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*J Immunol* published online 17 July 2013
http://www.jimmunol.org/content/early/2013/07/17/jimmunol.1301043

Supplementary Material  http://www.jimmunol.org/content/suppl/2013/07/17/jimmunol.1301043.DC1

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ERAAP and Tapasin Independently Edit the Amino and Carboxyl Termini of MHC Class I Peptides

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

Effective CD8+ T cell responses depend on presentation of a stable peptide repertoire by MHC class I (MHC I) molecules on the cell surface. The overall quality of peptide–MHC I complexes (pMHC I) is determined by poorly understood mechanisms that generate and load peptides with appropriate consensus motifs onto MHC I. In this article, we show that both tapasin (Tpn), a key component of the peptide loading complex, and the endoplasmic reticulum aminopeptidase associated with Ag processing (ERAAP) are quintessential editors of distinct structural features of the peptide repertoire. We carried out reciprocal immunization of wild-type mice with cells from Tpn- or ERAAP-deficient mice. Specificity analysis of T cell responses showed that absence of Tpn 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 Tpn, respectively, defined the characteristic amino and carboxyl termini of canonical MHC I peptides. Thus, the optimal pMHC I repertoire is produced by two distinct peptide editing steps in the endoplasmic reticulum. The Journal of Immunology, 2013, 191: 000–000.

Presentation of endogenous peptides by MHC class I (MHC I; peptide–MHC I complexes [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 Ag 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 Ag receptors. Because circulating CD8+ T cells make only transient contacts with APCs, effective CD8+ T cell responses are critically dependent on presentation of an optimally stable pMHC I repertoire.

To elicit robust CD8+ T cell responses, we selected peptides entering the Ag presentation pathway to yield high-affinity pMHC I that will persist on the cell surface. In addition to a characteristic length of 8–10 aa, 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 ER by the 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 (Tpn) and calreticulin, the thiol oxidoreductase ERp57, β2-microglobulin, and the MHC I H chain. Among these components, Tpn is critical for the formation and function of the PLC (8–10). Tpn interacts directly with TAP, the MHC I H 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 Tpn-deficient mice (16, 17) and in several MHC I molecules in human cells (9, 18). Furthermore, the loss of Tpn results in presentation of suboptimal pMHC I (9, 11, 17, 19–22). Thus, Tpn is the key mediator of peptide loading in the PLC. Nevertheless, the molecular features of the peptide cargo affected by Tpn remain unknown.

The ER aminopeptidase associated with Ag 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 wild-type (WT) mice (25–29). Analysis of CD8+ T cell responses elicited in WT mice by ERAAP-deficient cells showed that classical as well as nonclassical 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 because of extra N-terminal residues (30). How ERAAP edits peptides presented by MHC I and whether editing occurs within the PLC is not known. Because Tpn physically brings together PLC components, cells without Tpn lack a functional PLC. We reasoned that the peptide editing events in the PLC might be evident in cells lacking Tpn or ERAAP.

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

Materials and Methods

Mice

ERAAP-deficient (25), Tpn-deficient (16), TAP1-deficient (32), and Kb and Dd double-deficient (KbDd dko) mice (33) have been described elsewhere. C57BL/6J mice were purchased from The Jackson Laboratory. ERAAP- and Tpn-deficient mice were crossed to generate ERAAP and Tpn double-deficient (ERAAP−/−Tpn−/−) 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 Abs used for flow cytometry analysis were purchased from BD Biosciences: anti–H-2Kb (AF6-88.5), anti–H-2Dd (25-9-17), anti-CD8 (53-6.7), anti-CD4 (RM4-5), and anti–IFN-γ (XMG1.2). Abs CD16/32 (Fc block, clone 93) and anti–H-2Dd (28-14-8) were purchased from eBioSciences. For in vivo depletions, purified anti-NK1.1 Ab (PK136) from BioXcell was used. To block presentation by MHC I to T cell lines, we used the following culture supernatants: anti–H-2Kb (5F1.5), anti–H-2Db (B22.249), and anti-CD8 (RM4-5). Samples were subjected to manual spectral validation (36). To analyze the amino acid conservation by mass spectrometry, anti–H-2Kb (Y3) and anti–H-2Db (B22.249) were used.

Cell lines and DNA constructs

ERAAP and TAP double-deficient (ERAAP−/−TAP−/−) fibroblasts were previously reported, and immortalized ERAAP−/− Tpn−/− 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, 1A2, 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 chlorophenol red-β-d-galactopyranoside (Roche). Splenocytes from indicated mice treated with 200 ng/ml LPS (Sigma) for 14–16 h were used as APCs for T cell hybridomas. The ES-X9[SHL8] construct containing ER-localization sequence by tandem mass spectrometry

Peptide sequencing by mass spectrometry, anti–H-2Kb (AF6-88.5) and B22.249 (anti–H-2Dd). Samples were subject to fast protein liquid chromatography-HPLC fractionation, and sequence identification by nanochip electrospray ionization-quadrupole time of flight 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 large groups of more than seven peptides, we used WebLogo program (http://weblogo.berkeley.edu/).

Results

ERAAP and Tpn differentially influence pMHC I repertoire

ERAAP and Tpn are both ER-resident editors of the peptide repertoire presented by MHC I. To assess the relative contributions of ERAAP and Tpn to the overall peptide repertoire, we measured pMHC I surface expression by flow cytometry (Fig. 1A–C). Spleen cells from WT, Tpn−/−, ERAAP−/−, ERAAP−/−Tpn−/−, or KdMHC I (Fig. 1A, 1B), as well as the MHC II molecule Aβ as a negative control (Fig. 1C). Compared with WT C57BL/6 mice, loss of ERAAP diminished MHC expression by ~20%, whereas loss of Tpn with or without ERAAP expression diminished MHC expression by ~90% (16, 17, 34). However, Tpn-deficient cells expressed relatively more pMHC I compared with cells lacking TAP1, the peptide transporter, or cells completely lacking Kd, as well as Dd MHC I. Thus, ERAAP and especially Tpn were required for maintaining normal level of pMHC I expression on the cell surface.

We next assessed the influence of ERAAP and Tpn 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 WT B6, Tpn-deficient (Tpn−/−),
or ERAAP-deficient (ERAAP<sup>−/−</sup>) mice (Fig. 2). The absence of ERAAP affected pMHC I presentation differentially (23, 25, 31), ranging from no detectable change in the pK<sup>b</sup> ligand recognized by the 27.5Z hybridoma to an almost complete loss of the pMHC I ligands recognized by the LPAZ and 11p9Z hybridomas. In contrast, 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 Tpn was generally deleterious for all the pMHC I tested. Taken together, these observations show that normal expression of pMHC I was influenced by ERAAP and even more so by Tpn.

**Absence of Tpn causes selective loss of pMHC I ligands**

To further define the specific changes that occurred in the pMHC I repertoire because of loss of Tpn, we took advantage of the immune systems’ ability to detect differences between self and nonself. If certain pMHC I were absent in Tpn-deficient mice, specific CD8<sup>+</sup> T cells would not be tolerized to them and would respond to the novel pMHC I expressed by WT cells. We immunized Tpn<sup>−/−</sup> mice with WT spleen cells expressing the normally diverse pMHC I repertoire. After 10 d, splenocytes from recipient mice were restimulated for a week with WT spleen cells. The cultures were then analyzed for presence of CD8<sup>+</sup> T cells that produced IFN-γ when stimulated with spleen cell APCs of the indicated genotype. The Tpn<sup>−/−</sup> anti-WT CD8<sup>+</sup> T cells responded strongly to WT APCs but not to self APCs (Fig. 3A, 3B), showing that Tpn-deficient mice perceived normal pMHC I as foreign in WT cells. We infer that Tpn deficiency caused the loss of many pMHC I normally present in WT 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<sup>+</sup> T cells, we used spleen cells from K<sup>b</sup>D<sup>b</sup> dko mice as APCs. The Tpn<sup>−/−</sup>
anti-WT T cells did not respond to K\(^{b}\)D\(^{b}\) dko spleen cells, indicating that Tpn-dependent peptides were presented by K\(^{b}\) and D\(^{b}\) MHC I in WT cells, rather than nonclassical MHC I molecules (Fig. 3B).

**Tpn-deficient cells also express novel immunogenic pMHC I**

To determine whether loss of Tpn 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-\(\gamma\) in response to Tpn\(^{-/-}\), but not to self WT APCs, indicating presence of novel pMHC I in Tpn\(^{-/-}\) cells (Fig. 4A, 4B).

Tpn 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 Tpn 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-\(\gamma\) (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, although blocking the A\(^{b}\) MHC II molecule or the K\(^{b}\) MHC I molecule did not inhibit IFN-\(\gamma\) production in any of the five lines tested, blocking with the anti-D\(^{b}\) Ab inhibited T cell responses more effectively than blocking with anti-K\(^{b}\) (Fig. 4C). The possible contribution of CD8\(^{+}\) T cells restricted by other nonclassical 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 Abs. Together, these results show that loss of Tpn 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, we used WT or Tpn\(^{-/-}\) splenocytes as APCs after treatment with BFA, an inhibitor of ER-Golgi transport, for either 2 or 4 h (Fig. 4D). Although BFA 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 Tpn are not required for peptide trimming by ERAAP**

The loss and gain of novel pMHC I in either Tpn 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 Tpn (Fig. 5A, 5B). We transfected ERAAP\(^{-/-}\)TAP\(^{-/-}\) or ERAAP\(^{-/-}\)Tpn\(^{-/-}\) fibroblasts with N-terminally extended SHL8 precursors in the presence or absence of WT 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 coexpression of ERAAP, the precursor peptide was no longer detected, and two peaks corresponding to the precisely cleaved SHL8 octapeptide and the KSHL8 nonapeptide were found (Fig. 5A, lower panel). The SHL8 and KSHL8 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 Tpn-deficient cells (Fig. 5B). Thus, in the ER, ERAAP could trim antigenic precursors to their final products in the absence of TAP or Tpn. The results show directly that expression of either TAP or Tpn, and therefore an intact PLC, is not required for N-terminal trimming of antigenic precursors by ERAAP.

**Tpn- and ERAAP-deficient cells express unique, nonoverlapping pMHC I**

To further assess the relationship between peptide editing by ERAAP and Tpn, we examined the potential overlap between novel peptides generated in the absence of Tpn or ERAAP. T cell lines generated in WT mice by immunization with Tpn 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). In contrast, the WT anti-Tpn\(^{-/-}\) lines recognized only Tpn-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 Tpn have distinct and nonoverlapping roles in editing peptides for presentation on MHC I.

To further rigorously establish the distinction between the immunologically distinct Tpn or ERAAP-dependent ligands, we

**FIGURE 3.** Tpn-deficient mice elicit CD8\(^{+}\) T cell response to pMHC I expressed by WT cells. (A) Intracellular IFN-\(\gamma\) produced by Tpn\(^{-/-}\) anti-WT CD8\(^{+}\) T cell lines in response to WT or Tpn\(^{-/-}\) APCs. (B) Tpn\(^{-/-}\) anti-WT CD8\(^{+}\) T cell IFN-\(\gamma\) response against WT, Tpn\(^{-/-}\), TAP\(^{-/-}\), or K\(^{b}\)D\(^{b}\) 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 and B) Numbers indicate percent IFN-\(\gamma\) cells in the CD8\(^{+}\) T cell gate.
assessed the ability of WT mice to eliminate ERAAP<sup>−/−</sup> or Tpn<sup>−/−</sup> target cells in vivo (Fig. 7). We primed WT mice with splenocytes from ERAAP<sup>−/−</sup>, Tpn<sup>−/−</sup>, 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<sup>−/−</sup>, and Tpn<sup>−/−</sup> spleen cells as targets (Fig. 7A, 7B). We depleted NK cells in host mice before immunization and challenge to obviate the possible effect of these cells in targeting Tpn<sup>−/−</sup> or ERAAP<sup>−/−</sup> cells with lower MHC I expression (17, 40). To distinguish the three populations of donor cells recovered from host animals in vivo, we labeled each target cell population with a different fluorescent dye as shown schematically (Fig. 7A). After 20 h, 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.

FIGURE 4. Tpn-deficient cells elicit CD8<sup>+</sup> T cell responses in WT mice. (A) IFN-γ<sup>+</sup> response of WT anti-Tpn<sup>−/−</sup> CD8<sup>+</sup> T cell lines against WT or Tpn<sup>−/−</sup> APCs. Numbers indicate percent IFN-γ<sup>+</sup> cells of total CD8<sup>+</sup> T cells. (B) WT anti-Tpn<sup>−/−</sup> CD8<sup>+</sup> T cell responses against WT, Tpn<sup>−/−</sup>, or TAP<sup>−/−</sup> APCs. (A and B) Numbers indicate percent IFN-γ<sup>+</sup> cells in the CD8<sup>+</sup> T cell gate. (C) WT anti-Tpn<sup>−/−</sup> CD8<sup>+</sup> T cell responses against WT APCs previously treated with blocking Abs to MHC I K<sup>b</sup> (α-K<sup>b</sup>) or D<sup>b</sup> (α-D<sup>b</sup>), or to MHC II A<sup>b</sup> (α-A<sup>b</sup>). Percentages of CD8<sup>+</sup> IFN-γ<sup>+</sup> cells were normalized to no Ab control (No Ab). (D) IFN-γ<sup>+</sup> production by WT anti-Tpn<sup>−/−</sup> (left) and Tpn<sup>−/−</sup> anti-WT (right) CD8<sup>+</sup> T cell lines against splenocyte APCs treated with BFA for 2 or 4 h before coculture with T cells. Percent of CD8<sup>+</sup> IFN-γ<sup>+</sup> was normalized to untreated (−). Data are representative of three independent experiments (A, B) or are pooled from two independent experiments (C, D).

FIGURE 5. Peptide trimming by ERAAP does not require Tpn or TAP. (A and B) The ES-X9[SHL8] construct was cotransfected with ERAAP cDNA or with empty vector into (A) ERAAP<sup>−/−</sup> TAP<sup>−/−</sup> or (B) ERAAP<sup>−/−</sup> Tpn<sup>−/−</sup> fibroblasts. Cell lysates were fractionated by RP-HPLC and trypsinized to release SHL8 peptides before detection with B3Z hybridoma in the presence of L cells expressing H-2K<sup>b</sup>. Synthetic SHL8 and KSHL8 peptide run under identical conditions verified the HPLC fraction numbers. Data are representative of three independent experiments.
Mice primed with ERAAP<sup>−/−</sup> cells efficiently eliminated ERAAP<sup>−/−</sup> targets but did not influence the recovery of Tpn-deficient or self-WT cells (Fig. 7C). In contrast, WT mice primed with Tpn<sup>−/−</sup> splenocytes eliminated Tpn<sup>−/−</sup> targets, but not ERAAP<sup>−/−</sup> 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<sup>−/−</sup> or Tpn<sup>−/−</sup> targets suggests that these responses are mediated by the adaptive immune system. The in vitro and in vivo assessment of WT anti-Tpn<sup>−/−</sup> and WT-anti ERAAP<sup>−/−</sup> T cell lines demonstrates that the unedited peptide repertoires in cells deficient in ERAAP versus Tpn were distinct without any detectable overlap.

**Tpn-deficient cells present peptides lacking canonical consensus motif**

The earlier findings showed that loss of Tpn 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<sup>−/−</sup> 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 Tpn is not known. To define the Tpn-dependent changes in the peptide repertoire, we isolated K<sub>b</sub> and Db pMHC I from WT as well as Tpn<sup>−/−</sup> 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 Db and K<sub>b</sub>, respectively. In contrast, the lower pMHC I expression in Tpn-deficient cells allowed recovery of fewer peptides; 63 and 22 peptides bound to Db and K<sub>b</sub>, 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<sup>−/−</sup>-deficient splenocytes were also found in WT mice (Fig. 8A, Supplemental Fig. 1A, Supplemental Tables I, II). Remarkably, comparison of the unique

**FIGURE 6.** Immunogenic pMHC I expressed by Tpn or ERAAP-deficient cells do not overlap. T cell responses against indicated splenocyte APCs. (A) Tpn<sup>−/−</sup> anti-WT T cell response, (B) WT anti-Tpn<sup>−/−</sup> T cell response, and (C) WT anti-ERAAP<sup>−/−</sup> T cell response against spleen cell APCs derived from WT, Tpn<sup>−/−</sup>, or ERAAP<sup>−/−</sup> mice. Immunizations to induce specific CD8<sup>+</sup> T cells and intracellular cytokine staining to detect IFN-γ production are described earlier. 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, B) independent experiments.

**FIGURE 7.** Different ligands are used for rejection of Tpn- or ERAAP-deficient cells by WT mice in vivo. T cells from WT mice primed 7 d earlier with male WT, Tpn<sup>−/−</sup>, or ERAAP<sup>−/−</sup> splenocytes were assessed for their ability to specifically eliminate WT, Tpn<sup>−/−</sup>, or ERAAP<sup>−/−</sup> female targets in vivo. Targets were given distinct labels so they could be compared in the same host mouse: WT = CFSE (high dose); Tpn<sup>−/−</sup> = CFSE (low dose); ERAAP<sup>−/−</sup> = CMAC. (A) Schematic indicating the populations that represent ERAAP<sup>−/−</sup>, Tpn<sup>−/−</sup>, and WT. (B) Representative FACS plots of labeled targets before (input) and after (output) challenge. Input refers to proportions of each labeled cell type before challenge, whereas output refers to the labeled populations identified 20 h posttransfer. (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).
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, Supplemental Fig. 1B). Second, the canonical asparagine (N) residue at the p5 position of Dβ bound peptides was consistently absent in peptides produced in absence of Tpn (Fig. 8C, 8D). A loss of the conserved phenylalanine or tyrosine residues (F/Y) at the p5 anchor position was less obvious in peptides bound to Kβ (Supplemental Fig. 1C, 1D).

The most striking difference in the amino acid sequences was found in the C-terminal (Pβ) position of Kβ and Dβ 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, 8D, Supplemental Fig. 1C, 1D). However, in the Tpn⁻/⁻ samples, a higher frequency of abnormal amino acids was identified at PΩ. These included Lys (K), Ser (S), Asn (N), Pro (P), and Ala (A) for Dβ (Fig. 8C, 8D), and Thr (T) and Pro (P) for Kβ (Supplemental Fig. 1C, 1D). Thus, the ability to choose the appropriate C-terminal amino acid, a key determinant of pMHC I stability, was lost in the absence of Tpn.

We verified the MHC I binding characteristics of a few representative peptides by assessing their ability to stabilize Dβ or Kβ 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 Kβ and Dβ binding peptides (Supplemental Fig. 2A–D). From Tpn-deficient mice, even the peptides that contained both anchor residues, such as FSPLNPVRV (Dβ) and SLNRFIPL (Kβ), were suboptimal binders compared to the native peptides even when added to the surface of TAP-deficient cells lacking Tpn (Fig. 8C, 8D). A loss of the conserved phenylalanine residue at the Pβ anchor position was obvious in peptides bound to Tpn⁻/⁻ cells. A loss of the conserved phenylalanine residue at the Pβ anchor position was obvious in peptides bound to Tpn⁻/⁻ cells. No loss of other anchor residues, such as P2 and P5, was identified at the Pβ position of Kβ and Dβ peptides eluted from Tpn⁻/⁻ cells. However, in the Tpn⁻/⁻ samples, a higher frequency of abnormal amino acids was identified at PΩ. These included Lys (K), Ser (S), Asn (N), Pro (P), and Ala (A) for Dβ (Fig. 8C, 8D), and Thr (T) and Pro (P) for Kβ (Supplemental Fig. 1C, 1D). Thus, the ability to choose the appropriate C-terminal amino acid, a key determinant of pMHC I stability, was lost in the absence of Tpn.

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We verified the MHC I binding characteristics of a few representative peptides by assessing their ability to stabilize Dβ or Kβ 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 Kβ and Dβ binding peptides (Supplemental Fig. 2A–D). From Tpn-deficient mice, even the peptides that contained both anchor residues, such as FSPLNPVRV (Dβ) and SLNRFIPL (Kβ), were suboptimal binders compared to the native peptides even when added to the surface of TAP-deficient cells lacking Tpn (Fig. 8C, 8D). A loss of the conserved phenylalanine residue at the Pβ anchor position was obvious in peptides bound to Tpn⁻/⁻ cells. No loss of other anchor residues, such as P2 and P5, was identified at the Pβ position of Kβ and Dβ peptides eluted from Tpn⁻/⁻ cells. However, in the Tpn⁻/⁻ samples, a higher frequency of abnormal amino acids was identified at PΩ. These included Lys (K), Ser (S), Asn (N), Pro (P), and Ala (A) for Dβ (Fig. 8C, 8D), and Thr (T) and Pro (P) for Kβ (Supplemental Fig. 1C, 1D). Thus, the ability to choose the appropriate C-terminal amino acid, a key determinant of pMHC I stability, was lost in the absence of Tpn.

The second, the canonical asparagine (N) residue at the p5 position of Kα and Dα peptides eluted from WT cells was consistently absent in peptides produced in absence of Tpn (Fig. 8C, 8D). A loss of the conserved phenylalanine or tyrosine residues (F/Y) at the p5 anchor position was less obvious in peptides bound to Kα (Supplemental Fig. 1C, 1D). Thus, the ability to choose the appropriate C-terminal amino acid, a key determinant of pMHC I stability, was lost in the absence of Tpn.

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 aa, 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 (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.

**FIGURE 8.** MHC I in WT and Tpn-deficient cells present unique peptides. The H-2Dβ MHC I were immunoprecipitated from WT and Tpn-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 Tpn-deficient cells. (B–D) Analysis of the unique peptides from WT and Tpn-deficient cells. (B) The average lengths of peptides recovered from WT or Tpn-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-2Dβ peptides. (D) Logo representation of H-2Dβ peptides eluted from WT or Tpn-deficient cells. Peptides are grouped according to their lengths, and the numbers of peptides in each group are 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).
The MHC I molecules are loaded with their peptide cargo in the ER within the 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 Tpn (16, 17). Tpn 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 Tpn, but not other PLC components (20). Likewise, thorough in vitro reconstitution in microsomes, Tpn mediated the binding of high-affinity peptide to MHC I (44). Taken together with the severe reduction of pMHC I expression on the surface when Tpn 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 Tpn. We elicited CD8+ T cell responses in WT mice immunized with spleen cells from Tpn-deficient mice and vice versa. Because the immune response has evolved to distinguish nonself from self, the CD8+ T cell responses are exquisitely specific for nonself pMHC I. Importantly, the T cell can reveal changes in the pMHC I repertoire caused by alterations in Tpn expression that are not readily detected by any other assay. The specificity of the CD8+ T cell responses showed that the absence of Tpn, 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 MHC I expressed by Tpn-deficient cells were also less stable, suggesting that the presented peptides were structurally distinct from their WT counterparts.

Interestingly, different structural changes in the pMHC I repertoire were caused by the loss of Tpn rather than by loss of ERAAP. We compared the effects of ERAAP versus Tpn 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 cell responses elicited by Tpn 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 Tpn 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 Tpn or the peptide transporter, TAP. Together, these results strongly support the notion that Tpn and ERAAP edit different aspects of the pMHC I repertoire.

Mass spectrometry analysis of peptides eluted from ERAAP and Tpn-deficient spleen cells revealed the molecular basis for the structural differences in the unedited pMHC I repertoires. In the absence of Tpn, the most striking changes occurred at the C terminus where noncanonical amino acids at ΠΩ were far more frequent than in WT cells. In contrast, many more KΩ and DΩ 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 Tpn in ERAAP-deficient cells, but generation of the appropriate N termini required ERAAP. Thus, Tpn 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 Tpn is the ultimate arbitrator for ensuring that peptides with appropriate C-terminal residue are presented. Consistent with a role for Tpn-mediated editing at the C termini of peptides, T134K mutation in the α2 helix of MHC I was found to affect Tpn–MHC I interactions in human cells (46). Although the molecular mechanism by which Tpn edits peptides is still unclear, putative models for peptide optimization via Tpn include accelerated dissociation of unfavorable peptides (40, 44, 47) or maintenance of the MHC I in a peptide-receptive conformation until bound by a high-affinity peptide (48). Notably, Tpn–MHC interactions occurred with the C-terminal end of the MHC I peptide binding groove.

In conclusion, Tpn 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 nonoverlapping, it should be interesting to determine why Tpn or ERAAP are differentially targeted for immune evasion by viruses (49–51) and in cancer (40, 52).

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

Disclosures
The authors have no financial conflicts of interest.

References


