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Cytosolic processing governs TAP-independent presentation of a critical melanoma antigen

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Abstract

Cancer immunotherapy has been flourishing in recent years with remarkable clinical success. But as more patients are treated, a shadow is emerging that has haunted other cancer therapies: tumors develop resistance. Resistance is often caused by defects in the MHC class I (MHC I) antigen presentation pathway critical for CD8 T cell-mediated tumor clearance. TAP and tapasin, both key players in the pathway, are frequently downregulated in human cancers, correlating with poor patient survival. Reduced dependence on these factors may promote vaccine efficiency by limiting immune evasion. Here, we demonstrate that $PMEL_{209-217}$, a promising phase 3 trial-tested anti-melanoma vaccine candidate, is robustly presented by various TAP- and/or tapasin-deficient cell lines. This striking characteristic may underlie its potency as a vaccine. Surprisingly, cytosolic proteasomes generate the peptide even for TAP-independent presentation, while tripeptidyl peptidase 2 (TPP2) efficiently degrades the epitope. Consequently, inhibiting TPP2 substantially boosts $PMEL_{209-217}$ presentation, suggesting a possible strategy to improve the therapeutic efficacy of the vaccine.

Keywords

TAP; HLA-A2; MHC; tapasin; PMEL; antigen presentation; proteasome; TPP2

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AUTHOR CONTRIBUTIONS

RML and NV conceived and designed the experiments. RML, NV, and VF performed experiments. RML, NV, VF, BV, and PC analyzed the data and interpreted the results. RML and NV wrote the paper with contributions from VF, BV, and PC.

DISCLOSURES

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INTRODUCTION

Cancer is the second leading cause of death in the United States with more than 600,000 Americans projected to die in 2018 alone. Cancer immunotherapy activates and mobilizes the patients' immune system against their tumor and has recently emerged as a forefront strategy to fight the disease. In particular, monoclonal antibodies targeting the immune checkpoints PD-1 or CTLA-4 induce long-term regression of metastatic tumors in a significant fraction of patients and can dramatically prolong survival (reviewed in (1, 2)). Patients not responding to treatment often bear tumors with a lower pre-existing T cell infiltrate or which lack an immune-active tumor microenvironment (3, 4). Such patients might benefit from combination therapies utilizing cancer vaccines whose efficacy would subsequently be boosted by immune checkpoint inhibitors.

Cancer vaccines are based on the *in vivo* activation of CD8+ cytolytic T lymphocytes (CTL). If CTLs recognize MHC I-presented antigenic peptides at the surface of cancer cells, they will kill these targets, thereby eradicating the tumor. A large number of tumor-expressed T cell epitopes have been identified (5), some of which are currently used as vaccines in immunotherapeutic trials. A better understanding of the expression profiles, processing and presentation pathways of the respective antigens will be crucial to select the most promising targets for the next generation of immunotherapies. To this end, it is important to determine which antigenic peptides are generated in tumors and which vaccines would most efficiently limit the development of immune escape variants.

Peptides recognized by CTLs typically originate from the degradation of intracellular proteins by the proteasome, which comprises three catalytic subunits: β1, β2, and β5 (2). In immune cells or cells stimulated with interferon-γ (IFN-γ), three other catalytic subunits are induced – LMP2 , MECL-1, and LMP7 – which replace the subunits β1, β2, and β5, respectively, to form immunoproteasomes. Intermediate proteasomes containing only one or two immunosubunits (β1, β2, LMP7 and LMP2, β2, LMP7) also exist. Standard, intermediate, and immunoproteasomes all have unique cleavage specificities, generating different sets of peptides from the same protein precursors (6, 7). Other cytosolic proteases such as tripeptidyl peptidase 2 (TPP2), insulysin, or nardilysin acting independently or in concert with the proteasome are also involved in the production of some antigenic peptides (8).

Peptides produced in the cytosol are subsequently transported into the lumen of the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP). TAP is a heterodimer composed of two subunits, TAP1 and TAP2, both of which are essential for peptide translocation (8). Inside the ER, the chaperone tapasin binds the N-terminal domains (N-domains) of the TAP chains (9) and bridges TAP to MHC I via its lumenal domain. Moreover, tapasin recruits two additional proteins: the thiol oxidoreductase ERp57, captured via a disulfide bond, and the chaperone calreticulin, which binds simultaneous to ERp57 and MHC I (8). The resulting complex consisting of one TAP heterodimer, two tapasin-ERp57 conjugates, and one or two MHC I-calreticulin units is known as the peptide-loading complex (PLC) (10, 11). Within the PLC, antigenic peptides are inserted into the binding groove of MHC I with tapasin acting as a crucial editor skewing

the peptide repertoire towards high affinity ligands (12). Some peptides require N-terminal trimming via ER-associated aminopeptidases ERAP1 and/or ERAP2 (13-15). Once a ligand with sufficient affinity is captured, MHC I dissociates from the PLC and migrates to the plasma membrane. Various quality control pathways act along the secretory route (16-18), further ensuring that only optimally loaded MHC I molecules reach the cell surface. At the plasma membrane, peptide antigens are presented to CTLs.

Immunotherapeutic treatment can lead to the development of tumor escape variants no longer recognized by the immune system. This frequently occurs through loss of IFNγ signaling in the tumor (19, 20) leading to a reduction in antigen presentation by impairing the coordinated upregulation of the antigen processing machinery (21). Alternatively, components of the MHC I antigen processing and presentation machinery, such as β_2-microglobulin (β_2m), are often found directly mutated in non-responder or relapsing patients treated by immunotherapy (20, 22, 23).

The central role of the classical MHC I antigen presentation pathway in tumor clearance is also highlighted by the large number of cancers in which TAP (24-32) and/or tapasin (33-35) are downregulated or not expressed at all. Both molecules are key players in the process and their loss or downmodulation in tumors causes immune evasion and is frequently associated with a poor prognosis. While reduced expression of components in the MHC I antigen presentation pathway is often reversible by IFNγ treatment (21), structural defects that cannot be corrected by cytokine application have been observed for both TAP (36) and tapasin (37, 38). Interestingly, loss of TAP not only reduces MHC I antigen presentation but is associated with the presentation of an altered repertoire of self-epitopes, some of which are not presented by TAP-sufficient cells (39, 40). This reflects a growing number of non-classical mechanisms of MHC I antigen presentation, which have been described in recent years, partially compensating defects in the classical pathway (41). Targeting tumor antigens that are presented both in the presence and in the absence of TAP and/or tapasin (*i.e.* via classical and non-classical routes) provides a rationale for the development of novel immunotherapeutic vaccines designed to limit the possibility of tumor escape variants. However, to our knowledge, only three tumor-associated T cell epitopes have been shown to be presented in a TAP-independent manner in human cancers (39, 42, 43) and dependence on tapasin has only rarely been investigated (44, 45).

Among known melanoma antigens, $PMEL_{209\text{-}217}$ has been shown to be one of the most potent peptides when used as a vaccine (typically in an anchor residue-modified form to enhance HLA-A2 binding) (46). It is derived from the pigmentation-associated melanocyte differentiation factor PMEL (also called gp100 or Pmel17), which is expressed in > 90% of metastatic melanomas. A recent phase 3 trial demonstrated a significant increase in objective clinical responses in stage IV or locally advanced stage III melanoma patients, when the vaccine was combined with high-dose IL-2 compared to IL-2 alone (47). This trial also found a statistically significant positive effect of $PMEL_{209\text{-}217}$ on progression-free survival and a strong trend towards increased overall survival (47). Even though there have been setbacks with this vaccine (48), a recent study suggested that $PMEL_{209\text{-}217}$ is superior when compared against other melanoma peptide vaccines (46). However, the underlying mechanism is unclear. We hypothesized its potency may be explained if the peptide were

less dependent on the classical MHC I pathway, making it less likely that tumors can evade its presentation. To this end, we investigated whether and to what extent $PMEL_{209-217}$ presentation in melanoma cells can circumvent TAP or tapasin.

MATERIALS AND METHODS

Cell lines and cell culture

Buf1280 is a human melanoma cell line lacking TAP1- and HLA-A2 expression (36). LG2-MEL-220 (Mel220) is a human PMEL-deficient, HLA-A2-negative melanoma cell line positive for TAP1 expression (49, 50). M553 is a tapasin-deficient, HLA-A2-negative human melanoma cell line and its tapasin-CFP transfectant has been described (51). EB81-MEL2.7 is a human HLA-A2-positive melanoma cell line autologous to CTL7 and was obtained by limiting dilution cloning of EB81-MEL.2 cells (52). T2 is an HLA-A2-positive lymphoblastoid cell line lacking both TAP genes (53). CTL clones EB81-CGE 606 C/2.1 (CTL7) (54), as well as CTL IVSB and CTL 210/9 (55) have been described. Buf1280, Mel220, M553, EB81-MEL2.7, and T2 cells were cultured in IMDM (Sigma) / 10% FCS (HyClone) containing non-essential amino acids (Gibco), GlutaMax (Gibco), and penicillin/streptomycin (Gibco). Transfectants were grown in medium additionally containing 2 mg/ml G418 and/or 1.5 μg/ml puromycin. Cell lines lentivirally transduced with shRNAs were cloned by limiting dilution.

Treatment with pharmacological drugs

Unless otherwise noted, for pharmacological assays target cells were subjected to the following treatments at 37 °C for 2.5 hrs before measuring their recognition by CTLs: 20 μM lactacystin, 20 μM AAF-CMK (Biomol), 5 μM epoxomicin, 100 μM cycloheximide, 10 μg/ml brefeldin A, 100 μM butabindide (Tocris), 10 μM PAQ-22, 250 μM primaquine, and 2 nM concanamycin B. Drugs were purchased from Sigma unless otherwise specified.

Antibodies

Mouse monoclonal antibodies HC-A2 (HLA-A2) (56), BB7.2 (folded HLA-A2) (57), and 148.3 (TAP1) (58) have been described. Mouse monoclonal antibody ab54685 (TPP1) was purchased from Abcam. Rabbit polyclonal antibody Pep13h (PMEL C-terminus) (59) has been described. Rat monoclonal antibody 9G10 (Grp94, SPA-850) was purchased from Enzo Life Sciences. Rabbit polyclonal antibodies ab3329 (LMP7) (Abcam), 14120–1-AP (TPP2) (Proteintech), ab93341 (Derlin-1) (Abcam), ab15038 (Sec61β) (Abcam), AP2184A (Hrd1) (Abgent), 4108 (LC3A/B) (Cell Signaling Technology), and PA3–900 (calreticulin) (ThermoFisher) were purchased from the indicated suppliers. FITC-anti-HLA-A2 (551285), FITC-anti-CD107a (555800), FITC-anti-CD107b (555804), and APC-anti-CD8 (555369) antibodies were purchased from BD Biosciences.

Vector constructs

PMEL, IR-wt, and PMELΔ190–208 in pBMN-IRES-neo (50), as well as TAP1 in pLPCX (10) have been described. HLA-A2 in pBMN-IRES-neo was generated by cloning the HLA-A2 cDNA into pBMN-IRES-neo via EcoRI-sites. Quikchange mutagenesis was employed to clone HLA-A2 mutant T134K using HLA-A2 in pBMN-IRES-neo as template in

combination with primer pair 5’- GGACCTGCGCTCTTGGAAGGCGGCGGACATGG C-3’/5’-GCCATGTCCGCCGCCTTCCAAGAGCGCAGGTCC-3’. Quikchange mutagenesis was employed to clone the $PMEL_{209-217}$ minigene construct (pep209) using PMEL in pBMN-IRES-neo as a template in combination with primer pair 5’-GGAATTCCA CCATGATTACTGACCAGGTGCCTTTCTCCGTCTGAGCGGCG-3’/5’-CGCCGCTCAGA CGGAGAAAGGCACCTGGTCAGTAATCATGGTGGAATTCC-3’. PMEL-ΔSS was cloned into the expression vector as an EcoRI-EcoRI fragment after PCR-amplifying PMEL with primer pair 5’-AATGAATTCCACCATGGCTACAAAAGTACCCAG-3’/5’-TGAATTCGCCGCTCAGACCTGCTGCCC-3’. An HLA-A2-IRES-ICP47 construct to simultaneously render Mel220 cells HLA-A2 positive and TAP-inhibited was cloned by an overlap PCR using the following two fragments generated by a standard Pfu-driven reaction: (1) a 1810 bp fragment generated with primer pair 5’-CG GATCCCAGTGTGGTGGTAGG-3’/5’-CCATTTCCAGGGCCCACGACATGGTATTATCA TCGTGTTTTTCAAAGG-3’ and HLA-A2 in pBMN-IRES-neo as template and a (2) 310 bp fragment generated with primer pair 5’-CCTTTGAAAAACACGATGATAATACCATGT CGTGGGCCCTGGAAATGG-3’/5’-TATCGATGCGAACCCCAGAGTCCCGCTCAACGGGTTACCGGATTACGGG-3’ and ICP47 in pLPCX as template. The resulting overlap fragment was cloned into a pBMN vector as BamHI/ClaI fragment. LMP7 was cloned from IFNγ-stimulated buf1280 cells using primer pair 5’-ATCTCTGGGTGCTGGGCGGTC-3’/5’-GCTGCCACCACCACCATTATTGATTG-3’ in a standard RT-PCR and transferred into EcoRI-cleaved pBMN-IRES-puro as an MfeI/MfeI fragment amplified using primer pair : 5’-TTCAATTGCTCTGGGTGCTGGGCGGTCAT-3’/5’-AACAATTGGCTGCCACCACCACCATTATTG-3’. Lentiviral pLKO1.puro-based shRNA constructs targeting TPP2 were purchased from Sigma (shRNA construct #1 targets sequence 5’-CGCCTTAAAGACCTTCCATTT-3’; shRNA construct #5 targets sequence 5’-GCTGGATTCTAGTGACATTTA-3’). Lentiviral pLKO1.puro-based shRNA constructs targeting TPP1 were purchased from Sigma (shRNA construct #3 targets sequence 5’-CCTCGTCTGTTAAGTGTGAAT-3’; shRNA construct #5 targets sequence 5’-CCCTCTGTGATCCGTAAGCGA-3’). Lentiviral pLKO1.puro-based shRNA constructs specific for Hrd1 (construct #1), Derlin-1 (construct #2), and Sec61β (construct #3) were constructed to target sequences 5’-GAGACAGTTTCAGATGATT-3’, 5’-GCCAGCAGACTATTTATTCAT-3’, 5’-CCCAACATTTCTTGGACCAAA-3’, respectively. All vectors were sequenced before retroviral transduction (60) into Mel220, T2, or buf1280 cells and selected with appropriate antibiotics.

RT-PCR

Reverse transcription (RT) was performed on total RNA. Expression of PMEL, tyrosinase, and Melan-A was measured by a standard Taq-driven PCR. Primer pairs specific for tyrosinase, Melan-A, and PMEL were 5 ‘-GGATAGCGGATGCCTCTCAAAG-3’/5’-CCCAAGGAGCCATGACCAGAT-3’, 5 ‘-CTGACCCTACAAGATGCCAAGAG-3’/5’-ATCATGCATTGCAACATTTATTGATGGAG-3’, and 5’-ATGTGGAACAGGCAGCTGTAT-3’/5’-TTCAAGGGAAGATGCAGGCATCG-3’. PCR products were analyzed on an ethidium bromide-stained 1% agarose gel.

T cell assays

To measure T cell activation via degranulation, 60000 to 250000 target cells were co-cultured with the 50000 CTLs for 1h in the presence of FITC-labeled anti-CD107a and anti-CD107b antibodies (both at 1:50) in a total of 200 μl per well of a 96- or 48-well plate. Subsequently, BFA (Sigma) was added (10 μg/ml) and cells were incubated for another 3.5h. Finally, cells were harvested, CTLs were stained with APC-labeled anti-CD8 and T cell activation was assessed by flow cytometry measuring the surface exposure of CD107 caused by degranulation. To this end, propidium iodide (Sigma) was added for life-gating before 10000 CD8-positive, propidium iodide-negative events were acquired using a FACSCalibur or FACSVerse flow cytometer (BD Biosciences). Within this population, the percentage of CD107a/b-positive cells (activated CTLs post degranulation) was determined. To measure target cell lysis via europium release, the Europium Kit (Perkin Elmer) was used following the manufacturer's instructions. The IFNγ ELISA to measure T cell activation was carried out as described (44).

Western blotting

Western blotting was carried out as described (61). Briefly, 5×10^6 cells were lysed for 1h in 500 μl PBS / 1% Triton-X100 containing Complete protease inhibitor cocktail (Roche) on ice. Lysates separated by SDS-PAGE were blotted onto Immobilon-P PVDF Transfer Membrane (Millipore). Immunoblots were probed with indicated antibodies.

RESULTS

$PMEL_{209-217}$ can be presented by HLA-A2 in a TAP-independent manner

The human melanoma cell line buf1280 expresses PMEL (50), but lacks the expression of TAP1- and HLA-A2, because of frameshift mutations in both genes (36) (Table I). Since TAP transporter activity requires both TAP1 and TAP2 (8) and TAP2 stability requires physical presence of TAP1 (62, 63), neither TAP subunit is detectable at steady state and TAP is completely inactive in buf1280 cells (36). Therefore, buf1280 cells are a powerful tumor cell model system to investigate TAP-independent antigen presentation. To assess whether the HLA-A2-restricted epitope $PMEL_{209-217}$ is a TAP-independent antigen, we analyzed its presentation in buf1280 derivative cell lines using the $PMEL_{209-217}$-specific CTL clone EB81-CGE 606 C/2.1 (CTL7) (54). The buf1280-derived target cells examined in these experiments were stably transduced with either HLA-A2 alone or with both HLA-A2 and TAP1 (Fig. 1A, Table I). As expected, CTL7 efficiently recognized autologous tumor cells (EB81-MEL2.7) (Table I) but did not recognize untransduced, HLA-A2-negative buf1280 cells (Fig. 1B). However, CTL7 recognized TAP-deficient, HLA-A2-transduced buf1280(A2) cells in both T cell activation (Fig. 1B) and target cell lysis (Fig. 1C) assays, demonstrating that $PMEL_{209-217}$ can be presented by HLA-A2 independently of TAP. Transduction of buf1280(A2) cells with TAP1, which restores TAP activity (36), further enhanced T cell recognition (Fig. 1B). This suggests that $PMEL_{209-217}$ can be presented through both TAP-dependent and TAP-independent pathways.

The stability of cell surface-exposed HLA-A2-$PMEL_{209-217}$ complexes was measured in Brefeldin-A (BFA) experiments (17) using buf1280(A2) cells. This revealed a short surface

half-life consistent with $PMEL_{209-217}$ being a low affinity epitope (64, 65) (Fig. 1D). Sensitivity to BFA also suggests that TAP-independent presentation of $PMEL_{209-217}$ requires functional secretion.

TAP-independent presentation of $PMEL_{209-217}$ requires cytosolic processing

Residues 209-217 are part of the PMEL luminal domain exposed to potentially protease-rich secretory and endocytic environments while the molecule traffics to melanosomes (66). Moreover, these residues are part of the melanosomal PMEL amyloid core (67). Thus, proteolytic liberation of the epitope during early trafficking or during breakdown of aging PMEL fibrils could potentially underlie TAP-independent presentation of $PMEL_{209-217}$. We therefore addressed requirements within the PMEL protein itself necessary for TAP-independent $PMEL_{209-217}$ presentation.

To this end, we employed the human PMEL-free melanoma cell line Mel220 (49) stably transduced with HLA-A2 and the potent viral TAP-inhibitor ICP47 (68) (Table I). As expected, Mel220(A2; ICP47) cells were only recognized by CTL7 if they were also transfected with PMEL (Suppl. Fig. S1A). Since ICP47 was highly active in these cells (Suppl. Fig. S1B), these results established TAP-independent $PMEL_{209-217}$ presentation in a second human melanoma system and also confirmed antigen specificity of the CTL clone. A previously described PMEL mutant, which fails to target to melanosomes but instead forms non-fibrillar aggregates in lysosomes (IR-wt) (50) also efficiently delivered the epitope in this system, suggesting that melanosomal degradation of PMEL amyloid fibrils is not the source of the TAP-independent epitope (Suppl. Fig. S1A/B). Strikingly, even a completely ER-retained PMEL mutant (Δ190–208) (50) gave rise to the epitope, strongly suggesting that TAP-independent presentation of $PMEL_{209-217}$ does not require access of the protein to secretory or endocytic compartments (Suppl. Fig. S1C/D). Therefore, endocytic processing and/or recycling are unlikely to explain TAP-independent presentation of $PMEL_{209-217}$.

Next, we tested whether PMEL insertion into ER membranes was required for TAP-independent presentation of $PMEL_{209-217}$. To this end, we examined whether Mel220(A2; ICP47) cells transduced with a cytosolically expressed PMEL mutant lacking the signal sequence (ΔSS) (Suppl. Fig. S1E) would be recognized by CTL7. These experiments demonstrated that co-translational translocation of PMEL into the ER membrane is not necessary for TAP-independent $PMEL_{209-217}$ presentation (Fig. 2A/B). This result was further confirmed in T2 cells, in which both TAP1 and TAP2 genes are fully deleted (Table I), which additionally ruled out that presentation of $PMEL_{209-217}$ might depend on residual low TAP activity (Fig. 2C). Taken together, this strongly suggests that even TAP-independent presentation of $PMEL_{209-217}$ utilizes peptide that has been processed in the cytosol. To confirm that TAP-independent presentation of $PMEL_{209-217}$ occurs when the peptide is produced in the cytosol, we transduced a cytosolic minigene encoding the antigenic peptide (preceded by a start methionine (pep209)) into T2 cells or TAP-deficient Mel220(A2; ICP47) cells. In both cell systems, $PMEL_{209-217}$ was presented extremely efficiently, demonstrating that cytosolic production of the peptide drives its TAP-independent presentation (Fig. 2D/E).

TAP-independent presentation was observed for $PMEL_{209\text{-}217}$ and another well-characterized TAP-independent epitope ($tyrosinase_{1–9}$) but not for the classically TAP-dependent epitope $tyrosinase_{369–377}$ (43) (Fig. 2F/G). This confirmed that TAP-independent presentation was a specific property of the PMEL-derived epitope, thus ruling out systematic leakage of all peptides into the ER. Moreover, experiments with mixed target cell cultures, in which half of the cells expressed HLA-A2 but not PMEL and the other half expressed PMEL but not HLA-A2, showed that the process was cell-autonomous and did not involve loading of antigens released from dying cells (Fig. 2H). Altogether, these results demonstrate that $PMEL_{209\text{-}217}$ destined for TAP-independent presentation is generated in the cytosol and, hence, must cross a cellular membrane by a mechanism independent of TAP in order to gain access to HLA-A2.

The proteasome generates $PMEL_{209\text{-}217}$ for TAP-independent presentation

Proteasomal generation of $PMEL_{209\text{-}217}$ has been extensively characterized. While the standard proteasome promotes the formation of the epitope, the immunoproteasome subunit LMP7 can cleave inside the peptide, leading to its destruction (6, 7). To assess whether TAP-independent presentation of $PMEL_{209\text{-}217}$ requires proteasomal activity, we treated buf1280(A2) cells with proteasome inhibitors lactacystin or epoxomicin and used them as target cells in T cell assays. Both treatments dramatically reduced T cell recognition (Fig. 3A/B), suggesting that the proteasome generates the $PMEL_{209\text{-}217}$ peptide in TAP-deficient cells for MHC I-mediated presentation. TAP-independent presentation of $PMEL_{209\text{-}217}$ was also sensitive to treatment with cycloheximide, indicating that the process requires protein synthesis (Fig. 3B). In line with an involvement of the proteasome, stimulation of buf1280(A2) cells with interferon-γ (IFNγ), which induces the immunoproteasome, reduced $PMEL_{209\text{-}217}$ presentation (Fig. 3C/D/E). Furthermore, overexpression of the immunoproteasome subunit LMP7 (Fig. 3E/F), which forces its incorporation into the proteasome at the cost of the standard subunit β5 (Fig. 3G), significantly reduced TAP-independent $PMEL_{209\text{-}217}$ presentation (Fig. 3D/H).

Tripeptidyl peptidase 2 degrades $PMEL_{209\text{-}217}$ in the cytosol

Tripeptidyl peptidase 2 (TPP2) is a cytosolic protease involved in MHC class I antigen processing. TPP2 promotes the formation of some epitopes (69, 70) while limiting others, but its global effect on MHC class I-presented antigens is likely negative (71, 72). AAF-CMK inhibits the activity of various proteases including TPP2, TPP1, and cytosolic puromycin-sensitive amino peptidase (PSAP) (73, 74). We found that in the presence of AAF-CMK, presentation of $PMEL_{209\text{-}217}$ by buf1280(A2) cells was significantly enhanced without a major effect on global HLA-A2 levels (Fig. 4A/B). Not surprisingly, HLA-A2 expression by the target cells was required for the drug to increase T cell recognition (Fig. 4C). The effect of AAF-CMK was abrogated in the presence of proteasome or protein synthesis inhibition (Fig. 3A/B), indicating that the compound did not cause enhanced formation of the PMEL epitope but rather prevented its degradation. Moreover, for the drug to augment presentation of $PMEL_{209\text{-}217}$, functional secretion was required (Fig. 4D). When surface-MHC class I molecules were removed by acid-wash (17), AAF-CMK-treated cells recovered higher T cell recognition, which was completely blocked by brefeldin A (BFA)

(Fig. 4E, *left panel*), but the kinetics of recovery were similar in the absence and presence of the inhibitor (Fig. 4E, *right panel*).

The knockdown of TPP1 had no effect on TAP-independent $PMEL_{209-217}$ presentation and the respective cells remained sensitive to AAF-CMK (Suppl. Fig. S2A/B). Treatment with the PSAP inhibitor PAQ-22 (75) also did not enhance $PMEL_{209-217}$ presentation and AAF-CMK sensitivity was maintained (Suppl. Fig. S2C). This suggests that neither TPP1 nor PSAP play a major role in degrading the epitope. In contrast, and similar to AAF-CMK (Fig. 4A-D), the specific TPP2 inhibitor butabindide (76) significantly enhanced TAP-independent $PMEL_{209-217}$ presentation, while TPP2-silenced cells lacked sensitivity to the drug (Fig. 4F/G and Suppl. Fig. S2D). Similarly, TPP2 knockdown cells, expressing either of two different TPP2-specific shRNAs, displayed enhanced $PMEL_{209-217}$ presentation at steady state and the cells largely lost their sensitivity to AAF-CMK (Fig. 4H). Butabindide and AAF-CMK likely act on the same target, because they affected TAP-independent $PMEL_{209-217}$ presentation to a similar extent and the two drugs produced no additive effect (Suppl. Fig. S2E). Altogether, this suggests that TPP2 is the protease that degrades $PMEL_{209-217}$ in the cytosol.

Presentation of $PMEL_{209-217}$ is independent of tapasin

To examine whether $PMEL_{209-217}$ presentation depends on tapasin, we analyzed the recognition by CTL7 of the tapasin-deficient human melanoma cell line M553 transfected with HLA-A2 alone or co-transfected with HLA-A2 and tapasin (Table I). Both cell lines were strongly recognized by the CTL, but the presence of tapasin caused enhanced recognition (Fig. 5A). Thus, although both TAP and tapasin significantly promote $PMEL_{209-217}$ presentation, neither factor is absolutely required for vigorous recognition of PMEL-expressing cells by CTL7.

In a previous study, a subset of epitopes had been reported whose presentation survived the lack of TAP or the lack of tapasin individually, but not the loss of both factors simultaneously (77). Therefore, we tested whether $PMEL_{209-217}$ presentation persists in cells in which neither TAP nor tapasin provide support for HLA-A2 loading. To this end, we analyzed the recognition of TAP-deficient buf1280 cells expressing the HLA-A2 mutant T134K, which does not associate with tapasin (78-81), by CTL7.

Surprisingly, although wildtype HLA-A2 is a rather TAP-independent MHC class I allele (82, 83) (Fig. 1A/B, *white bars*), its tapasin binding-deficient mutant T134K was dramatically reduced, in fact almost not detectable, at the plasma membrane of TAP-deficient buf1280(T134K) cells (Fig. 5B, *white bars*). However, HLA-A2 T134K reached significant surface levels when TAP1 was stably introduced into these cells, restoring TAP transporter activity (Fig. 5C/D, *white bars*). Thus, although global surface expression of HLA-A2 is not particularly dependent on either TAP or tapasin alone, simultaneous loss of support from both molecules severely collapses surface-HLA-A2.

Strikingly, even though only minute levels of HLA-A2 were detectable at the cell surface of buf1280(T134K) cells, $PMEL_{209-217}$ continued to be recognized by CTL7 (Fig. 5B/D/E). Moreover, when these cells were treated with AAF-CMK, T cell activation even exceeded

the recognition of tapasin-sufficient buf1280(A2) cells expressing wildtype HLA-A2 (Fig. 5E). This suggests that presentation of $PMEL_{209-217}$ is extraordinarily resistant to simultaneous loss of TAP and tapasin. Such resistance may contribute to the superior qualities of this epitope as a vaccine (46).

$PMEL_{209-217}$ presentation does not depend on Hrd1/Derlin-1-mediated ER-associated degradation (ERAD)

Cytosolically generated peptides, such as $PMEL_{209-217}$ must cross a membrane in order to gain access to MHC I. How this occurs in TAP-deficient cells is poorly characterized, although some pathways have been proposed (41). One possibility is that an alternative peptide transporter exists in the ER membrane compensating for the loss of TAP. Obvious transporter candidates in such a scenario would be the molecules discussed as potential ERAD channels, Hrd1 (84) and Derlin-1 (85, 86), as well as the translocon Sec61. To investigate a possible role for these proteins in TAP-independent $PMEL_{209-217}$ presentation, we generated buf1280(A2) cell clones with a stable knockdown of Hrd1 (Fig. 6A, *left panel*) or Derlin-1 (Suppl. Fig. S3A/B) and assessed their recognition by CTL7. These clones consistently displayed higher HLA-A2 surface levels, likely reflecting reduced degradation of this MHC class I allele as a consequence of reduced ERAD in the cell (*white bars* in Fig. 6A, *right panel* and Suppl. Fig. S3C). Concomitantly, these clones were better recognized by CTL7 indicating that neither Hrd1 nor Derlin-1 are necessary for TAP-independent $PMEL_{209-217}$ presentation (*black bars* in Fig. 6A, *right panel* and Suppl. Fig. S3C). Interestingly, these results also argue against Hrd1/Derlin-1-mediated ERAD as a source for the generation of the $PMEL_{209-217}$ peptide in buf1280(A2) cells. Unfortunately, cells are not viable without Sec61, which makes assessing its possible involvement in TAP-independent peptide transport a difficult task. However, the translocon component Sec61β is not essential, but involved in some, although not all functions of the channel (87). Nevertheless, a set of stable Sec61β knockdown clones showed no significant defect in $PMEL_{209-217}$ presentation (Suppl. Fig. S3D-F, *black bars*), excluding at least an involvement of this particular Sec61 component in the process.

$PMEL_{209-217}$ presentation requires endocytic recycling

Autophagy has been discussed as a potential mechanism delivering cytosolic peptides into endosomes for capture by recycling MHC I in TAP-deficient cells (88). Interestingly, treatment of buf1280(A2) cells with concanamycin B (ConB), a specific inhibitor of vacuolar-type H^+-ATPase, significantly augmented $PMEL_{209-217}$ presentation (Fig. 6B, *black bars*) without affecting global surface-HLA-A2 levels (Fig. 6B, *white bars*). This is consistent with a scenario in which neutralization of endosomal pH protects the peptide antigen from degradation. We note that a similar phenomenon had been reported previously for a murine TAP-independent epitope (89). However, this scenario would predict that after capturing its peptide cargo, $PMEL_{209-217}$-loaded HLA-A2 would employ endocytic recycling to present its antigenic ligand to CTL7. In line with such a mechanism, primaquine, a specific inhibitor of endocytic recycling, significantly suppressed $PMEL_{209-217}$ presentation in buf1280(A2) cells (Fig. 6C, *black bars*) – again without affecting global surface-HLA-A2 (Fig. 6C, *white bars*). Interestingly, CTL7 recognition was comparably low in primaquine-treated cells irrespective of whether these cells had been co-

treated or not with AAF-CMK (Fig. 6C). This indicates that peptide protection in the cytosol through inhibition of TPP2 drives higher $PMEL_{209-217}$ presentation only when endocytic recycling is allowed to occur. Altogether, these data are consistent with a model in which cytosolic $PMEL_{209-217}$ is delivered into endosomes by a yet uncharacterized mechanism and there gains access to recycling HLA-A2 leading to TAP-independent presentation.

Surprisingly, conventional macroautophagy, a potential candidate for such a mechanism, is unlikely to be involved in the process. Specifically, the autophagy inducer rapamycin did not augment CTL7 recognition of buf1280(A2) cells (Suppl. Fig. S4A). In fact, if anything, at very high concentrations rapamycin suppressed $PMEL_{209-217}$ presentation (Suppl. Fig. S4B), although this might reflect toxicity of the drug at these levels. Moreover, the autophagy inhibitor 3-methyladenine (3-MA) did not affect $PMEL_{209-217}$ presentation at any concentration tested (Suppl. Fig. S4C/D). In line with this, knockdown of the key autophagy gene ATG5 did not significantly affect $PMEL_{209-217}$ presentation (Suppl. Fig. S4E/F, *black bars*). A number of possible mechanisms remain that could deliver the cytosolic $PMEL_{209-217}$ peptide into endosomal compartments for HLA-A2 loading. These potential mechanisms include TAP-L-mediated import into lysosomes (90), ATG5-independent macroautophagy (91), microautophagy (92), and chaperone-assisted autophagy (93).

DISCUSSION

$PMEL_{209-217}$ has long been known as a potential melanoma vaccine. Early work by Rosenberg and colleagues identified the epitope and demonstrated that TILs isolated from a subset of melanoma patients recognized the antigen (94). These groundbreaking studies also found a significant correlation between the reactivity of such TILs against PMEL and clinical responses in the context of adoptive T cell transfer therapy (94). Subsequently, a phase 2 trial using an anchor residue-optimized derivative of $PMEL_{209-217}$ ($PMEL_{209-217/T210M}$) in combination with high-dose IL-2 reported tumor regression in 42% of treated metastatic melanoma patients (95). This suggested a substantial potency of the peptide as a successful vaccine. Analyzing a large cohort of 684 metastatic melanoma cases treated at the Surgery Branch of the National Cancer Institute that had received either high-dose IL-2 alone or IL-2 in conjunction with various vaccines, further underscored this potential. In fact, the study identified $PMEL_{209-217/T210M}$ as a highly effective ingredient giving rise to almost twice the number of clinical objective responses than IL-2 alone or IL-2 in combination with other vaccines (46). This was corroborated in an extensive randomized phase 3 trial involving 185 patients, which demonstrated increased potency of the $PMEL_{209-217/T210M}$/high dose IL-2 combination over treatment with IL-2 alone (47). Specifically, $PMEL_{209-217/T210M}$ significantly extended progression-free survival, displayed a trend towards increased overall survival, and almost tripled the number of clinical responses (16% versus 6%) (47). Although there have been setbacks with this vaccine as well (48), taken together, above studies support the view that $PMEL_{209-217}$ is a particularly effective antigen. Our finding that the peptide is highly flexible with respect to its route of antigen presentation and is presented through TAP-dependent, tapasin-dependent, but also TAP/tapasin-independent pathways provides a possible explanation for its potency.

Strikingly, specific CTLs recognize $PMEL_{209-217}$ even on target cells in which neither TAP nor tapasin provide support for MHC I loading (Fig. 5B/D/E, *black bars*). This is important, because various epitopes have been described that can bypass the need for TAP or tapasin individually but cannot overcome the lack of both factors simultaneously (77). In line with this, we show that the tapasin-binding mutant T134K is almost completely absent from the surface of TAP-deficient buf1280 cells (Fig. 5B/D/E, *white bars*), while HLA-A2-T134K efficiently reaches the plasma membrane in TAP-sufficient counterparts (Fig. 5D, *white bars*). In fact, surface levels of the T134K mutant in buf1280 cells are so drastically reduced that it is surprising that anything at all is presented in this situation (Fig. 5B/D/E, *white bars*). For this reason, we note that we included untransfected HLA-A2-negative buf1280 cells as unrecognized negative controls in all above experiments (Fig. 5B/D/E). Together this suggests that the vast majority of TAP-independent HLA-A2 epitopes is not presented without tapasin assistance, while the vast majority of tapasin-independent epitopes has no access to MHC I without TAP. $PMEL_{209-217}$ appears to be exceptional in its ability to cause notable T cell recognition even in such an unfavorable environment. The behavior of HLA-A2 may at least in part reflect the interdependence of TAP and tapasin, because tapasin not only organizes the PLC and edits the peptide cargo loaded onto MHC I but also stabilizes and mediates TAP heterodimerization (12, 45, 63). Tumors frequently display coordinated downregulation of the antigen presentation machinery (21) or multiple independent defects in the MHC I pathway (38). Thus, simultaneous loss or downmodulation of TAP and tapasin – alongside other factors – is a scenario that is often encountered in cancer patients and should be taken into consideration for the design of future vaccines. We postulate that the success of $PMEL_{209-217}$ as a vaccine is at least in part attributable to its high capacity to be presented not only via the classical, TAP/tapasin-dependent MHC I pathway, but also through non-classical routes.

Loss of IFNγ signaling is emerging as a major mechanism of resistance to modern checkpoint blockade immunotherapies (19, 20). Given that many components of the MHC I antigen processing machinery including TAP and tapasin are IFNγ-inducible, antigens exhibiting persistent presentation in TAP^{low}/$tapasin^{low}$ cells may be particularly well suited for use as vaccines in combination with such therapies. In this context, it is disappointing that $PMEL_{209-217}$ did not further improve the efficiency of anti-CTLA-4 treatment in a recent trial (48). The absence of activity in this particular study is baffling and unexplained at present and may reflect loss of antigen (96), lack of $CD4^+$ T cell help (97), or choice of adjuvant (98, 99) among other factors. In principle, some of these potential pitfalls can be addressed by using multi-epitope vaccines and/or extended, longer peptides additionally containing MHC class II-epitopes. Moreover, higher efficacy of cancer vaccines might be achieved by changing the mode of delivery of the vaccine. Peptide and protein-based vaccines are often not optimal to induce strong CTL responses, while viral vector-based vaccines designed to express specific antigenic peptides can be highly immunogenic and induce strong CTL-mediated anti-tumor responses (100).

We believe $PMEL_{209-217}$ remains a strong candidate for such therapies. Unlike fully personalized vaccines based exclusively on mutant neoepitopes (23, 101), PMEL expression is shared between a majority of patients (102), rendering the peptide a potential off-the-shelf drug with known reliable potency that might be added to personalized vaccines. Moreover,

as an unmutated self-peptide, $PMEL_{209-217}$ presentation is independent of the high mutagenicity caused, for instance, by mismatch repair deficiency that was recently shown to predict the response to PD-1 blockade therapy (103). In case of cancers that do not generate a lot of mutated neoepitopes, conserved peptides like $PMEL_{209-217}$ may be the only available option.

Tumor cells may not only be equipped with proteases that can generate $PMEL_{209-217}$ but may additionally express proteases that efficiently degrade the peptide, thereby limiting its presentation. In this context, we identify TPP2 as a cytosolic enzyme counteracting proteasome-mediated formation of the epitope. TPP2 inhibition (Fig. 4A/B/G and Suppl. Fig. S2E) or silencing (Fig. 4F/H) sharply increases CTL recognition, strongly suggesting that the protease degrades the peptide in the cytosol. Particularly, in a background mimicking a TAP/tapasin-defective environment the effect of TPP2 inhibition is dramatic (Fig. 5E, *black bars*), even though HLA-A2 surface levels remain minimal (Fig. 5E, *white bars*). Although TPP2 has been reported to productively generate a certain subset of epitopes (69, 70), its overall net effect on antigen presentation is believed to be negative (71, 72). Our findings are in line with this trend. Importantly, we also reveal a possible Achilles' heel of $PMEL_{209-217}$-mediated immunotherapy. Our results predict that upregulation of TPP2 in the tumor might substantially limit the presentation of $PMEL_{209-217}$, potentially allowing the tumor to evade recognition by CTLs and continue to grow. In this context, it would be interesting to examine whether TPP2 levels are augmented in progressing metastases of $PMEL_{209-217}$-treated patients. If this were to be the case and/or to impede basal TPP2 activity to boost the peptide's presentation, blockade of TPP2 during $PMEL_{209-217}$ therapy may be a strategy worth considering.

At present, we have little information about the nature of the non-classical pathway of $PMEL_{209-217}$ presentation aside from the formation of the epitope in the cytosol (Fig. 2A-E) through the proteasome (Fig. 3A/B). As a charged molecule additionally containing several polar residues, the peptide is highly unlikely to passively cross the ER (or another cellular) membrane, as some strongly hydrophobic, TAP-independent antigens have been reported to do (77). Moreover, $PMEL_{209-217}$ is not derived from a signal sequence (signal sequences are a common source for TAP-independent, HLA-A2-presented epitopes (39, 41, 43, 104)). Our observation that neutralization of endosomal pH vigorously boosts $PMEL_{209-217}$ presentation (Fig. 6B) while inhibition of endocytic recycling blocks the process (Fig. 6C) suggests an involvement of endocytic compartments in the mechanism. Given our results, the most likely scenario is that some as yet uncharacterized process, likely not conventional macroautophagy (Suppl. Fig. S4A-F), imports cytosolic $PMEL_{209-217}$ into the endosomal system where the peptide accesses recycling HLA-A2 leading to its subsequent presentation at the cell surface. Interestingly, the non-classical MHC I pathway employed by $PMEL_{209-217}$ seems to possess a certain specificity with respect to the nature of its peptide ligands (Fig. 2F). Characterizing the associated machinery that drives the process and revealing its ligands may identify novel promising candidates for anti-tumor vaccines that share favorable features with $PMEL_{209-217}$. Our current efforts are focused on this important goal.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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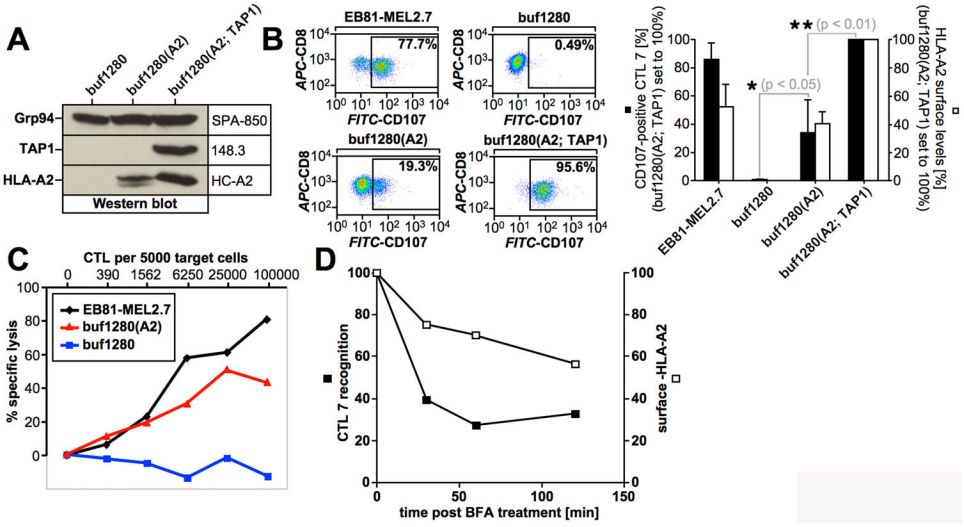

Figure 1, TAP-independent presentation of PMEL$_{209-217}$.

(A) Western blot analysis of buf1280 transfectants. **(B)** CTL7 recognition of target cells. Degranulated CTLs (CD107a/b^{+}CD8^{+}propidium iodide^{-}) are shown in the gate and their percentage among total CTLs (CD8^{+}propidium iodide^{-}) is depicted in each dot plot (*left panels*). Average CTL activation in four independent degranulation assays (*black bars*) are shown in the bar diagram (*right panel*). For each experiment, HLA-A2 surface levels on target cells were determined (*white bars*). Error bars represent the standard deviation from the mean. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. **(C)** CTL7-mediated target cell lysis measured using a europium release assay. **(D)** buf1280(A2) cells were treated with BFA (5 μg/ml) for 30 min, 1h, or 2h before they were used as targets in a T cell activation assay as in Fig. 1B. CTL7 degranulation (*black squares*) and HLA-A2 surface levels (*white squares*) are depicted.

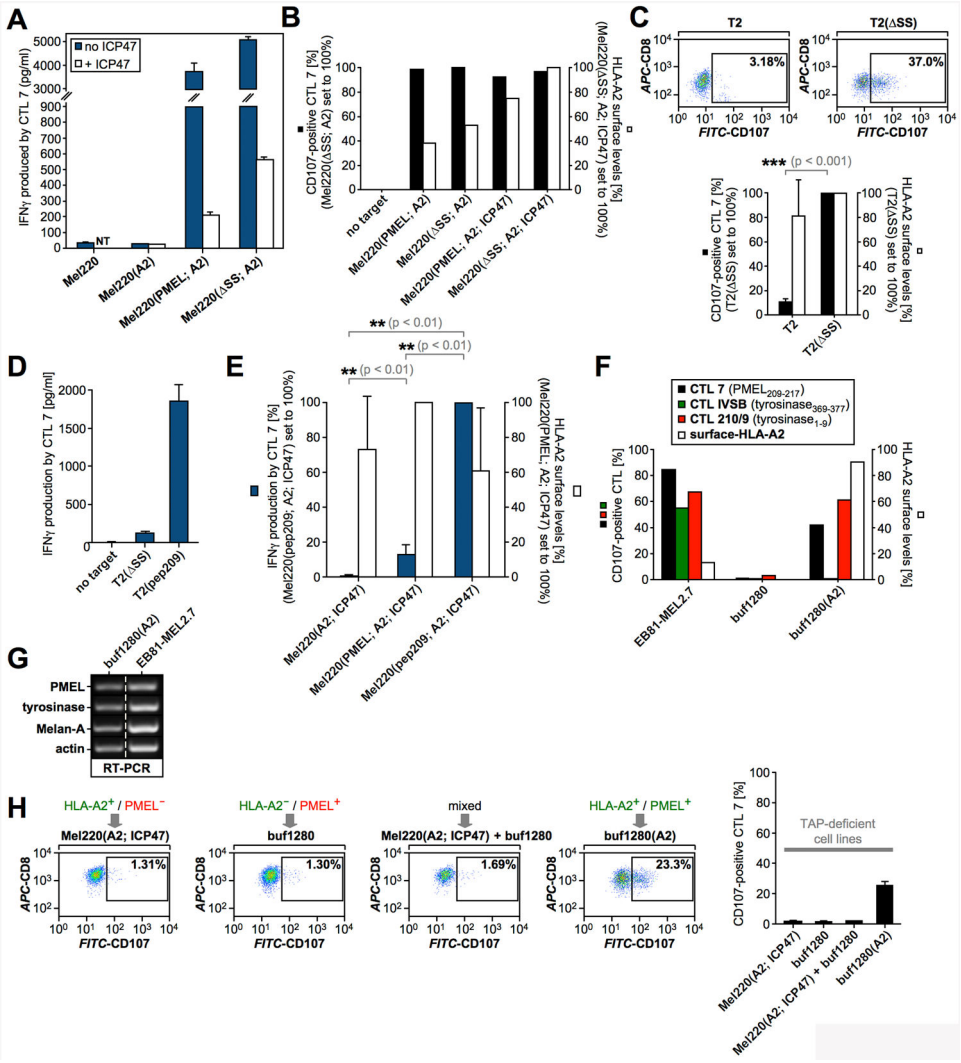

Figure 2, Cytosolic processing of PMEL$_{209-217}$.

(A) CTL7 activation measured by IFN**γ** ELISA in response to TAP-sufficient (*blue bars*) or TAP-inhibited (*white bars*) target cells (NT, not tested). **(B, C)** CTL7 degranulation measured in response to the indicated cell lines is shown as in Fig. 1B. CTL7 activation (*black bars*) and HLA-A2 surface levels on target cells (*white bars*) are depicted in bar diagrams. The experiment in Fig. 2C was repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments. A paired two-tailed t-test was performed for statistical evaluation. Surface-HLA-A2 levels were measured in only two of these three experiments (*white bars*). **(D, E)** CTL7 activation measured by IFN**γ** ELISA (*dark blue bars*). The experiment in Fig. 2E was repeated four times. Error bars represent the standard deviation from the mean of these four independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Surface-HLA-A2 levels were measured by flow cytometry in only two of these four experiments (*white bars*). Relatively low T cell recognition of T2(ΔSS) cells in Fig. 2D reflects consistently lower sensitivity of the IFN**γ** ELISA compared to LAMP degranulation (Fig. 2C). **(F)** T cell activation of clone CTL7 (*black bars*), tyrosinase$_{1-9}$-specific clone CTL

210/9 (*red bars*) and tyrosinase$_{369\text{-}377}$-specific clone CTL IVSB (*green bars*) measured using the degranulation assay in response to the indicated cell lines. HLA-A2 surface levels are shown in white bars. **(G)** Expression of melanocyte differentiation antigens analyzed by RT-PCR. The dashed line indicates where an irrelevant lane was removed. **(H)** CTL7 activation measured in response to the indicated cell lines or cell line mixtures. Degranulation is quantified in the rightmost panel. Error bars represent the standard deviation from the mean of two independent experiments.

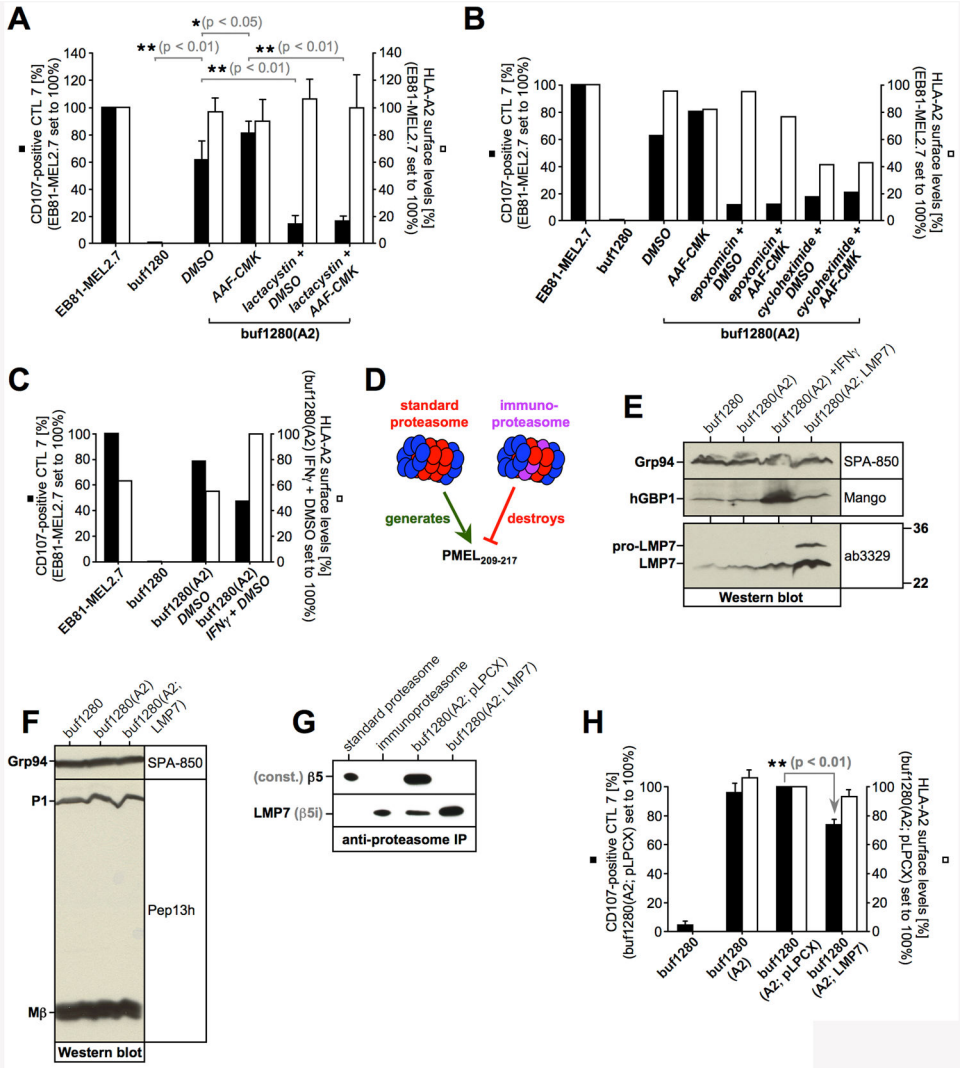

Figure 3, PMEL$_{209\text{-}217}$ destined for TAP-independent presentation is generated by the standard proteasome.

(A, B, C, H) CTL7 degranulation is shown as in Fig. 1B. CTL7 activation (*black bars*) and HLA-A2 surface levels on target cells (*white bars*) are depicted in bar diagrams. The experiment in Fig. 3A was repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Data from this experiment was also included in Fig. 3C and 4D. The experiment in Fig. 3H was repeated four times (pLPCX, empty vector). Error bars represent the standard deviation from the mean of these four independent experiments (the control cell line buf1280(A2) was included only three times). A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Data from this experiment was also included in Fig. 5D and 6C. **(D)** The standard proteasome promotes the generation of epitope PMEL$_{209\text{-}217}$, while the immunoproteasome cleavage-destroys it. **(E)** Expression of the immunoproteasome subunit LMP7 in cells stably transduced with LMP7 or empty vector (pLPCX) analyzed by Western blotting. **(F)** Western blotting demonstrates that LMP7 expression does not affect endogenous PMEL expression.

The PMEL ER form (P1) and the PMEL-Mβ fragment are labeled. **(G)** Proteasomes immunoisolated with antibody MCP21 from cells stably transduced with LMP7 or empty vector (pLPCX). Western blots were probed with antibodies against LMP7 or the standard proteasome subunit β5.

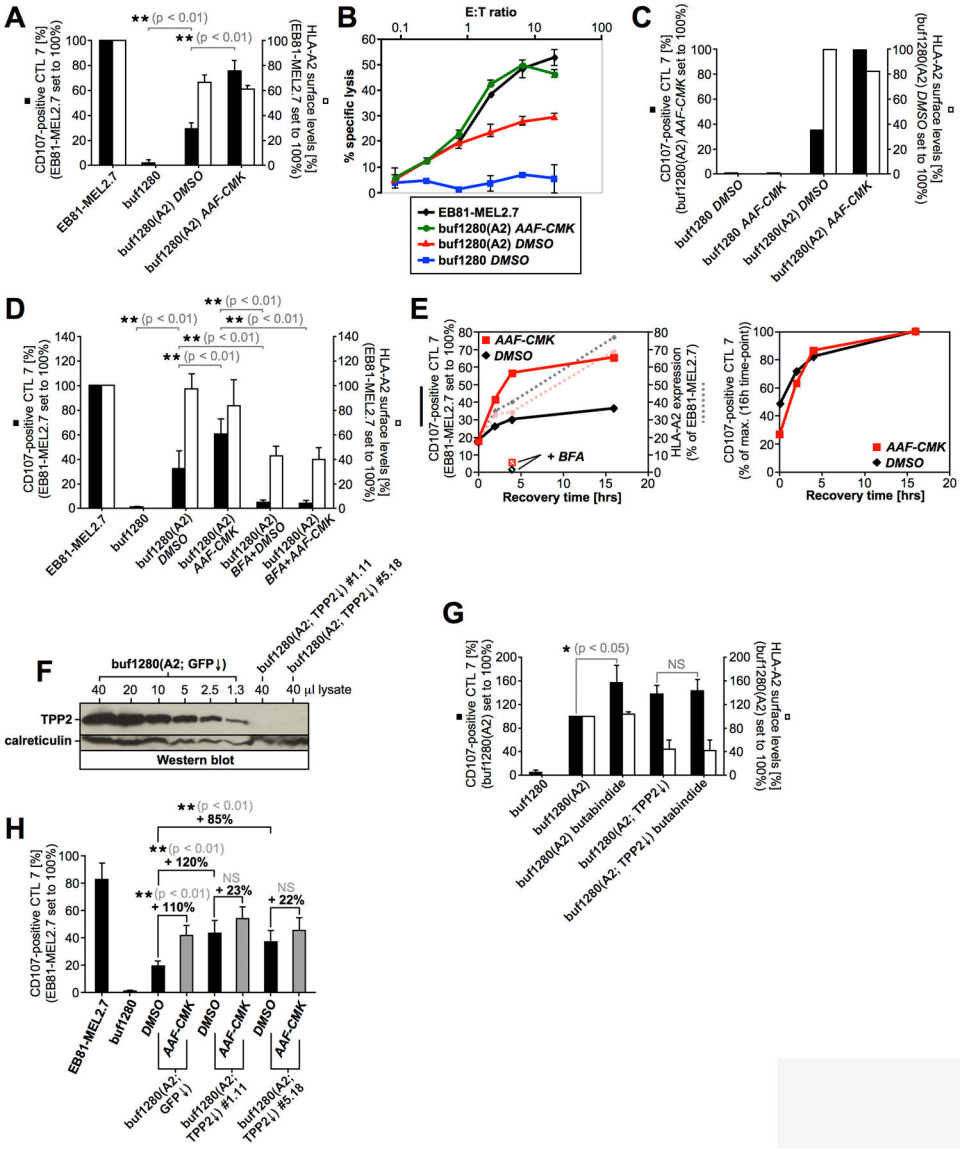

Figure 4, Tripeptidyl peptidase 2 degrades PMEL$_{209\text{-}217}$ in the cytosol.

(A, C, D, G) CTL7 degranulation is shown as in Fig. 1B. CTL7 activation (*black bars*) and HLA-A2 surface levels on target cells (*white bars*) are depicted in bar diagrams. The experiment in Fig. 4A was repeated four times. Error bars represent the standard deviation from the mean of these four independent experiments (surface-HLA-A2 levels (*white bars*) were measured in only three of these four experiments). A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. The experiment in Fig. 4D was repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Data from this experiment was also included in Fig. 3A and 5E. The experiment in Fig. 4G was repeated four times. Error bars represent the standard deviation from the mean of these four independent experiments. A Repeated Measures ANOVA with Dunnett's post test was performed for statistical evaluation (NS = not significant). **(B)**

CTL7-mediated target cell lysis of buf1280 transfectants treated or not with AAF-CMK (20 μM) was measured using a europium release assay. **(E)** buf1280(A2) cells were acid-washed to remove surface-MHC class I and incubated at 37 °C for the indicated times (0h, 2h, 4h, 16h) in the presence (*red lines*) or absence (*black lines*) of AAF-CMK (20 μM). HLA-A2 surface levels were determined (*dotted lines*) and cells were used as targets in a T cell activation assay as in Fig. 1B (*full lines*). Where indicated, samples were additionally treated with 10 μg/ml BFA. T cell recognition was normalized with respect to untreated autologous tumor cells (EB81-MEL2.7) (*left panel*), or maximal response (both 16h time-points set to 100%) (*right panel*). **(F)** Various amounts of a buf1280(A2; GFP↓) control lysate were loaded on an SDS-PAGE gel together with 40 μl of a lysate derived from TPP2-silenced buf1280(A2; TPP2↓) clones #1.11 (shRNA construct #1) and #5.18 (shRNA construct #5). **(H)** CTL7 degranulation measured in response to buf1280(A2) cell lines stably transduced with TPP2-specific shRNA constructs #1 (buf1280(A2; TPP2↓) #1.11) or #5 (buf1280(A2; TPP2↓) #5.18) or a GFP-specific control shRNA construct (buf1280(A2; GFP↓). Error bars represent the standard deviation from the mean of five independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation (NS = not significant).

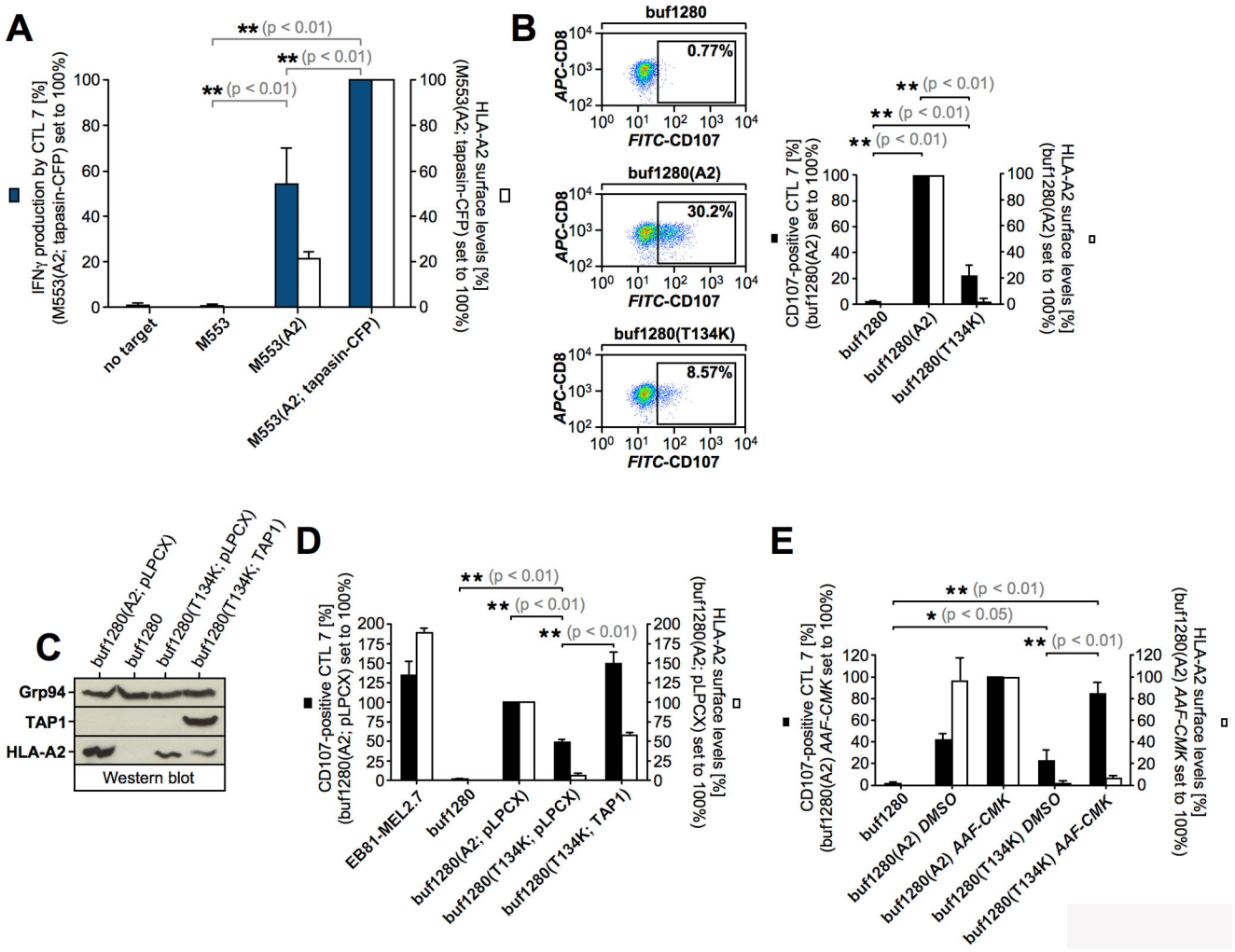

Figure 5, TAP-independent PMEL$_{209\text{-}217}$ presentation occurs in the absence of tapasin assistance.

(A) CTL7 activation measured by IFNγ ELISA (*dark blue bars*). The experiment was repeated three times and the error bars represent the standard deviation from the mean of these three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Surface-HLA-A2 levels were measured by flow cytometry (*white bars*). **(B, D, E)** CTL7 degranulation is shown as in Fig. 1B. CTL7 activation (*black bars*) and HLA-A2 surface levels on target cells (*white bars*) are depicted in bar diagrams. The experiment in Fig. 5B was repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments (surface-HLA-A2 levels (*white bars*) were measured in only two of these three experiments). A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. The experiment in Fig. 5D was repeated three times (pLPCX, empty vector). Error bars represent the standard deviation from the mean of these three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Data from this experiment was also included in Fig. 3H and 6C. The experiment in Fig. 5E was repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments.

A One-way ANOVA with Dunnett's post test was performed for statistical evaluation. Data from this experiment was also included in Fig. 4D. **(C)** Expression of HLA-A2 and TAP1 in cells stably transduced with wildtype HLA-A2 or HLA-A2 T134K and co-expressing either empty vector (pLPCX) or TAP1 was analyzed by Western blotting.

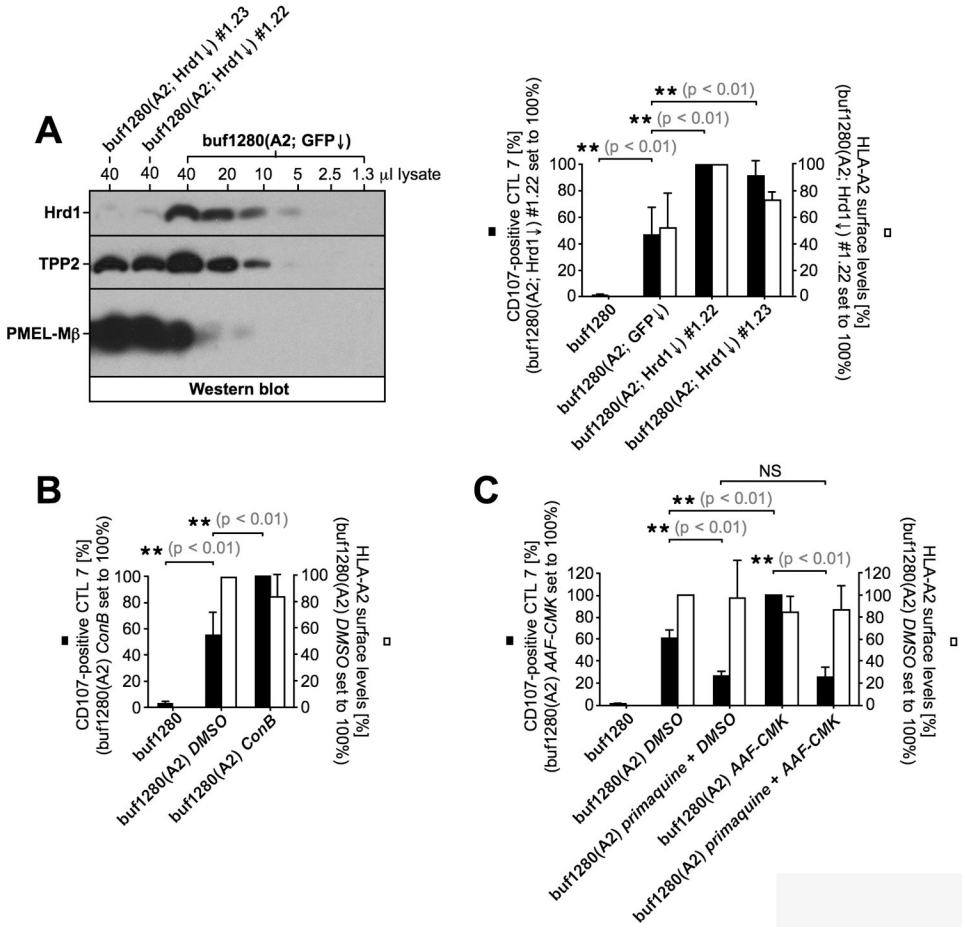

Figure 6, TAP-independent PMEL$_{209\text{-}217}$ presentation requires endocytic recycling but not Hrd1-dependent ERAD.

(A) Various amounts of a buf1280(A2; GFP↓) control lysate were loaded on an SDS-PAGE gel together with 40 µl of a lysate derived from Hrd1-silenced buf1280(A2; Hrd1↓) clones #1.22 and #1.23 (shRNA construct #1) (*left panel*). CTL7 degranulation measured in response to buf1280(A2) cell lines stably transduced with Hrd1-specific shRNA construct #1 (buf1280(A2; Hrd1↓) #1.22 and buf1280(A2; Hrd1↓) #1.23) or a GFP-specific control shRNA construct (buf1280(A2; GFP↓). Error bars represent the standard deviation from the mean of three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation (*right panel*). **(B, C)** CTL7 degranulation is shown as in Fig. 1B. CTL7 activation (*black bars*) and HLA-A2 surface levels on target cells (*white bars*) are depicted in bar diagrams. Both experiments were repeated three times. Error bars represent the standard deviation from the mean of these three independent experiments. A One-way ANOVA with Dunnett's post test was performed for statistical evaluation (NS = not significant). Some data shown in Fig. 6C was also included in Fig. 3A, 4D, and 5D.

Table I,

Selected target cell lines used in T cell assays.

M553, Mel220, buf1280, and EB81-MEL2.7 are human melanoma cell lines. T2 is a human lymphoblastoid cell line.

	HLA-A2	TAP	tapasin	PMEL
buf1280	−	−	+	+
buf1280(A2)	transduced	−	+	+
buf1280(A2; TAP1)	transduced	transduced	+	+
M553	−	+	−	+
M553(A2)	transduced	+	−	+
M553(A2; tapasin-CFP)	transduced	+	transduced	+
Mel220	−	+	+	−
Mel220(PMEL; A2)	transduced	+	+	transduced
Mel220(PMEL; A2; ICP47)	transduced	inhibited	+	transduced
EB81-MEL2.7	+	+	+	+
T2	+	−	+	−
T2(ΔSS)	+	−	+	transduced *
T2(pep209)	+/+	−/−	+/+	transduced **

* cytosolic signal-sequence-deficient PMEL,

** cytosolic $PMEL_{209\text{-}217}$ minigene