Published in final edited form as:
J Immunol. 2013 August 15; 191(4): 1547–1555. doi:10.4049/jimmunol.1301043.

ERAAP and Tapasin independently edit the amino and carboxyl termini of MHC class I peptides

Takayuki Kanaseki[*,†,1], **Kristin Camfield Lind**[*,1], **Hernando Escobar**[‡], **Niranjana Nagarajan**[*], **Eduardo Reyes-Vargas**[‡], **Brant Rudd**[‡], **Alan L. Rockwood**[‡], **Luc Van Kaer**[§], **Noriyuki Sato**[†], **Julio C. Delgado**[‡], and **Nilabh Shastri**[*]

[*]Division of Immunology and Pathogenesis, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720, U.S.A

[†]Department of Pathology, Sapporo Medical University, Sapporo, Hokkaido 060-8556, Japan

[‡]ARUP Institute for Clinical and Experimental Pathology, Department of Pathology, University of Utah School of Medicine, Salt Lake City, UT 84108, U.S.A

[§]Department of Pathology, Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, U.S.A

Abstract

Effective CD8+ T cell responses depend upon presentation of a stable peptide repertoire by MHC I molecules on the cell surface. The overall quality of pMHC I is determined by poorly understood mechanisms that generate and load peptides with appropriate consensus motifs onto MHC I. Here we show that both tapasin, a key component of the peptide loading complex, and ERAAP, the ER aminopeptidase associated with antigen processing, are quintessential editors of distinct structural features of the peptide repertoire. We carried out reciprocal immunization of wild-type mice with cells from tapasin- or ERAAP-deficient mice. Specificity analysis of T cell responses showed that absence of tapasin or ERAAP independently altered the peptide repertoire by causing loss as well as gain of new pMHC I. Changes in amino acid sequences of MHC bound-peptides revealed that ERAAP and tapasin respectively defined the characteristic amino and carboxy termini of canonical MHC I peptides. Thus, the optimal pMHC I repertoire is produced by two distinct peptide editing steps in the ER.

Introduction

Presentation of endogenous peptides by MHC I (pMHC I) on the cell surface enables the immune system to detect and eliminate infected or transformed cells. The peptides are generated from intracellular proteins and loaded onto MHC I by the antigen processing pathway (1, 2). The pathway begins in the cytoplasm where antigenic precursors are fragmented to produce a pool of intermediate peptide fragments. The fragments are transported into the endoplasmic reticulum (ER) where they are loaded onto MHC I molecules. The resulting pMHC I are exported to the cell surface to serve as potential ligands for recognition by the CD8+ T cell antigen receptors. Because circulating CD8+ T cells make only transient contacts with antigen presenting cells (APCs), effective CD8+ T

[2]Address correspondence to Dr Nilabh Shastri, Division of Immunology, LSA 421, Department of Molecular and Cell Biology, University of California, Berkeley, CA 94720-3200, Tel: (510)-643-9197, FAX: (510)-643-9230, nshastri@berkeley.edu.
[1]These authors contributed equally to this work

[1]This work was supported by grants from the National Institutes of Health to N.S

cell responses are critically dependent upon presentation of an optimally stable pMHC I repertoire.

To elicit robust $CD8^+$ T cell responses, peptides entering the antigen presentation pathway are selected to yield high-affinity pMHC I that will persist on the cell surface. In addition to a characteristic length of 8–10 amino acids, the peptides presented by MHC I on the cell surface are uniquely defined by the presence of conserved consensus motifs. The set of peptides bound by a given MHC I molecule shares conserved amino acids located at discrete positions, called anchor residues, that allow peptide binding to the MHC I (3). Amino acid substitutions at these anchor positions resulted in loss of stable interactions between peptides and MHC I that in turn inhibited CD8+ T cell responses.

The pool of peptides for MHC I presentation is produced from endogenously synthesized proteins fragmented mainly by the multicatalytic proteasome (4) as well as other proteases (5, 6). These models suggest that cytoplasmic proteolysis is primarily responsible for generating the canonical C-termini of antigenic peptides. The intermediate peptide fragments are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (7). Upon entering the ER, the peptides encounter the peptide loading complex (PLC) that facilitates loading of optimal peptide onto MHC I (8, 9). The PLC consists of TAP, the chaperones tapasin and calreticulin, the thiol oxidoreductase ERp57, β2 microglobulin and the MHC I heavy chain. Among these components, tapasin is critical for the formation and function of the PLC (8–10). Tapasin interacts directly with TAP, the MHC I heavy chain and ERp57, thereby bringing the PLC components together and keeping the empty MHC I close to the source of incoming peptides (9–15). Consistent with its central function in the PLC, surface expression of MHC I molecules is profoundly diminished in tapasin-deficient mice (16, 17) and in several MHC I molecules in human cells (9, 18). Furthermore, the loss of tapasin results in presentation of suboptimal pMHC I (9, 11, 17, 19–22). Thus, tapasin is the key mediator of peptide loading in the PLC. Nevertheless, the molecular features of the peptide cargo affected by tapasin remain unknown.

The ER aminopeptidase associated with antigen processing (ERAAP) has emerged as yet another editor of the pMHC I repertoire in the ER (23, 24). The loss of ERAAP caused profound changes in the pMHC I repertoire relative to WT mice (25–29). Analysis of CD8+ T cell responses elicited in WT mice by ERAAP-deficient cells showed that classical as well as non-classical MHC I presented a distinct, highly immunogenic, peptide repertoire (26, 27, 30, 31). Furthermore, examination of the sequence of presented peptides in ERAAP-deficient cells by mass-spectrometry revealed that the peptides were longer, often due to extra N-terminal residues (30). How ERAAP edits peptides presented by MHC I and whether editing occurs within the PLC is not known. Because tapasin physically brings together PLC components, cells without tapasin lack a functional PLC. We reasoned that the peptide editing events in the PLC might be evident in cells lacking tapasin or ERAAP.

Here we analyzed the peptide editing functions of tapasin and ERAAP required for generating the optimal pMHC I repertoire. We examined peptide editing events in cells lacking tapasin or ERAAP. By immunological, biochemical and molecular analyses we find that ERAAP and tapasin independently edited the N- and C-termini of the peptide repertoire presented by MHC I on the cell surface.

METHODS

Mice

ERAAP-deficient mice (25), tapasin-deficient mice (16), TAP1-deficient mice (32) and K^b-D^b double-deficient mice (33) have been described elsewhere. C57BL/6J mice were purchased from the Jackson Laboratory. ERAAP-deficient mice and tapasin-deficient mice were crossed to generate ERAAP and tapasin double-deficient mice. Use of all mice was done with the approval of the Animal Care and Use Committee of the University of California at Berkeley.

Antibodies

The following antibodies used for flow cytometry analysis were purchased from BD Biosciences: anti-H-$2K^b$ (AF6–88.5); anti-H-$2A^b$ (25–9–17); anti-CD8α (53-6.7); anti-CD4 (RM4–5) and anti-IFN-γ (XMG1.2). Antibodies CD16/32 (Fc Block, clone 93) and anti-H-$2D^b$ (28-14-8) were purchased from ebiosciences. For *in vivo* depletions, purified anti-NK1.1 antibody (PK136) from BioXcell was used. To block presentation by MHC I to T cell lines, the following culture supernatants were used: anti-H-$2K^b$ (5F1.5), anti-H-$2D^b$ (B22.249) or anti-H-$2A^b$ (M5/114). In the immuno-affinity purification of pMHC I complexes for mass spectrometry, anti-H-$2K^b$ (Y3) and anti-H-$2D^b$ (B22.249) were used.

Cell Lines and DNA constructs

ERAAP and TAP double-deficient fibroblasts were previously reported and immortalized ERAAP and tapasin double-deficient fibroblast cell lines were generated in the same manner as previously described (25). The use and generation of β-galactosidase (lacZ)–inducible T cell hybridomas B3Z, 30NXZ, 1AZ, 11P9Z, LPAZ, 27.5Z, and BEKo8Z have been described elsewhere (25, 31). Activation of T cell hybridomas was determined by measurement of cleavage of the lacZ substrate CPRG (Chlorophenyl red-β-D-galactopyranoside; Roche). Splenocytes from indicated mice treated with 200ng/ml LPS (Sigma) for 14–16 hours were used as APCs for T cell hybridomas. The ES-X9[SHL8] construct containing ER-localization sequence followed by N-terminally extended antigenic SIINFEHL (SHL8) peptide was previously described (34).

Peptide extraction and reverse phase-HPLC analysis

The preparation, fractionation and detection of the peptide extracts has been described (34). Briefly, the peptides were eluted from the cells by 10% formic acid, fractionated by reverse phase-HPLC, treated with trypsin and assayed as described above. Synthetic peptides were prepared by D. King (University of California at Berkeley).

Immunizations and Cytotoxic T Lymphocyte Lines (CTLs)

To generate T cell lines, female tapasin-deficient or wild-type mice were immunized intraperitoneally with 2×10^7 spleen cells from male wild-type or tapasin-deficient mice, respectively. Ten days after immunization, spleen cells were restimulated *in vitro*. Cultures contained 20 U/ml (first two restimulations) or 50 U/ml (subsequent restimulations) of hIL-2 (BD Biosciences) and irradiated spleen cells from female mice of the same genotype used for immunization. Additional restimulations were done every 7–10 days.

Measurement of Intracellular IFN-γ production by CTLs

The $CD8^+$ T cell responses of the immunized mice were measured by assessment of intracellular IFN-γ production. Spleen cell APCs were treated with 200 ng/ml of lipopolysaccharide (LPS, Sigma) overnight and $CD4^+$ and $CD8^+$ cells were depleted using magnetic beads (Dynal Biotech, invitrogen) prior to incubation with restimulated $CD8^+$ T

cells. 1μl/ml brefeldin A (GolgiPlug (BD Biosciences)) was added after one hour and total incubation of $CD8^+$ T cells and APCs was 5 hours. Cells were stained for surface markers followed by intracellular staining for IFN-γ. Cells were analyzed by flow cytometry using a LSR II (BD) and with FlowJo Software (Treestar). All plots were first gated on live cells based on forward/side scatter, followed by gating on CD8+ and IFN-γ+. For blocking of particular MHC I molecules, APCs were first incubated for 30 minutes on ice with antibody supernatant prior to addition of $CD8^+$ T cells as described above. For analysis of pMHC I surface stability, APCs were incubated for 2 or 4 hours at 37 °C in medium containing 8mg/ml brefeldin A (Sigma) followed by addition of $CD8^+$ T cells and staining as above.

In vivo cytotoxicity assay

WT female mice, depleted of NK cells at least 36 hours prior to every immunization, were primed intraperitoneally with 2×10^7 male WT, $ERAAP^{-/-}$ or $Tpn^{-/-}$ splenocytes. Mice were challenged on day seven with a 1:1:1 mix of labeled $ERAAP^{-/-}$, $Tpn^{-/-}$ and WT female APCs. Target cell labels: $ERAAP^{-/-}$ labeled with 20μM CellTracker Blue CMAC (7-Amino-4-Chloromethylcoumarin, Invitrogen); $Tpn^{-/-}$ targets labeled with 0.125 μM CFSE (low-dose); WT targets labeled with 1.25μM CFSE (high-dose) (Carboxyfluorescein succinimidyl ester, Invitrogen). 20 hours after challenge with labeled cells, host mice were sacrificed and splenocytes analyzed by flow cytometry to determine percentage of cells remaining. Plots were gated on live cells prior to anaysis of target populations. Percent Specific Lysis was calculated as follows: 100 x (1- (ratio of target output)/ (ratio of target input)) where input is determined prior to injection of targets and output represents targets recovered after challenge. To calculate ratios: (% population of interest)/ (%$ERAAP^{-/-}$+ %$Tpn^{-/-}$ +%WT).

Large-scale peptide sequencing by MS/MS

Peptide sequencing using immunoaffinity purification of pMHC complexes from detergent-solubilized spleen lysates was performed as reported previously(35). Briefly, freshly isolated spleen cells from 25 C57BL/6 and 50 tapasin-deficient mice were lysed and pMHC I was immuno-purified using mAbs Y3 (anti–H-$2K^b$) and B22.249 (anti–H-$2D^b$). Samples were subject to FPLC- HPLC fractionation, and sequence identification by nanochip ESI-QTOF mass spectrometer. Peptides were identified with high confidence using an initial search with Spectrum Mill algorithm followed by expert manual spectral validation(36). To analyze the amino acid conservation of groups more than seven peptides, we used WebLogo program (http://weblogo.berkeley.edu/).

Results

ERAAP and Tapasin differentially influence pMHC I repertoire

ERAAP and tapasin are both ER-resident editors of the peptide repertoire presented by MHC I. To assess the relative contributions of ERAAP and tapasin to the overall peptide repertoire, we measured pMHC I surface expression by flow cytometry (Fig 1a–c). Spleen cells from WT, $Tpn^{-/-}$, $ERAAP^{-/-}$, ERAAP and tapasin double-deficient ($ERAAP^{-/-}Tpn^{-/-}$), TAP-deficient ($TAP^{-/-}$) or K^b and D^b double-deficient (K^bD^b dko) strains were stained for D^b and K^b MHC I (Fig 1a–b), as well as the MHC II molecule A^b as a negative control (Fig 1c). Compared to wild-type C57BL/6 (WT) mice, loss of ERAAP diminished MHC expression by about 20%, while loss of tapasin with or without ERAAP expression diminished MHC expression by about 90%(16, 17, 34). However, tapasin deficient cells expressed relatively more pMHC I compared to cells lacking TAP1, the peptide transporter, or cells completely lacking K^b as well as D^b MHC I. Thus, ERAAP and especially tapasin were required for maintaining normal level of pMHC I expression on the cell surface.

We next assessed the influence of ERAAP and tapasin on the generation of specific peptides bound to MHC I. We measured the presentation of a panel of endogenously processed peptides on the surface of spleen cells from wild-type B6, tapasin-deficient ($Tpn^{-/-}$) or ERAAP-deficient ($ERAAP^{-/-}$) mice (Fig 2). The absence of ERAAP affected pMHC I presentation differentially(23, 25, 31); ranging from no detectable change in the pK^b ligand recognized by the 27.5Z hybridoma to an almost complete loss of the pMHC I ligands recognized by the LPAZ and 11p9Z hybridomas. On the other hand, presentation of pMHC I ligands recognized by 1AZ, 30NXZ and BEko8Z hybridomas was markedly enhanced on surface of ERAAP-deficient cells. In contrast, loss of tapasin was generally deleterious for all the pMHC I ligands tested. Taken together these observations show that normal expression of pMHC I was influenced by ERAAP and even more so by tapasin.

Absence of tapasin causes selective loss of pMHC I ligands

To further define the specific changes that occurred in the pMHC I repertoire due to loss of tapasin, we took advantage of the immune systems' ability to detect differences between self- and non-self. If certain pMHC I were absent in tapasin-deficient mice, specific $CD8^+$ T cells would not be tolerized to them and would respond to the novel pMHC I expressed by wild-type cells. We immunized $Tpn^{-/-}$ mice with wild-type spleen cells expressing the normally diverse pMHC I repertoire. After ten days, splenocytes from recipient mice were restimulated for a week with WT spleen cells. The cultures were then analyzed for presence of $CD8^+$ T cells that produced IFN-γ when stimulated with spleen cell antigen presenting cells (APCs) of the indicated genotype. The $Tpn^{-/-}$ anti-WT $CD8^+$ T cells responded strongly to WT APCs but not to self APCs (Fig 3a–b), showing that tapasin-deficient mice perceived normal pMHC I as foreign in wild-type cells. We infer that tapasin-deficiency caused the loss of many pMHC I normally present in wild-type cells. Typical of peptides presented by MHC I, these pMHC I required TAP for their presentation (Fig 3b).

To establish the ligand specificity of the responding CD8+ T cells, we used spleen cells from K^b and D^b (K^bD^b dko) double-deficient mice as APCs. The $Tpn^{-/-}$ anti-WT T cells did not respond to K^bD^b dko spleen cells indicating that tapasin-dependent peptides were presented by K^b and D^b MHC I in WT cells, rather than non-classical MHC I molecules (Fig 3b).

Tapasin-deficient cells also express novel immunogenic pMHC I

To determine whether loss of tapasin editing also resulted in presentation of novel pMHC I we immunized WT mice with $Tpn^{-/-}$ cells. The recipient T cells were restimulated *in vitro* and analyzed for responses to WT or $Tpn^{-/-}$ APCs. WT anti-$Tpn^{-/-}$ T cells produced IFN-γ in response to $Tpn^{-/-}$, but not to self- WT APCs, indicating presence of novel pMHC I in $Tpn^{-/-}$ cells (Fig 4a–b).

Tapasin expression appears to affect TAP stability and could thus influence peptide transport into the ER(37). Therefore, it was possible that the peptides presented in the absence of tapasin could be independent of TAP transport (38, 39). However, when we used $TAP^{-/-}$ cells as APCs, WT anti-$Tpn^{-/-}$ T cells did not produce IFN-γ (Fig 4b), indicating that peptide transport is required for presentation of these pMHC I and that this presentation is not a consequence of TAP-deficiency. Furthermore, while blocking the A^b MHC class II molecule or the K^b MHC I molecule did not inhibit IFN-γ production in any of the five lines tested, blocking with the anti-D^b antibody inhibited T cell responses more effectively than blocking with anti-K^b (Fig 4c). The possible contribution of $CD8^+$ T cells restricted by other non-classical MHC I to the overall $CD8^+$ T cell response is presently unclear. Alternatively, some ligands may represent novel pMHC I conformations that are not recognized by conventional anti-MHC I antibodies. Together, these results show that loss of tapasin not

only caused a profound loss of pMHC I, it also allowed generation of new and immunologically distinct pMHC I.

In $Tpn^{-/-}$ mice, many K^b and D^b are less stable on the cell surface than in their WT counterparts(16). To test the stability of the immunogenic pMHC I in $Tpn^{-/-}$ cells, WT or $Tpn^{-/-}$ splenocytes were used as APCs following treatment with brefeldin A, an inhibitor of ER-Golgi transport, for either two or four hours (Fig 4d). While brefeldin A treatment of WT APCs did not affect expression of pMHC I recognized by the $Tpn^{-/-}$ anti-WT T cells, treatment of $Tpn^{-/-}$ APCs caused a dramatic loss of WT-anti-$Tpn^{-/-}$ stimulating ligands. Thus, even though pMHC I expressed by $Tpn^{-/-}$ cells were markedly less stable than those expressed by WT cells they were nevertheless highly immunogenic.

PLC components TAP and tapasin are not required for peptide trimming by ERAAP

The loss and gain of novel pMHC I in either tapasin or ERAAP-deficient cells suggested the function of these two editors may be linked. For example, peptide trimming could be more effective if ERAAP interacted with the PLC that could provide ERAAP with access to incoming peptides and empty MHC I. To directly assess the role of PLC in determining ERAAP function, we analyzed peptide processing in cells lacking the key PLC components TAP or tapasin (Fig 5a–b). We transfected ERAAP and TAP double-deficient ($ERAAP^{-/-}TAP^{-/-}$) or ERAAP and tapasin double-deficient ($ERAAP^{-/-}Tpn^{-/-}$) fibroblasts with N-terminally extended SHL8 precursors in the presence or absence of wild-type ERAAP. The peptides extracted from transfected cells were fractionated by HPLC to separate the untrimmed precursor from the trimmed peptide products. The HPLC fractions were assayed for presence of antigenic peptides containing SHL8 with or without the N-terminal extension using the SHL8/K^b-specific B3Z hybridoma as described earlier (34). In $ERAAP^{-/-}TAP^{-/-}$ cells without ERAAP (vector alone), the N-terminally extended precursor peptide was the predominant peptide species (Fig 5a, **upper panel**). Upon co-expression of ERAAP, the precursor peptide was no longer detected, and two peaks corresponding to the precisely cleaved SHL8 octapeptide and the KSHL8 nonapeptides were found (Fig 5a, **lower panel**). The SHL8 and K-SHL8 peptides respectively represent the final products presented by the K^b and D^b MHC I present in these cells. Likewise, the same precursor and processed peptides were detected in presence or absence of ERAAP in tapasin-deficient cells (Fig 5b). Thus, in the ER, ERAAP could trim antigenic precursors to their final products in the absence of TAP or tapasin. The results show directly that expression of either TAP or tapasin, and therefore an intact PLC, is not required for N-terminal trimming of antigenic precursors by ERAAP.

Tapasin- and ERAAP-deficient cells express unique, non-overlapping pMHC I

To further assess the relationship between peptide editing by ERAAP and tapasin we examined the potential overlap between novel peptides generated in the absence of tapasin or ERAAP. T cell lines generated in WT mice by immunization with tapasin or ERAAP-deficient cells were tested for responses to various APCs. The $Tpn^{-/-}$ anti-WT $CD8^+$ T cell lines responded to both WT and $ERAAP^{-/-}$ APCs equally well, suggesting that both cells presented the unique pMHC I that were lost in $Tpn^{-/-}$ mice (Fig 6a). On the other hand, the WT anti-$Tpn^{-/-}$ lines recognized only tapasin-deficient cells, but did not recognize either WT or $ERAAP^{-/-}$ cells (Fig 6b). Likewise, WT anti-$ERAAP^{-/-}$ lines recognized $ERAAP^{-/-}$ APCs but did not respond to either WT or $Tpn^{-/-}$ APCs (Fig 6c). The lack of cross-reactivity between T cells specific for $Tpn^{-/-}$ or $ERAAP^{-/-}$ APCs showed that ERAAP and tapasin have distinct and non-overlapping roles in editing peptides for presentation on MHC I.

To further rigorously establish the distinction between the immunologically distinct tapasin or ERAAP-dependent ligands, we assessed the ability of WT mice to eliminate ERAAP$^{-/-}$ or Tpn$^{-/-}$ target cells *in vivo* (Fig 7). We primed WT mice with splenocytes from ERAAP$^{-/-}$, Tpn$^{-/-}$ or WT mice as a negative control. Seven days later, the mice were challenged with a cell mixture containing an equal number of WT, ERAAP$^{-/-}$ and Tpn$^{-/-}$ spleen cells as targets (Fig 7a,b). We depleted NK cells in host mice prior to immunization and challenge to obviate the possible effect of these cells in targeting Tpn$^{-/-}$ or ERAAP$^{-/-}$ cells with lower MHC I expression (17) (40). To distinguish the three populations of donor cells recovered from host animals *in vivo, e*ach target cell population was labeled with a different fluorescent dye as shown schematically (Fig 7a). After 20 hours, spleens from host WT mice were analyzed for the presence of each labeled cell population (Fig 7b, **OUTPUT**). A decrease in the percentage of cells recovered relative to the WT (self) targets indicates elimination of individual populations by the immune system of WT hosts.

Mice primed with ERAAP$^{-/-}$ cells efficiently eliminated ERAAP$^{-/-}$ targets, but did not influence the recovery of tapasin-deficient or self-WT cells (Fig 7c). In contrast, WT mice primed with Tpn$^{-/-}$ splenocytes eliminated Tpn$^{-/-}$ targets but not ERAAP$^{-/-}$ or WT targets. Finally, there was no specific loss of any of these target cells in mice primed with self-WT cells. Furthermore, the requirement for prior immunization for the *in vivo* elimination of ERAAP$^{-/-}$ or Tpn$^{-/-}$ targets suggests that these responses are mediated by the adaptive immune system. The *in vitro* and *in vivo a*ssessment of WT anti-Tpn$^{-/-}$ and WT-anti ERAAP$^{-/-}$ T cell lines demonstrates that the unedited peptide repertoires in cells deficient in ERAAP versus tapasin were distinct without any detectable overlap.

Tapasin-deficient cells present peptides lacking canonical consensus motif

The above findings showed that loss of tapasin results in the presentation of a novel set of peptides on the cell surface that are immunogenic to WT T cells and are distinct from the unedited peptides presented by ERAAP-deficient cells. Our previous analysis of the unedited peptides in ERAAP$^{-/-}$ spleen cells had found that the novel peptides were longer in length and varied at their N-termini (30). Whether specific structural changes also occurred in peptides produced in absence of tapasin is not known. To define the tapasin-dependent changes in the peptide repertoire, we isolated K^b and D^b pMHC I from WT as well as Tpn$^{-/-}$ splenocytes, eluted the bound peptides and determined their amino acid sequences by tandem mass spectrometry. From WT cells, we identified 210 and 163 peptides bound to D^b and K^b respectively. In contrast, the lower pMHC I expression in tapasin-deficient cells allowed recovery of fewer peptides; 63 and 22 peptides bound to D^b and K^b respectively. We did not find any obvious differences in the intracellular localization of the source proteins for these peptides (data not shown). Many peptides in Tpn$^{-/-}$-deficient splenocytes were also found in WT mice (Fig 8a; Fig S1a; Supplementary Table I–II). Remarkably, comparison of the unique peptides in Tpn$^{-/-}$ cells with their WT counterparts revealed significant differences. First, peptides in Tpn$^{-/-}$ cells were markedly longer than those in WT cells (Fig 8b; Fig S1b). Second, the canonical asparagine (N) residue at the p5 position of D^b bound peptides was consistently absent in peptides produced in absence of tapasin (Fig 8c–d). A loss of the conserved phenylalanine or tyrosine residues (F/Y) at the p5 anchor position was less obvious in peptides bound to K^b (Fig S1c–d).

The most striking difference in the amino acid sequences was found in the C-terminal (PΩ) position of K^b and D^b peptides eluted from Tpn$^{-/-}$ samples. Typically, the C-terminal position is occupied by an aliphatic amino acid; Met (M), Ile (I), Leu (L) or Val (V), as seen in peptides found in WT cells (Fig 8c–d and S1c–d). However, in the Tpn$^{-/-}$ samples, a higher frequency of abnormal amino acids was identified at PΩ. These included Lysine (K), Ser (S), Asn (N), Pro (P) and Ala (A) for D^b (Fig 8c–d) and Thr (T) and Pro (P) for K^b (Fig

S1c–d). Thus, the ability to choose the appropriate C-terminal amino acid - a key determinant of pMHC I stability - was lost in the absence of tapasin.

We verified the MHC I binding characteristics of a few representative peptides by assessing their ability to stabilize D^b or K^b on the surface of TAP-deficient RMA/s cells. Each peptide bound to the respective MHC I, although the binding was lower and the decay was faster than the canonical K^b and D^b binding peptides (Fig S2a–d). From tapasin-deficient mice, even the peptides that contained both anchor residues, such as FSPLNPVRV (D^b) and SLNRFIPL (K^b), were suboptimal binders of MHC I. Thus, in contrast to the striking influence of ERAAP on the N-termini, tapasin primarily influenced the C-termini peptides presented by MHC I.

Discussion

We show here that generation of the optimal pMHC I repertoire required independent editing by tapasin and ERAAP. The absence of tapasin profoundly altered the pMHC I repertoire, causing loss of many pMHC I normally expressed in WT cells as well as gain of other novel pMHC I. Interestingly, the new pMHC I in $Tpn^{-/-}$ cells were immunologically distinct from those found in cells lacking the N-terminal peptide editor, ERAAP. The CD8+ T cells in WT mice elicited by either $ERAAP^{-/-}$ or $Tpn^{-/-}$ cells were highly specific and did not cross-react. Amino acid sequences of unedited peptides revealed that differences between the peptides in ERAAP versus tapasin-deficient cells were in their N- and C-terminal anchor residues, respectively. Thus, tapasin defines the C-terminus while ERAAP shapes the N-terminus of canonical MHC I peptides.

The MHC I molecules present an extraordinarily diverse set of peptides to allow effective immune surveillance of virtually all endogenous proteins (3). Nevertheless, the canonical peptides presented by a given MHC I molecule share certain key features; a length of 8–10 amino acids and the presence of conserved residues at the C-terminus and at an internal p2 or p5 position(41). These conserved amino acids are called anchor residues because their presence determines the stability of the pMHC I complex(42). Editing of the available peptide repertoire is crucial to ensure that only the stable pMHC I reach the surface that are capable of triggering $CD8^+$ T cell responses. To generate the optimal pMHC I repertoire thus requires the editing mechanisms to determine the appropriate C-termini, the length as well as the internal conserved residues. The extent to which these choices are determined by the shape of the peptide binding groove of the MHC I itself versus other key players in the pathway has remained unclear.

The MHC I molecules are loaded with their peptide cargo in the ER within the peptide-loading complex (PLC)(8). The crucial role of the PLC in peptide loading has been demonstrated by the severe loss of peptide-loaded MHC in cells without tapasin(16, 17). Tapasin is the key element that holds the PLC together and retains the MHC I molecules in the ER until loaded with appropriate peptides(43). When various antigenic peptides were assessed for binding MHC I in the ER and subsequent display on the cell surface, presentation efficiency was determined by peptide affinity in presence of tapasin but not other PLC components(20). Likewise, through *in vitro* reconstitution in microsomes, tapasin mediated the binding of high-affinity peptide to MHC class I(44). Taken together with the severe reduction of pMHC I expression on the surface when tapasin was lost, these observations suggest that most peptides require editing in the PLC. Which structural aspects of the canonical MHC I bound peptides determined the appropriate affinity threshold remained unclear.

We first used an immunological approach to characterize the changes in the peptide repertoire caused by loss of tapasin. We elicited CD8+ T cell responses in wild-type mice immunized with spleen cells from tapasin-deficient mice and *vice versa*. Because the immune response has evolved to distinguish non-self from self the CD8+ T cell responses are exquisitely specific for non-self pMHC I. Importantly, the T cell can reveal changes in the pMHC I repertoire caused by alterations in tapasin expression that are not readily detected by any other assay. The specificity of the CD8+ T cell responses showed that the absence of tapasin, like that of ERAAP described earlier (26), caused the loss of many WT pMHC I as well as gain of other immunogenic pMHC I. As in ERAAP-deficient cells, the new pMHC I expressed by tapasin-deficient cells were also less stable suggesting that the presented peptides were structurally distinct from their wild-type counterparts.

Interestingly, different structural changes in the pMHC I repertoire were caused by the loss of tapasin than by loss of ERAAP. We compared the effects of ERAAP versus Tapasin-deficiency to determine whether there were similarities in their editing functions. To examine a broad set of endogenous pMHC I, we compared the potential cross-reactivity of WT CD8+ T cells responses elicited by tapasin versus ERAAP-deficient cells. We did not detect cross-reactivity between the two groups, suggesting that the unedited peptide repertoires of ERAAP$^{-/-}$ and Tpn$^{-/-}$ cells did not overlap. The lack of overlap between pMHC I generated in absence of ERAAP or tapasin was also found *in vivo*, ruling out potential artifacts caused by *in vitro* cultures. Although both ERAAP$^{-/-}$ and Tpn$^{-/-}$ unedited pMHC I were potently immunogenic and caused *in vivo* rejection, mice primed with Tpn$^{-/-}$ did not reject ERAAP$^{-/-}$ targets and vice versa. Independent analysis of naturally processed peptides extracted from living cells further confirmed that ERAAP function did not require tapasin or the peptide transporter, TAP. Together, these results strongly support the notion that tapasin and ERAAP edit different aspects of the pMHC I repertoire.

Mass spectrometry analysis of peptides eluted from ERAAP and Tapasin -deficient spleen cells revealed the molecular basis for the structural differences in the unedited pMHC I repertoires. In the absence of tapasin, the most striking changes occurred at the C-terminus where non-canonical amino acids at PΩ were far more frequent than in WT cells. In contrast, many more K^b and D^b bound peptides in ERAAP-deficient splenocytes were extended by extra amino acids at the N-terminus or by potential bulges between the anchor residues, but the C-termini always contained the canonical residues(30). Appropriate C-terminal anchor residues could therefore be maintained in presence of tapasin in ERAAP-deficient cells, but generation of the appropriate N-termini required ERAAP. Thus, tapasin was important not only for integrity of the PLC, but also for its unique role in editing the C-termini of peptides presented by MHC I.

Previous studies have assumed that cytosolic enzymes, such as the proteasome, generated peptides with canonical C-terminal residues(4, 45). However, our data showed that tapasin is the ultimate arbitrator for ensuring that peptides with appropriate C-terminal residue are presented. Consistent with a role for tapasin-mediated editing at the C-termini of peptides, T134K mutation in the alpha 2 helix of MHC I was found to affect tapasin:MHC I interactions in human cells(46). While the molecular mechanism by which tapasin edits peptides is still unclear, putative models for peptide optimization via tapasin include accelerated dissociation of unfavorable peptides(44, 47, 48) or maintenance of the MHC I in an peptide-receptive conformation until bound by a high-affinity peptide(49). Notably tapasin-MHC interactions occurred with the C-terminal end of the MHC I peptide binding groove.

In conclusion, tapasin and ERAAP edit distinct aspects of the peptides loaded onto MHC I molecules in the ER and explain the canonical features of the MHC I peptide cargo. Because

the changes in pMHC I ligands due to failure of these editing steps are non-overlapping, it should be interesting to determine why tapasin or ERAAP are differentially targeted for immune evasion by viruses(50–52) and in cancer(47, 53).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank David King for peptide synthesis and Hector Nolla for assistance with flow cytometry.

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Abbreviations used in this paper

ER	endoplasmic reticulum
ERAAP	endoplasmic reticulum aminopeptidase associated with antigen processing
pMHC I	peptide-MHC I complexes
Tpn	tapasin

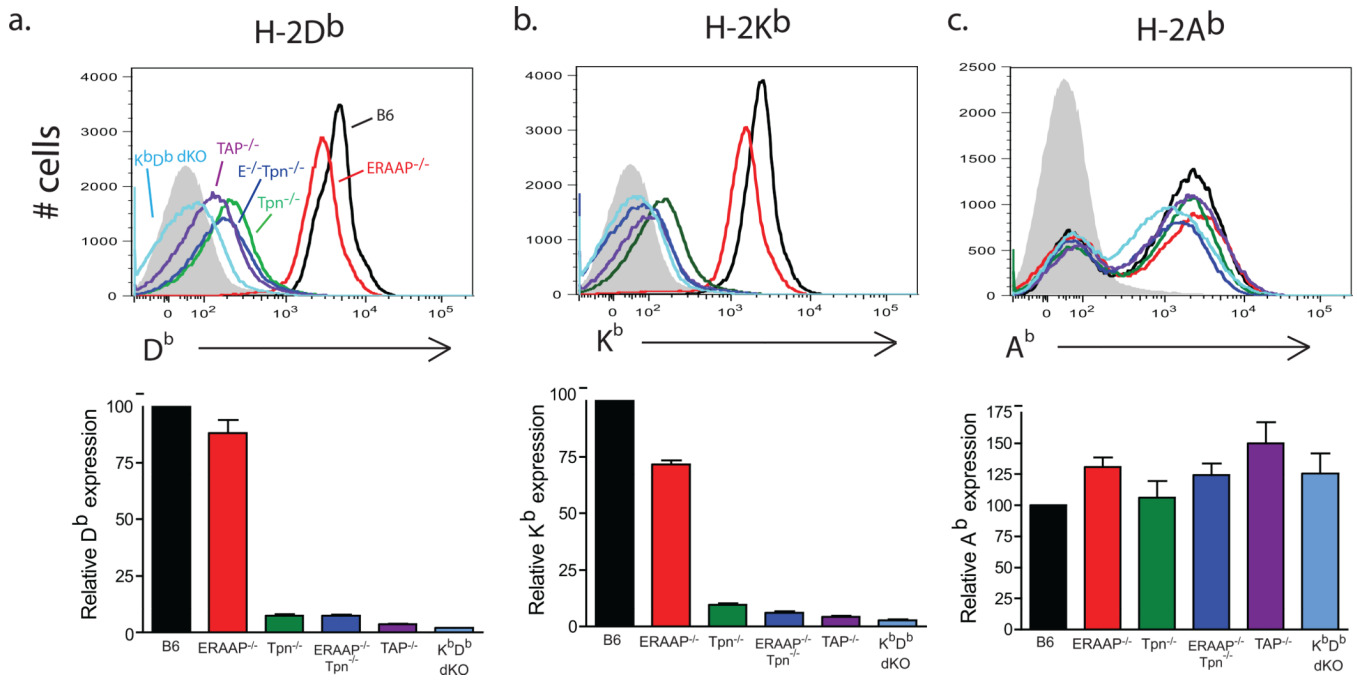

Figure 1. Tapasin and ERAAP differentially affect surface pMHC I expression

(a–c) Surface (a) H-2D^b, (b) H-2K^b and (c) H-2A^b expression on spleen cells derived from WT, ERAAP$^{-/-}$, Tpn$^{-/-}$, ERAAP$^{-/-}$Tpn$^{-/-}$, TAP$^{-/-}$, K^{b}D^b dKO mice. Bar graphs summarize the mean fluorescence intensity (MFI). FACS plots are gated on live cells. Top panels show representative data and bottom panels show data from three independent experiments.

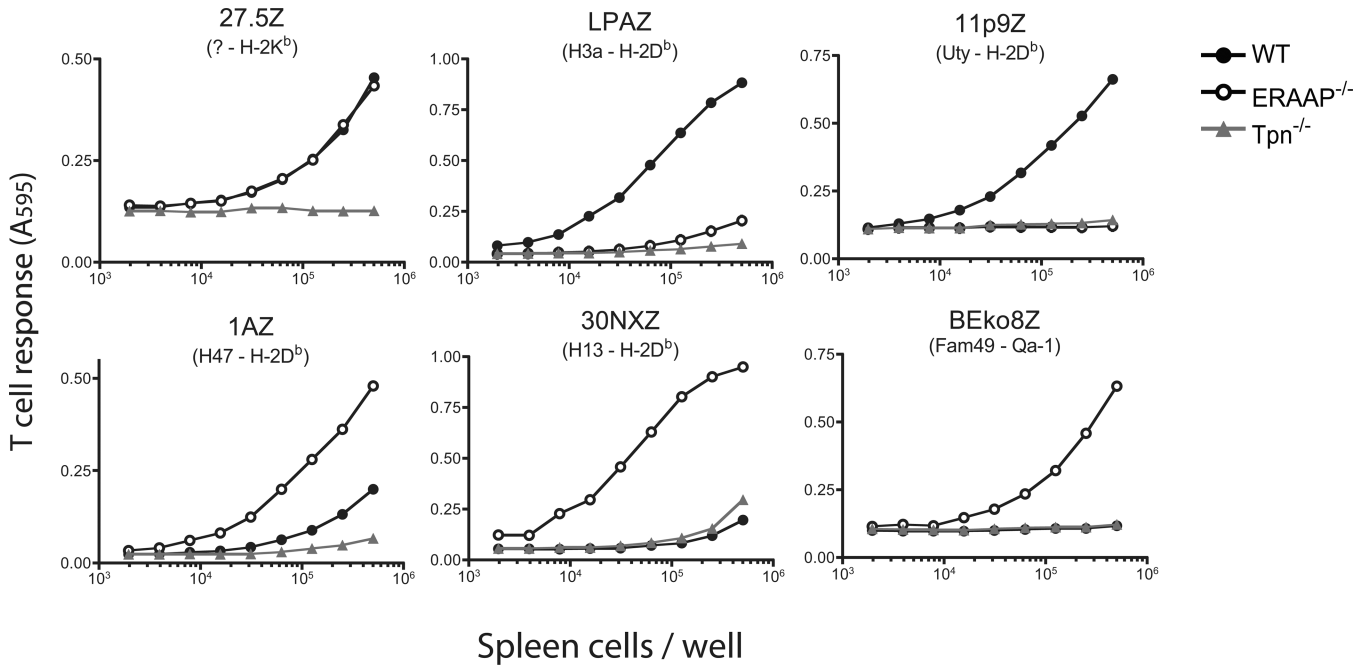

Figure 2. Tapasin and ERAAP differentially affect presentation of endogenous pMHC I by spleen cells

Splenocytes from indicated male mice were stimulated with 200ng/ml LPS overnight prior to incubation with CD8$^+$ T cell hybridomas 27.5Z, LPAZ, 11P9Z, 1AZ, 30NXZ, or BEKo8Z. Hybridomas specifically recognize peptides encoded by the indicated genes and presented on H-2D^b, H-2K^b, or Qa-1^b. Hybridoma response is determined by assessing conversion of the colorometric substrate CPRG to chlorophenol red measured at absorbance at 595 nm (A595). Data is representatives of three independent experiments.

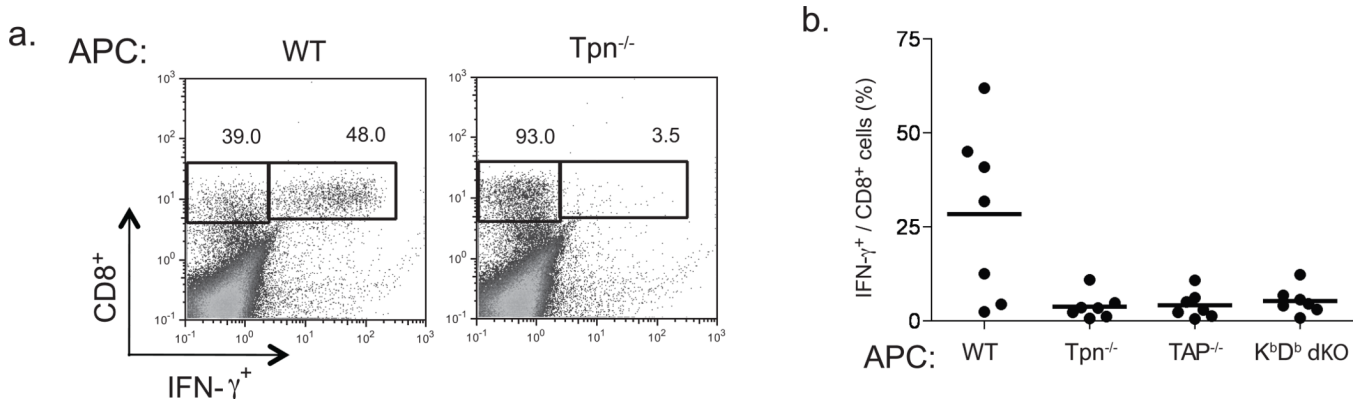

Figure 3. Tapasin-deficient mice elicit CD8+ T cell response to pMHC I expressed by WT cells

(a) Intracellular IFN-γ+ produced by $Tpn^{-/-}$ anti-WT $CD8^+$ T cell lines in response to WT or $Tpn^{-/-}$ APCs. **(b)** $Tpn^{-/-}$ anti-WT $CD8^+$ T cell IFN-γ+ response against WT, $Tpn^{-/-}$, $TAP^{-/-}$ or K^b and D^b double-deficient (K^bD^b dKO) APCs. Each point represents an individual mouse. Data are from one of two independent experiments (a) or pooled from two independent experiments (b). (a–b) Numbers indicate percent IFN-γ positive cells in the CD8+ T cell gate.

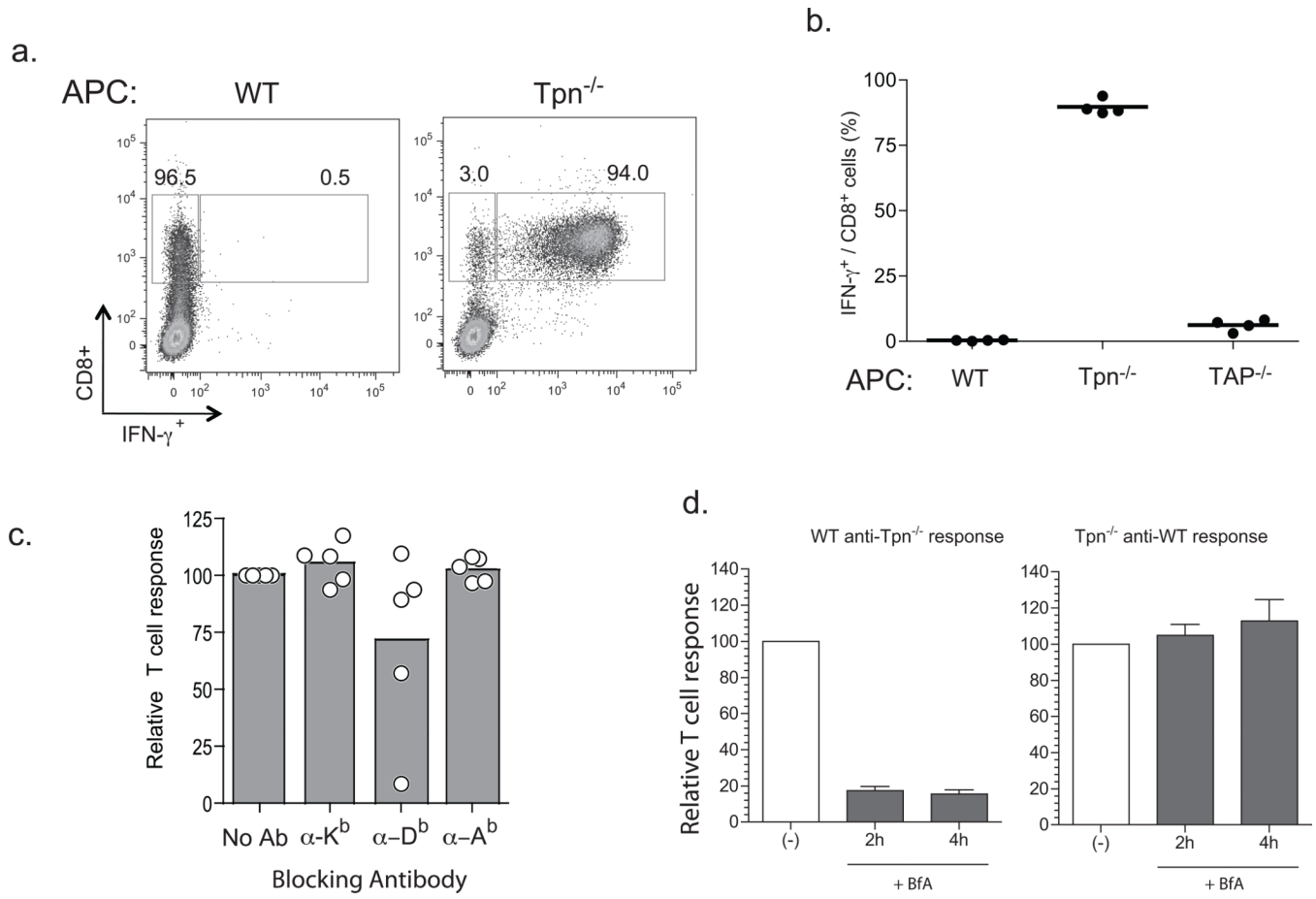

Figure 4. Tapasin-deficient cells elicit CD8$^+$ T cell responses in wild-type mice

(a) IFN-γ^+ response of WT anti-Tpn$^{-/-}$ CD8$^+$ T cell lines against WT or Tpn$^{-/-}$ APCs. Numbers indicate percent IFN-γ^+ positive cells of total CD8$^+$ T cells. **(b)** WT anti-Tpn$^{-/-}$ CD8$^+$ T cell responses against WT, Tpn$^{-/-}$ or TAP$^{-/-}$ APCs. (a–b) Numbers indicate percent IFN-γ positive cells in the CD8+ T cell gate. **(c)** WT anti-Tpn$^{-/-}$ CD8$^+$ T cell responses against WT APCs previously treated with blocking antibodies to MHC I K^b (α-K^b) or D^b (α-D^b) or to MHC II A^b (α-A^b). Percentages of CD8$^+$IFN-γ^+ cells were normalized to no antibody control (No Ab). **(d)** IFN-γ^+ production by WT anti-Tpn$^{-/-}$ (left) and Tpn$^{-/-}$ anti- WT (right) CD8$^+$ T cell lines against splenocyte APCs treated with brefeldin A (BfA) for 2 or 4 hours prior to co-culture with T cells. Percent of CD8$^+$ IFN-γ^+ was normalized to untreated (−). Data are representative of three independent experiments (a and b) or are pooled from two independent experiments (c and d).

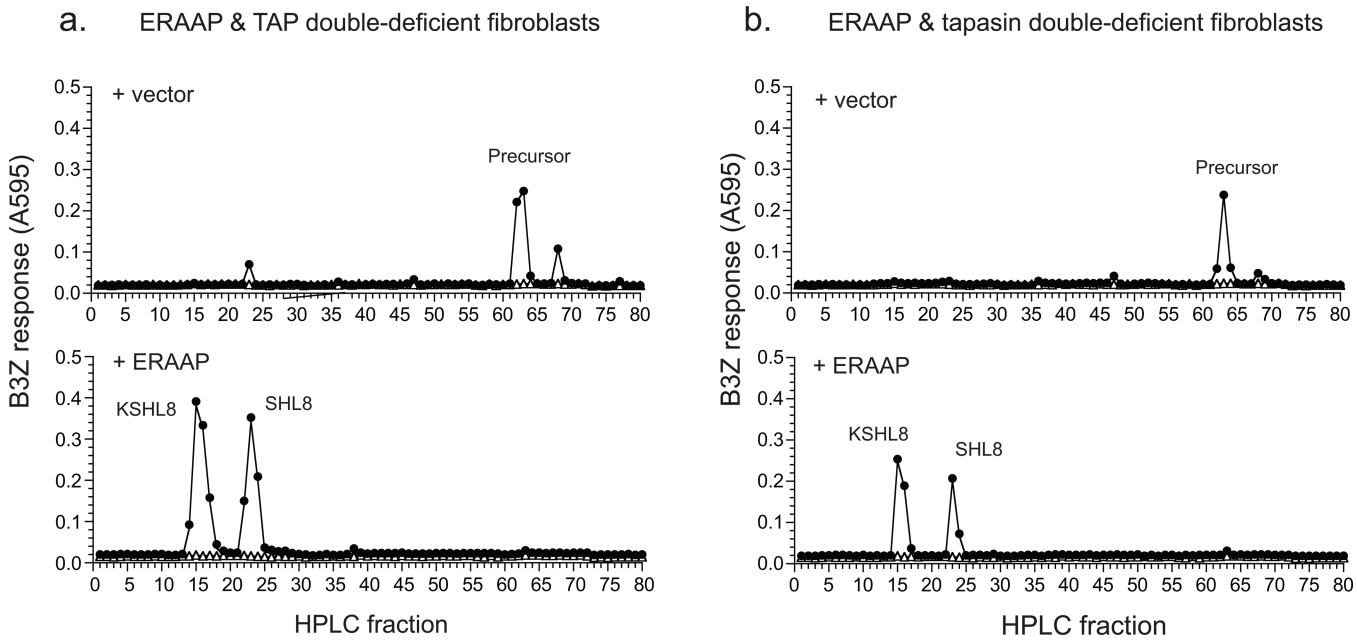

Figure 5. Peptide trimming by ERAAP does not require tapasin or TAP

(a,b) The ES-X9[SHL8] construct was co-transfected with ERAAP cDNA or with empty vector into **(a)** ERAAP and TAP double-deficient ($ERAAP^{-/-}TAP^{-/-}$) fibroblasts or **(b)** ERAAP and tapasin double-deficient ($ERAAP^{-/-}Tpn^{-/-}$) fibroblasts. Cell lysates were fractionated by RP-HPLC and trypsinized to release SHL8 peptides prior to detection with B3Z hybridoma in the presence of L-cells expressing H-$2K^b$. Synthetic SHL8 and KSHL8 peptide run under identical conditions verified the HPLC fraction numbers. Data are representative of three independent experiments.

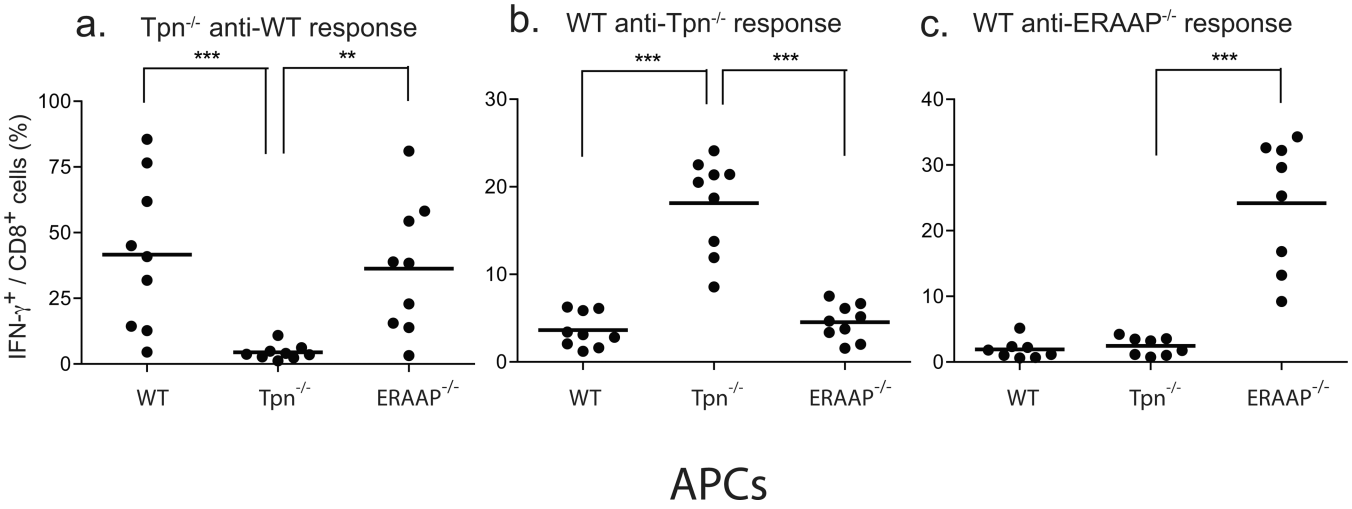

Figure 6. Immunogenic pMHC I expressed by tapasin or ERAAP-deficient cells do not overlap

T cell responses against indicated splenocyte APCs. **(a)** $Tpn^{-/-}$ anti-WT T cell response, **(b)** WT anti-$Tpn^{-/-}$ T cell response, and **(c)** WT anti-$ERAAP^{-/-}$ T cell response against spleen cell APCs derived from WT, $Tpn^{-/-}$ or $ERAAP^{-/-}$ mice. Immunizations to induce specific $CD8^+$ T cells and intracellular cytokine staining to detect IFN-γ production are described above. The p-values were calculated by Mann-Whitney U test. **p<0.01, ***p<0.001. Data are pooled from two (c) or three (a and b) independent experiments.

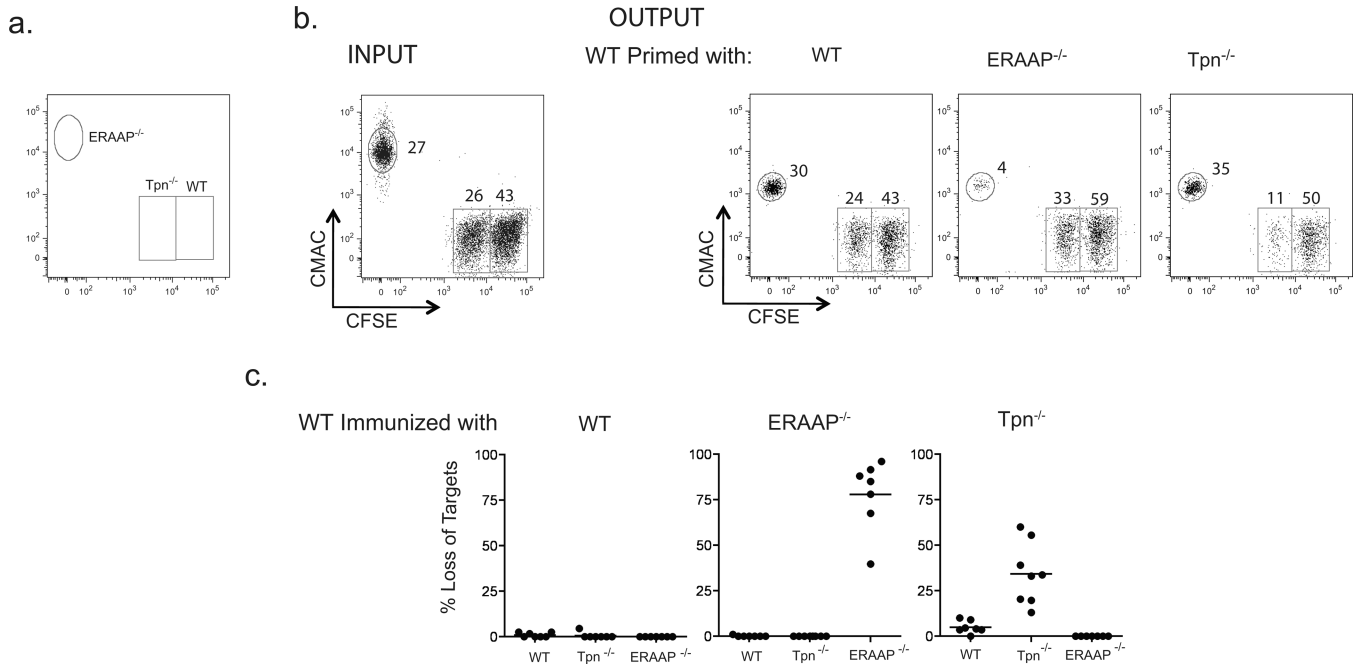

Figure 7. Different ligands are used for rejection of tapasin- or ERAAP-deficient cells by WT mice *in vivo*

T cells from WT mice primed seven days earlier with male WT, $Tpn^{-/-}$ or $ERAAP^{-/-}$ splenocytes were assessed for their ability to specifically eliminate WT, $Tpn^{-/-}$ or $ERAAP^{-/-}$ female targets *in vivo*. Targets were given distinct labels so they could be compared in the same host mouse: WT = CFSE (high-dose); $Tpn^{-/-}$ = CFSE (low-dose); $ERAAP^{-/-}$ = CMAC. **(a)** Schematic indicating the populations which represent $ERAAP^{-/-}$, $Tpn^{-/-}$ and WT **(b)** Representative FACS plots of labeled targets before (input) and after (output) challenge. Input refers to proportions of each labeled cell type prior to challenge while output refers to the labeled populations identified 20 hours post-transfer **(c)** Summary of *in vivo* killing assay from b. Negative loss (gain) is plotted as zero. Data are representative of two independent experiments (b) or are pooled from two independent experiments (c).

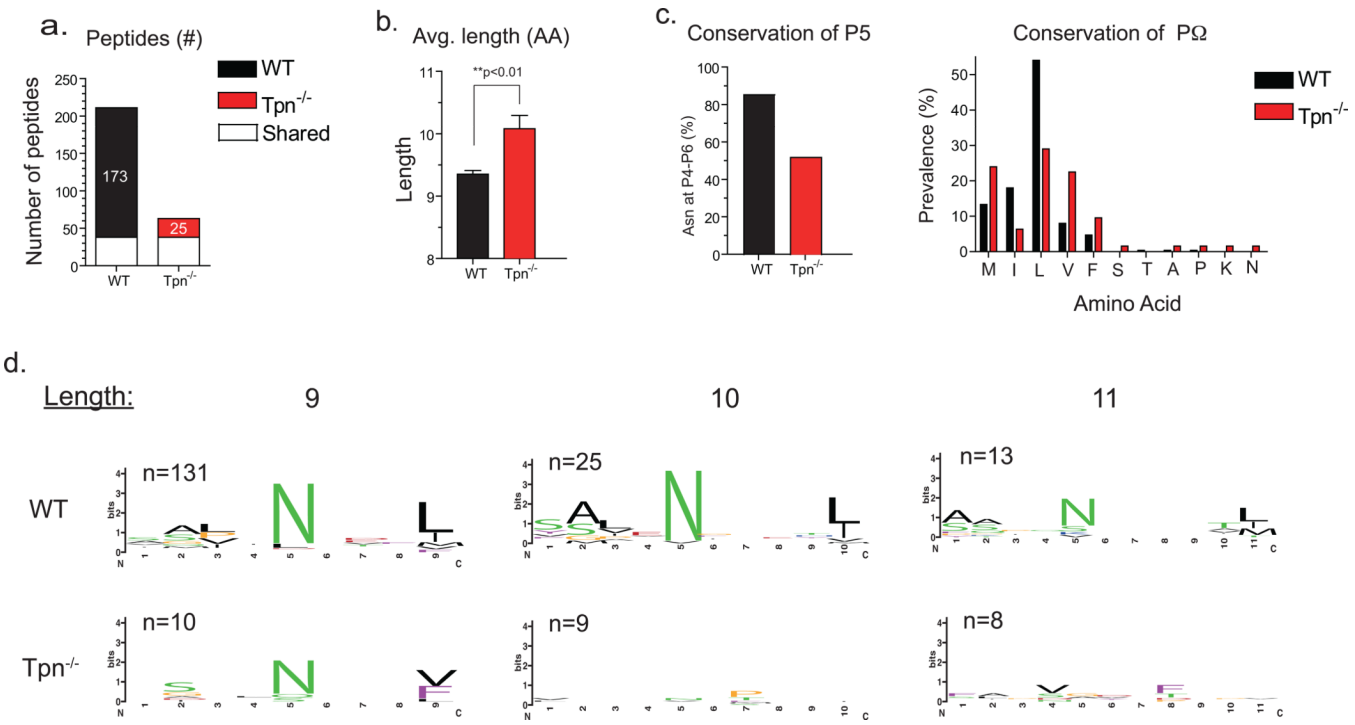

Figure 8. MHC I in WT and tapasin-deficient cells present unique peptides

The H-2D^b MHC I were immunoprecipitated from WT and tapasin-deficient spleen cells. Eluted peptides were sequenced by mass spectrometry and manually validated. **(a)** Numbers of distinct or shared peptides found in pMHC I expressed by WT or tapasin-deficient cells. **(b–d)** Analysis of the unique peptides from WT and tapasin-deficient cells. (**b**) The average lengths of peptides recovered from WT or tapasin-deficient cells. The indicated p-value was calculated by two-tailed t-test. **(c)** Conservation of p5 and C-terminal anchor residues (PΩ). Plots represent frequency of Asn (N) at P4–6 or the frequency of indicated amino acids at PΩ of H-2D^b peptides. **(d)** Logo representation of H-2D^b peptides eluted from WT or tapasin-deficient cells. Peptides are grouped according to their lengths, and the numbers of peptides in each group is indicated. The height of each bar is proportional to the degree of amino acid conservation and the height of each letter composing the column is proportional to its frequency at the given position. Amino acids are colored as follows: hydrophobic (black), aromatic (purple), acidic (red), basic (blue), neutral (green), and the others (orange).