 (HeLa<sub>A2</sub>) were grown in medium supplemented with 2 µg/mL puromycin and T2.27 cells in the presence of 0.1 mg/mL G418 [56, 57].
Figure 6. ERAP1 but not ERAP2 impinges upon the activation of the gp100[209-217]-specific CTL clones. (A) IFN-γ-production of gp100[209-217]-specific CTL clones incubated for 16 h with Ma-Mel-63a melanoma cells expressing HLA-A*02:01 and the gp100 antigen. Ma-Mel-63a cells were pretreated with 30 nM ERAP1, ERAP2, and control siRNA for 72 h. T2 cells served as negative and gp100[209-217]-peptide loaded T2 cells as positive control, the mean and the SD of three technical replicates are shown. Student's t-test; \( p < 0.01 \). (B) Ma-Mel-63a cell RNA was analyzed for down-regulation of ERAP1 and ERAP2. GAPDH served as loading control. (C) qPCR analysis of ERAP1 and ERAP2 down-regulation in Ma-Mel-63a cells. Relative expression was calculated in comparison to control siRNA treated cells and 18S rRNA served as housekeeping gene. The means and SD of three technical replicates are shown. (D) IFN-γ production of gp100[209-217] specific cytotoxic T cells incubated for 16 h with gp100 transfected HeLaA²⁺ cells (HeLa cells expressing HLA-A*02:01). HeLaA²⁺ cells were pretreated with siRNA against ERAP1 or ERAP2 as well as control siRNA for 72 h. T2 cells served as negative and gp100[209-217]-peptide loaded T2 cells as positive control, the mean and the SD of three technical replicates are represented. Student’s t-test; \( p < 0.01 \). (E) HeLa cell RNA was tested for down-regulation of ERAP1 and ERAP2 and overexpression of gp100. GAPDH served as loading control. (F) qPCR analysis of ERAP1 and ERAP2 down-regulation in HeLaA²⁺ cells. Relative expression was calculated in comparison to control siRNA treated cells and 18S rRNA served as housekeeping gene. The mean and SD of three technical replicates are shown. One out of two independent experiments is shown (A–F).

Immunoblotting and 2-dimensional gel electrophoresis

Western blot was performed as described [58]. Briefly, 25 µg protein of cell lysate or 100 µg for gp100 detection and 1 µg of purified 20S proteasome per lane were separated on SDS-PAGE, transferred to PVDF-membranes and were immunoblotted for proteasome subunits (all laboratory stock), β1i/LMP2 (Abcam), gp100 (Abcam), and GAPDH (Santa Cruz). For ERAP1/2 detection and quantification 50 or 100 µg protein of cell lysate were blotted and probed with ERAP1 (6H9, R&D Systems), ERAP2 (3F5, R&D Systems), and β-actin (Sigma-Aldrich). Membranes were developed with ECL (GE Healthcare). For absolute quantification of ERAP1/2 in human cell lines, the western blot signal was compared to recombinant ERAP1 (10, 50, 75, and 100 ng) or ERAP2 (5, 10, 20, and 50 ng), and was quantified using ImageJ software. ERAP content in cell lysates was calculated from the resulting standard curves.
To separate the subunits of the 20S proteasome complex, isoelectric focusing by carrier ampholytes was combined with SDS-PAGE. Proteasomes were applied to a carrier ampholyte isoelectric focusing gel. In the second dimension, proteins were loaded onto SDS-PAGE and stained with Coomassie brilliant blue G-250. Proteasome subunits could be identified based on their migration behavior in comparison to reference electrophoreses of selected proteasomes [59].

20S proteasome and recombinant ERAP1/2

20S proteasomes were purified from frozen melanoma cell lines and from human spleen and erythrocytes as described [60]. Recombinant human ERAP1 and ERAP2 were purchased from R&D Systems.

Peptides

The AHSSAFTITDQVPFSVSQLRALDGGNK gp100201-230 polypeptide as well as the gp100208-217 9mer ITDQVPFSV and the 10mer TITDQVPFSV, 11mer FTTITDQVPFSV, 12mer AFTITDQVPFSV, 13mer SAFTITDQVPFSV derive from the human melanocytic protein gp100\textsubscript{PMEL17} sequence. The HIVgp160\textsubscript{131-327} precursor peptide containing the gp160\textsubscript{219-327} epitope derives from the HIV gp160 glycoprotein. All peptides were synthesized using Fmoc solid phase chemistry.

In vitro digestion of short fluorogenic peptides

To control inhibition of peptidase activity in Ma-Mel-63a and UKRV-Mel-15a cells, cells exposed to leucinethiol (solved in 0.5 mM DTT) or DTT were lysed in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM MgCl\textsubscript{2}, 0.1% TritonX, and homogenized with a Dounce homogenizer. Specific peptidase activity was determined by incubation of 3 \mu g cell lysate with 50 \mu M H-Leu-AMC or H-Arg-AMC (Bachem) in 25 mM Tris-HCL (pH 7.5), 150 mM NaCl assay buffer for 30 min at 37°C, and computed as [nmol AMC/min/\mu g cell lysate].

Recombinant ERAP1 and ERAP2 enzymatic activity was tested by degrading the substrates H-Leu-AMC and H-Arg-AMC for ERAP1 and ERAP2, respectively. We used various substrate concentrations (10-200 \mu M) and 0.1 \mu g ERAPs in 25mM Tris (pH 7.5). The specific activity was 0.156 nmol/min/\mu g (ERAP1) and 0.05 nmol/min/\mu g (ERAP2) and comparable to the activities described by the manufacturer (R&D systems).

The fluorescence was measured by Tecan fluorometer using excitation 360 nm and emission 460 nm wavelength in a kinetics experiment (0–20 min). The free AMC calibration curve was done by measuring the fluorescence of free AMC at various concentrations (0–1 \mu M).
In vitro digestion of synthetic peptide substrate by purified 20S proteasome

The synthetic peptide gp100<sub>201-230</sub> (40 µM) was digested by 3 µg of purified 20S proteasomes in 100 µL TEAD buffer (20 mM Tris, 1 mM EDTA, 1 mM Na<sub>3</sub>, 1 mM DTT, pH 7.2) in kinetic experiments (0–8 h) at 37°C. For the assays performed with melanoma proteasomes we carried out two biological replicates, each of them measured 2–3 times by MS. For the assays performed with erythrocyte and spleen proteasomes we carried out one assay, measured twice by MS since the outcome recapitulated the results described previously [10]. Liquid chromatography–mass spectrometry (LC-MS) analyses of polypeptide digestion products were performed using the ESI-ion trap instrument DECA XP MAX (ThermoFisher Scientific, USA) as previously described [61]. Database searching was performed using SpliceMet’s ProteaJ, which allowed the identification of spliced and non-spliced peptide products [61]. Quantification of proteasome-generated peptides and computation of the substrate SCS was carried out by applying the QME (Quantification with Minimal Effort) method to the LC-MS analyses [37]. QME estimated the absolute content of peptide products based on their MS ion peak area measured in the digestion probe. The QME algorithm parameters were empirically computed in our previous study [37] and here applied. SCS describes the relative frequencies of proteasome cleavage after any given residue of the synthetic polypeptide substrates [37]. The SCS values shown in this study are the average of SCS measured over time [37].

Analysis of in vitro digestions of precursor peptides with ERAPs

Fifty micromolar of peptides were digested in vitro with 3 ng recombinant ERAP1 or recombinant ERAP2 in 20 µL assay buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 0.5 µg/mL albumin) at 37°C. For high enzyme concentrations, 25 µM of peptides were digested with 2 µg/mL ERAP1 or ERAP2 [18]. To inhibit ERAP1 activity, 3 ng recombinant enzyme were pre-incubated with 30 µM leucinethiol at room temperature for 20 min and then applied to in vitro processing experiments. For the analysis of the digestion products obtained from the gp100<sub>205-217</sub> precursor peptide, different ERAP1/ERAP2 ratios (pre-incubation 30 min at room temperature) were used, total enzyme concentration (3 ng/20 µL digestion volume) and peptide concentration (50 µM) were constant, recombinant ERAP1/ERAP2 ratio is indicated in Fig. 3. The digestion period was 4 h. Experiments were performed at least twice. Peptide products were identified by LC-MS/MS, as described for in vitro proteasome experiments (see above). Substrates’ abundance was quantified through the titration of synthetic peptides.

To analyze the substrate trimming dynamics of ERAP1, we applied a mathematical model describing Michaelis–Menten kinetics. We estimated the kinetic parameters of the model Km and kcat of the trimming of the synthetic substrates gp100<sub>205-217</sub>, gp100<sub>206-217</sub>, gp100<sub>207-217</sub>, gp100<sub>208-217</sub>, gp100<sub>209-217</sub> by purified ERAP1 in two of the kinetics experiments described elsewhere [35].

The model reactions can be depicted as follows: S + E ↔ [SE] → P + E, resulting in the ordinary differential equation model describing substrate degradation over time t: dS/dt = -E⋅kcat⋅S/(K_M+S), where S is the substrate, P is the sum of all products, E is the enzyme ERAP1; KM is the Michaelis–Menten constant; kcat describes the trimming of the substrate to products P (also maximal velocity). According to our model, although a large range of KM and kcat values could result in a good model fit with the experimental data, the KM and kcat were strongly correlated (Supporting Information Fig. 2B). Hence, we could compute ERAP1 efficiency as the ratio kcat/KM and then derive the parameter confidence distribution for KM and kcat (Supporting Information Fig. 2B). The parameters kcat, KM, and kcat/KM were estimated using exact Bayesian inference in a Markov Chain Monte Carlo scheme. The latter resulted in posterior parameter distributions (rather than single point estimates) and therefore we could also provide with an estimate of the parameter uncertainty. The medians and standard deviations (SD) of the marginal posterior parameter distributions are reported in Table 1.

Identification and quantification of ERAP1 in the lysates of different cell lines were performed as described before [62]. LC-MS runs were conducted as follows: samples were trapped and then analyzed by nonscale LC-MS/MS measurements using a Q Exactive Plus mass spectrometer coupled with an Ultimate 3000 RSLCnano (ThermoFisher Scientific). Protein identification and relative label-free quantification were performed using MaxQuant software version 1.6.0.1 [62] and Andromeda label-free quantification parameters were set to default. Spectra were matched to a human database (20 244 reviewed entries, downloaded from swissprot.org), a contaminant, and decoy database. In addition, protein identifications were calculated with FDR = 1% and proteins with one razor peptide per protein were used for identification. Lysates were digested twice and analyzed three times.

T-cell stimulation

For CTL-experiments PBLs obtained from healthy donors after informed consent and Ethical Committee (Charité) approval were transduced with the gp100<sub>209-217</sub>ITDQVPFSV peptide encoding plasmid encoding gp100<sub>MEL17</sub> (pcDNA3.1/myc-HIS/gp100mel [40])
for 24 h according to its manufacturer’s instructions (Lipofectamine 2000, Invitrogen), followed by co-incubation of target cells and gp100_{209-217}/T2OM specific T lymphocytes for 16 h. IFN-γ release in the supernatants was determined by human IFN-γ ELISA kit (Biologend). Co-incubation with T2 cells served as negative control, whereas T2 cells loaded with 1 µg/mL gp100_{209-217} peptide for 16 h were used as positive control. T-cell assays presented in this work were performed compliant to MIATA guidelines.

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Abbreviations: AIP: antigen processing and presentation pathway · ERAP: ER-aminopeptidase · gp100209-217/T210M: wild type and mutated sequence · gp100209-217/T210M*mutated sequence · LMP: low molecular weight protein · HeLaA2+*: HeLa cells expressing HLA-A*02:01 · LC-MS: liquid-chromatography–mass spectrometry · MECL-1: multicatalytic endopeptidase complex like · MS: mass spectrometry · PMEL/Pmel17: premelanosome protein · QME: quantification with minimal effort · SCS: site-specific cleavage strength · TPP2: tripeptidyl peptidase 2

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