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Analysis of the Role of Tripeptidyl Peptidase II in MHC Class I Antigen Presentation In Vivo[1]

Masahiro Kawahara,[2]* Ian A. York,[†] Arron Hearn,* Diego Farfan,* and Kenneth L. Rock[3]*

Previous experiments using enzyme inhibitors and RNA interference in cell lysates and cultured cells have suggested that tripeptidyl peptidase II (TPPII) plays a role in creating and destroying MHC class I-presented peptides. However, its precise contribution to these processes has been controversial. To elucidate the importance of TPPII in MHC class I Ag presentation, we analyzed TPPII-deficient gene-trapped mice and cell lines from these animals. In these mice, the expression level of TPPII was reduced by >90% compared with wild-type mice. Thymocytes from TPPII gene-trapped mice displayed more MHC class I on the cell surface, suggesting that TPPII normally limits Ag presentation by destroying peptides overall. TPPII gene-trapped mice responded as well as did wild-type mice to four epitopes from lymphocytic choriomeningitis virus. The processing and presentation of peptide precursors with long N-terminal extensions in TPPII gene-trapped embryonic fibroblasts was modestly reduced, but in vivo immunization with recombinant lentiviral or vaccinia virus vectors revealed that such peptide precursors induced an equivalent CD8 T cell response in wild-type and TPPII-deficient mice. These data indicate that while TPPII contributes to the trimming of peptides with very long N-terminal extensions, TPPII is not essential for generating most MHC class I-presented peptides or for stimulating CTL responses to several Ags in vivo. ***The Journal of Immunology,*** **2009, 183: 6069–6077.**

Cytotoxic T lymphocytes survey the MHC class I molecules on the surface of cells searching for ones that contain immunogenic peptides (1, 2). The MHC class I molecules display peptides derived from the cell's expressed genes. In most situations, these presented peptides are from normal cellular proteins and are ignored by CD8 T cells. However, if the abnormal peptides are present, for example, ones containing mutations or from viral proteins, then CTLs will recognize these complexes and be stimulated to destroy the abnormal cell. This process protects the host against tumors and viral infections.

Peptides that bind to MHC class I are produced from intracellular proteins as a byproduct of normal protein catabolism (3–5). The major protease responsible for the initial cleavage of cellular proteins into oligopeptides is the proteasome, a large particle in the cytosol and nucleus of cells. Most peptides produced by proteasomes are very rapidly hydrolyzed into amino acids by the concerted action of aminopeptidases and endopeptidases in the cytosol (6). However, a small fraction of peptides escape destruction and are transported by TAP into the endoplasmic reticulum (ER), where ones of the right size and sequence bind to newly assembled class I molecules (7). MHC class I molecules bind peptides that are of a precise size, which depending on the specific MHC class I molecule are between 8 and 10 aa. Less than 5% of the peptides produced by the proteasome are actually of the proper size to stably bind to any particular class I molecule (8). Proteasomes more frequently generate peptides (~10–20%) that are too long to bind to MHC class I molecules, but can serve as potential antigenic precursors (8). These long precursors can be converted to MHC class I-binding peptides by aminopeptidases, especially ER aminopeptidase 1 (ERAP1; ERAAP) (9–14), or may be completely degraded to amino acids by aminopeptidases and endopeptidases.

Where examined, most aminopeptidases preferentially degrade relatively short peptides and in vitro have little or no activity on peptides that are longer than ~16 aa (15, 16). An exception to this rule is tripeptidyl peptidase II (TPPII). TPPII (EC 3.4.14.10) is an abundant cytosolic aminopeptidase that sequentially removes tripeptides from the amino terminus of peptides, and it also has a poorly understood endoproteolytic activity (17, 18). TPPII is capable of degrading quite long peptides (at least as long as 41 aa) (17), and in vitro it is the major activity in cells that degrades peptides longer than 15 aa (16). However, since only ~10% of peptides produced by the proteasome are longer than 15 aa (8), the importance of this activity is not clear.

Several groups have reported a role for TPPII in MHC class I Ag presentation (16, 19–24). Most of these reports suggest a specialized role for TPPII in processing a limited number of presented peptides; however, one group suggested that in intact cells, proteasomes mainly generate very long peptides (in contrast to the behavior of purified proteasomes in vitro), and that TPPII is essential for processing these long peptides for Ag presentation (16). We have previously tested the role of TPPII in MHC class I Ag presentation in tissue culture, using small interfering RNA (siRNA) to eliminate TPPII from human (HeLa) cells (21). We

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[4] Abbreviations used in this paper: ER, endoplasmic reticulum; DC, dendritic cell; KO, knockout; LCMV, lymphocytic choriomeningitis virus; MEF, mouse embryonic fibroblast; siRNA, small interfering RNA; TPPII, tripeptidyl peptidase II; WT, wild type.

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found that under these conditions, overall MHC class I Ag presentation was only slightly affected, even though presentation of peptides derived from very long model peptides (14–17 residues) was markedly reduced in the absence of TPPII. We concluded that (as with purified proteasomes in vitro) proteasomes in intact cells mainly generate relatively short peptides that can be degraded by many intracellular peptidases, and that TPPII is not essential for MHC class I Ag presentation in tissue culture. In this study, we investigated whether TPPII is also dispensable for Ag presentation in primary and cultured mouse cells and intact mice.

Materials and Methods

Mice and PCR

Mice containing a "gene-trap" cassette that abrogates TPPII expression were purchased from Lexicon Pharmaceutical. These mice were produced by insertional mutagenesis of embryonic stem cells using a retroviral cassette containing a splice acceptor and a poly(A) signal. In these cells, the retroviral cassette was inserted between exons 2 and 3, so that a spliced TPPII containing exons 1 and 2 (98 out of 1262 aa) is expressed instead of full-length TPPII (see Fig. 1*A*). The presence of the gene-trap cassette was tested by genomic PCR using primers specific for a wild-type (mTPPIIg-F1, 5′-AGAATAGCCCATGTGCCAAC-3′; mTPPIIg-R1, 5′-CAACG AAACTTGCCTTCACA-3′) and a gene-trapped allele (mTPPIIg-F1, 5′-AGAATAGCCCATGTGCCAAC-3′; Lex-LTR2, 5′-ATAAACCCT CTTGCAGTTGCATC-3′), respectively. Real-time PCR of TPPII was performed on spleens, kidneys, and embryonic fibroblasts to quantify the knockdown level of full-length TPPII. We first used a TaqMan system (Applied Biosystems), in which the amplicon sequence was not provided by the manufacturer. Therefore, we also designed a set of defined primers that could be applied for a quantitative PCR SYBR Green assay. The primers used were TPPII ex11 forward (5′-GTGCCTAACTGGACATTGAG-3′) and TPPII ex12 reverse (5′-CAACATTATTTGCTTTCAGCCCTG-3′) for amplification around exons 11 and 12. The real-time PCR was performed on a MyiQ machine (Bio-Rad). We used the mice backcrossed with C57BL/6 mice (The Jackson Laboratory) for six generations in all experiments except the one using mouse embryonic fibroblasts (MEFs), in which we used the mice backcrossed for two generations, using MEFs derived from littermates as controls. In all experiments, we used age- and sex-matched mice for analysis. All mice were housed under specific pathogen-free conditions in the animal facility in the University of Massachusetts Medical School. Handling of the mice was performed according to institutional guidelines in the University of Massachusetts Medical School.

Cells

MEFs were produced from crosses of gene-trapped heterozygous mice as previously described (25). The genomic DNA extracted from the cells was analyzed by PCR to genotype each cell line. MHC class I Ag presentation was analyzed in homozygous gene-trapped, homozygous wild-type (WT), and heterozygous MEFs. MEFs were cultured in DMEM supplemented with 20% FBS in a 37°C/10% CO_2 incubator.

Mouse bone marrow-derived dendritic cells (DCs) were generated using standard protocols (26). Briefly, bone marrow cells were cultured in HCM media (RPMI 1640 (Invitrogen) supplemented with 10% FBS, 2 mM L-glutamine, 10 mM HEPES, 50 μM 2-ME, penicillin/streptomycin, and nonessential amino acids) in a 37°C/5% CO_2 incubator. On day 1, the cells were plated in the presence of 10 ng/ml mouse GM-CSF and 5 ng/ml mouse IL-4. On day 4, an additional 10 ng/ml mouse GM-CSF and 5 ng/ml mouse IL-4 were added to the culture media.

293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS and 2 mM L-glutamine.

Construction of plasmids and recombinant lentiviral vectors

We tested presentation of a model peptide SIINFEKL (S8L), the H-2K^b-restricted immunodominant epitope from chicken OVA. Construction of plasmids expressing N-extended S8L peptides has been previously described (21). Briefly, S8L preceded by N-terminal extensions of various lengths was inserted downstream of ubiquitin to generate a ubiquitin-peptide fusion protein. When expressed in cells, the N-terminal ubiquitin is rapidly cleaved by ubiquitin C-terminal hydrolases, yielding peptides with defined N-terminal residues (27). GFP was expressed from the same transcript as the ubiquitin-peptide using an internal ribosome entry site.

Recombinant lentiviral vectors expressing full-length OVA, ubiquitin, ubiquitin fused with S8L, and ubiquitin fused with 9-aa N-extended S8L were constructed by inserting each gene and an IRES-GFP cassette at an *Eco*RI-*Bam*HI site of FUGW (provided by Dr. E. Latz, University of Massachusetts Medical School, Worcester, MA) (28).

Peptides used in these experiments are summarized in Table I.

Table I. *Peptides expressed by plasmids*

Name of Peptide	Peptide Sequence	Length
S8L	SIINFEKL	8mer
N2S8L	LE SIINFEKL	10mer
N4S8L	EQLE SIINFEKL	12mer
N6S8L	GLEQLE SIINFEKL	14mer
N8S8L	VSGLEQLE SIINFEKL	16mer
N9S8L	EVSGLEQLE SIINFEKL	17mer
N10S8L	DEVSGLEQLE SIINFEKL	18mer

Transfection and transduction in vitro

MEFs were transiently transfected using FuGENE6 (Roche) as described previously (12). In some cases, MEFs were treated with 50 U/ml murine IFN-γ (BD Biosciences) 5 h after transfection.

For production of lentivirus, 293T cells were transiently transfected with each recombinant lentiviral vector together with packaging construct delta-8.91 and a VSV-G expression plasmid (gifts from Dr. E. Latz, University of Massachusetts Medical School, Worcester, MA) using TransIT-293 transfection reagent (Mirus Bio). Supernatant containing recombinant lentivirus was collected after 2 and 3 days of transfection and stored at −80°C until just before use. For in vivo injection, the supernatant was further concentrated by ultracentrifugation (29). Lentiviral titers (transducing units) were determined using NIH3T3 cells based on the GFP-positive cell ratio on day 2.

Bone marrow cells were transduced with recombinant lentivirus in the presence of 4 μg/ml polybrene (Sigma-Aldrich) on day 2. Half of the media was replaced on day 3 to reduce a toxic effect of polybrene on the cells. On day 7, cells were stained and analyzed by flow cytometry.

Handling of viral vectors was performed according to the guidelines of biosafety level 2^+ laboratories established by the Recombinant DNA Committee of the University of Massachusetts Medical School.

Poly(I:C) treatment of mice

Mice were injected i.p. with 200 μg of poly(I:C) (GE Healthcare) in a total volume of 100 μl of PBS. Spleens from the mice were harvested after 24 h and then stained for flow cytometric analysis.

Viral infection in vivo and peptide stimulation in vitro

Mice were injected i.p. with 5×10^4 PFU per mouse of lymphocytic choriomeningitis virus (LCMV) Armstrong (a gift from Dr. R. Welsh, University of Massachusetts Medical School, Worcester, MA) or with 1.25×10^6 PFU per mouse of recombinant vaccinia virus expressing full-length OVA, ubiquitin, ubiquitin fused with S8L, or ubiquitin fused with 9-aa N-extended S8L. Mice were injected in the footpad with 3.3×10^5 transducing units per mouse of recombinant lentivirus or with 4.8×10^5 PFU per mouse of recombinant vaccinia virus. Eight (LCMV and recombinant lentivirus) or seven (recombinant vaccinia virus) days later, splenocytes (LCMV and recombinant vaccinia virus) or lymphocytes from a draining lymph node (recombinant lentivirus) were harvested and incubated for 5 h with 5 μM of the appropriate peptide, or with 0.5 μg/ml anti-CD3ε (BD Biosciences), in the presence of GolgiPlug (BD Biosciences). Peptides that were used to stimulate IFN-γ production after LCMV infection were gp33 (KAVYNFATC), NP205 (YTVKYPNL), gp276 (SGVENPGGYCL), and NP396 (FQPQNGQFI). For recombinant vaccinia and lentivirus, S8L peptide was used. All peptides were synthesized by Anaspec. Cells were stained for CD8, CD44 (BD Biosciences), and intracellular IFN-γ (eBioscience) using commercial Abs, and analyzed by flow cytometry.

Abs and flow cytometry

The mAb 25.D1.16 (which recognizes S8L in combination with H-2K^b) (30), Y3 (anti-H-2K^b) (31), 28.14.8S (anti-H-2D^b) (32), and H36.4.5 (anti-influenza hemagglutinin; for isotype control) (a gift of W. Gerhard, The Wistar Institute, University of Pennsylvania) (33) were used as primary Abs in staining MEFs and lentivirus-transduced DCs for flow cytometry. After treatment with donkey anti-mouse F(ab′)$_2$ fragments conjugated to Cy5 (Jackson ImmunoResearch Laboratories), flow cytometry was performed on a FACSCalibur apparatus (BD Biosciences), followed by analysis with FlowJo software (Tree Star). Transfected cells were identified by

gating on GFP fluorescence. In the case of staining of DCs, PE-conjugated anti-CD11c Ab (eBioscience) was also used as a secondary Ab.

For staining cells isolated from spleens, bone marrows, and draining lymph nodes, AF6-88.5 (anti-H-2K^b), KH95 (anti-H-2D^b), and AF6-120.1 (anti-I-A^b), BB7.2 (anti-HLA-A2; for isotype control), and anti-Gr1 Abs conjugated to a fluorophore were used according to the manufacturer's directions (BD Biosciences). Other fluorophore-conjugated Abs against cell surface markers (CD4, B220, CD11c, CD86) were purchased from eBioscience. The cells were then analyzed by flow cytometry.

Western blotting

Tissue extracts were prepared in homogenization buffer (50 mM Tris (pH 7.4), 0.25 M sucrose, 5 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 10% glycerol) with complete protease inhibitor cocktail minitablets (Roche) using a dounce homogenizer on ice. The homogenate was spun at 325 × *g* for 10 min, and the supernatant was further spun at 16,000 × *g* for 20 min. The total amount of protein in the supernatant was quantified by BCA assay (Pierce). The supernatant was mixed with SDS/DTT sample buffer (New England Biolabs) and was heated to 100°C for 5 min. The resulting lysate was resolved by SDS-PAGE and was transferred to a nitrocellulose membrane. After the membrane was blocked with 5% milk in PBS containing 0.1% Tween 20, the blot was probed with chicken anti-TPPII Ab (Cedarlane Laboratories) or mouse anti-α-tubulin Ab (Abcam), followed by HRP-conjugated anti-chicken IgY (Promega) or HRP-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories), and detection was performed using an ECL system (Pierce Biotechnology).

Radiolabeling and immunoprecipitation

Thymocytes (25×10^6) were incubated in 700 μl of RMPI 1640 without methionine (Sigma-Aldrich) at 37°C and 5% CO_2 for 2.5 h, and then 500 μCi of L-[^{35}S]-methionine L-[^{35}S]-cysteine mix (EasyTag Express protein labeling mix; PerkinElmer) was added and the incubation continued for 4 h. Cells were spun down, washed with PBS, and lysed on ice by addition of 700 μl of ice-cold lysis buffer (Tris-buffered saline (pH 8.0), 1% (w/v) deoxycholic acid, 0.5% (v/v) Nonidet P-40) with protease inhibitors (complete protease inhibitor cocktail tablets; Roche). The resulting lysates were cleared by centrifugation through a 0.22-μm-pore cellulose acetate membrane (SpinX; Corning).

For immunoprecipitation, protein A-conjugated magnetic beads (15 μl/sample Dynabeads protein A; Invitrogen) were washed with lysis buffer (Tris-buffered saline (pH 8.0), 1% (w/v) deoxycholic acid, 0.5% (v/v) Nonidet P-40) and incubated 2 h at 4°C with rabbit anti-H-2K^b exon 8 (2 μl/sample; a gift from J. Yewdell, National Institutes of Health, Bethesda, MD) or rabbit anti-β_2-microglobulin (1 μl/sample; Dako) and then added to radiolabeled cell lysates. A third of each cell lysate volume was incubated with 15 μl of Ab beads overnight at 4°C with shaking. The beads were then washed three times with lysis buffer using a magnet (DynaMag-2; Invitrogen), resuspended in 50 μl/sample of sample buffer and reducing agent (XT sample buffer and reducing agent; Bio-Rad), and heated to 90°C for 10 min. Thirty microliters per sample was run in a 12% 30/0.8 acrylamide/bisacrylamide (Duracryl; Proteomic Solutions), 360 mM bis-Tris (Calbiochem/EMD Chemicals) gel. The running buffer was 50 mM MOPS, 50 mM Tris, 1 mM EDTA, 0.1% (w/v) SDS, and 5 mM sodium bisulfite. The gel was washed with distilled water, incubated for 1 h in scintillation phosphor solution (Autofluor, National Diagnostics), dried under vacuum, and autoradiography film was exposed to it at −80°C for 1–5 days. The films were developed and scanned, and the band intensities were quantified using ImageJ software (National Institutes of Health).

Results

TPPII gene-trapped mice are viable

Mice with a gene trap disrupting TPPII were obtained from Lexicon Pharmaceuticals. In the gene-trapped allele, the gene-trap cassette (containing a splice acceptor and a poly(A) signal) was inserted between exons 2 and 3, leading to expression of a truncated TPPII (98 out of 1262 aa) and impairment of expression of full-length TPPII (Fig. 1*A*). To evaluate the reduction in TPPII, TPPII mRNA levels were analyzed in tissues and cells by real-time quantitative PCR. TPPII mRNA levels derived in spleens, kidneys and MEFs from gene-trapped mice were reduced down to 3.2, 7.5, and 2.9% of WT mice, respectively. We also analyzed levels of TPPII mRNA by amplifying the active site region in exon 11 and found that mRNA levels in the gene-trapped MEFs were 4.9% of those

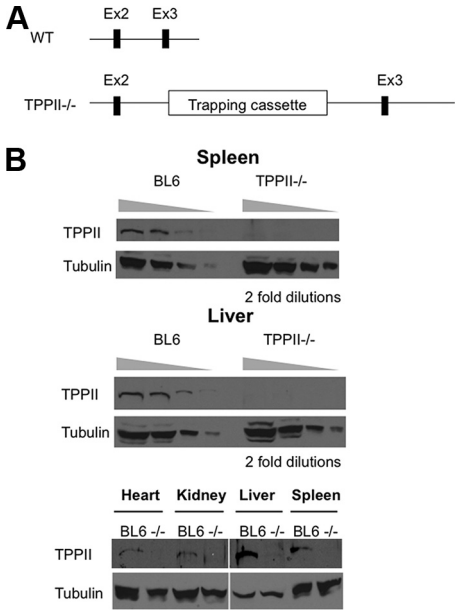

FIGURE 1. Construction and assessment of TPPII gene-trapped mice. *A*, Gene trap diagram. The TPPII gene-trapped mice were produced by insertional mutagenesis of embryonic stem cells using a retroviral cassette containing a splice acceptor and a poly(A) signal. In these cells, the retroviral cassette was inserted between exons 2 and 3, so that a spliced TPPII containing exons 1 and 2 (98 out of 1262 aa) is expressed instead of full-length TPPII. *B*, Undetectable expression level of the TPPII protein in TPPII gene-trapped mice. Tissue extracts were subjected to Western blotting either with anti-TPPII Ab or with anti-α-tubulin Ab.

in WT MEFs. These results indicate that the TPPII mRNA level is severely reduced (~95% reduction) in the gene-trapped mice; the residual mRNA level is possibly due to alternative splicing. To evaluate the effects of the reduction in mRNA levels on TPPII protein levels, we analyzed spleen, liver, heart, and kidney tissue from WT and gene-trapped mice by semiquantitative Western blotting. TPPII protein was present in WT mice but was undetectable in the gene-trapped animals, demonstrating that TPPII protein levels are reduced by at least 87.5% (Fig. 1*B*). These analyses indicated that the TPPII gene-trapped mice are markedly deficient in TPPII.

Mice with a gene trap disrupting TPPII (Fig. 1*A*) were viable and fertile, and they were normal in appearance and behavior. In a previous description of TPPII knockout (KO) mice, splenomegaly due to extramedullary hematopoiesis and evidence of chronic inflammation were observed (34). However, in our TPPII-deficient gene-trapped mice, we did not observe any evidence of extramedullary hematopoiesis or inflammation, including splenomegaly, increased splenic myeloid cells (GR-1$^+$) cells (Fig. 2), or abnormal histology (data not shown) or evidence of systemic cytokine effects (described below).

Surface MHC class I levels are increased on a subset of TPPII-deficient cells

In order for MHC class I molecules to be transported to the cell surface, they must first bind a peptide in the ER. Therefore, surface expression of MHC class I molecules is an indirect measure of overall peptide supply. Elimination of TPPII might either reduce the peptide supply to MHC class I if TPPII contributes to the degradation of MHC class I-binding peptides, or it might increase peptide supply if TPPII helps produce such peptides. We therefore measured MHC class I surface expression on splenic B and T cells, CD11c$^+$

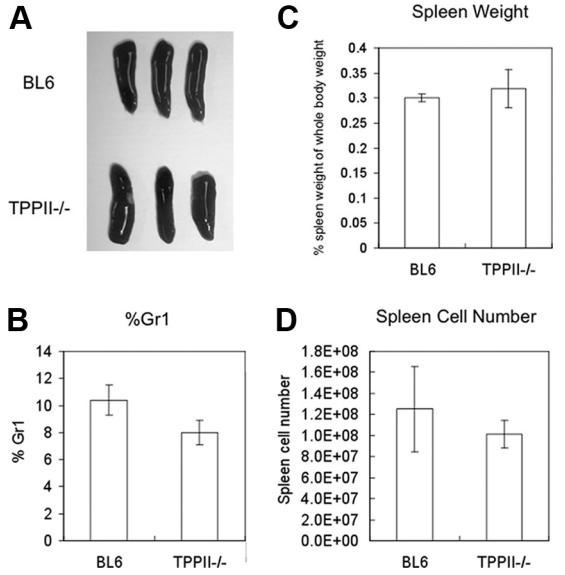

FIGURE 2. TPPII gene-trapped mice do not show extramedullary hematopoiesis. The bars represent averages with SDs ($n = 3$). *A*, Spleens of 5- to 6-mo-old BL6 and TPPII gene-trapped mice. *B*, Splenocytes were stained with anti-Gr1 Ab to identify splenic granulocytes. *C*, Comparison of the percentage of spleen weights of whole body weights. *D*, Comparison of number of splenocytes.

splenic DCs and thymocytes, as well as on bone marrow-derived DCs. The most striking finding in this analysis was an increase in MHC class I levels on thymocytes. H-2K^b and H-2D^b on all thymic subsets from TPPII gene-trapped mice were 20–30% higher than those on thymocytes from WT C57BL/6 mice (Fig. 3, *A* and *B*). There was also a trend for increased levels of MHC class I molecules on DCs (Fig. 3, *C* and *D*) and splenic lymphocytes (Fig. 4, *A*, *B*, *D*, and *E*) from TPPII-deficient mice, but in most cases this was not statistically significant. When mice were treated with the type I IFN inducer poly(I:C), MHC class I levels increased on peripheral lymphocytes from TPPII-deficient vs WT mice, but were not significantly different between the strains. I-A^b was similarly expressed on DCs and splenic B cells from TPPII gene-trapped mice compared with WT animals (Figs. 3, *E* and *F*, and 4, *C* and *F*).

TPPII gene-trapped mice respond normally to viral infection

To test the contribution of TPPII to immune responses to a viral infection, we examined the CTL responses to LCMV. C57BL/6 and TPPII gene-trapped mice were infected with LCMV. At the peak of the response on day 8 after infection, splenic lymphocytes were isolated, stimulated in vitro with individual LCMV epitopes, and stained for intracellular IFN-γ levels. The frequency of CTL producing IFN-γ in response to four LCMV epitopes in TPPII gene-trapped mice was not significantly different from those in C57BL/6 mice, suggesting that any contribution of TPPII to creating or destroying these presented peptides is not sufficient to affect immune responses in vivo (Fig. 5).

TPPII knockdown affects the trimming of long peptide precursors in some cell types

In our previous study using HeLa-Kb cells, we demonstrated that siRNA-mediated silencing of TPPII inhibited by ~50% the trimming of long peptide precursors (from 14 to 17 aa long) to generate MHC class I-presented peptides (21). To examine whether TPPII

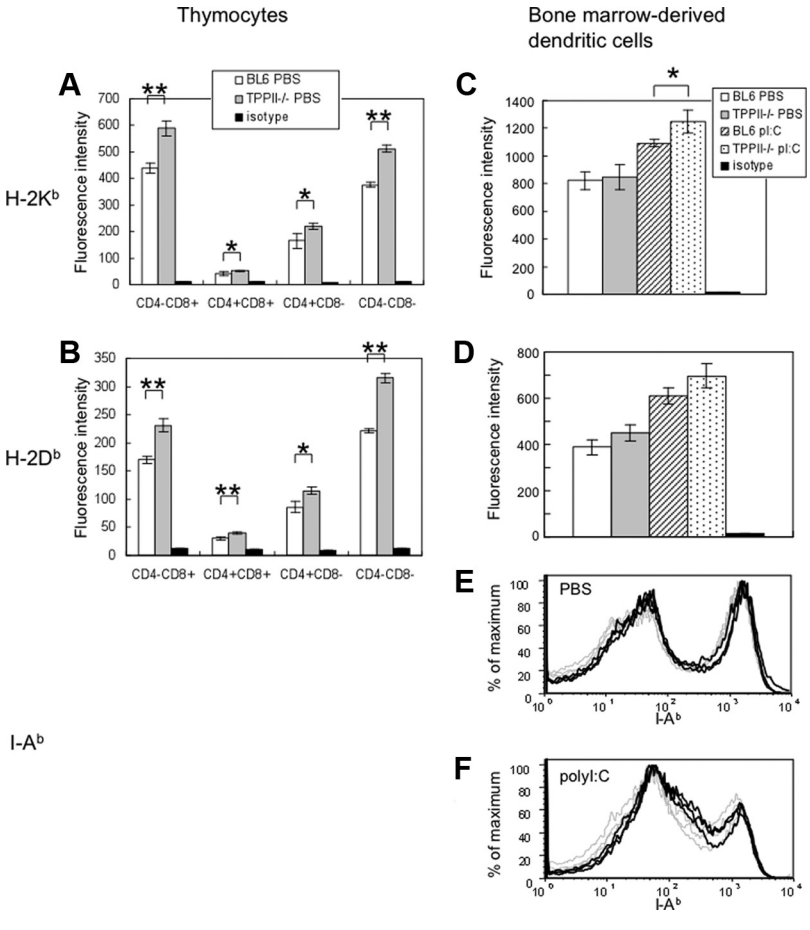

FIGURE 3. TPPII gene-trapped and WT thymocytes show differences in MHC class I, but not in MHC class II, levels. The mice were injected i.p. with PBS. After 24 h, thymocytes were stained with appropriate Abs and analyzed by FACS. Bone marrow cells were also harvested and cultured for 6 days, followed by addition of PBS or poly(I:C) (pI:C) and staining with appropriate Abs on day 7 and analysis by FACS. *A–D*, Expression levels of H-2K^b (*A* and *C*) and H-2D^b (*B* and *D*) expressed as geometric mean fluorescence intensity. The Student *t* test was used to determine statistical significance (*, $0.01 < p < 0.05$; **, $p < 0.01$). Error bars represent SDs ($n = 3$). Data are representative of three independent experiments. *E* and *F*, Flow cytometry traces of bone marrow-derived DCs (gated on CD11c^{high} cells) stained for I-A^b after 6 days of culture, followed by 24 h of treatment with (*E*) PBS or (*F*) poly(I:C). Bone marrow-derived DCs from WT mice are indicated by dashed light traces. Bone marrow-derived DCs from TPPII gene-trapped mice are indicated by solid dark traces.

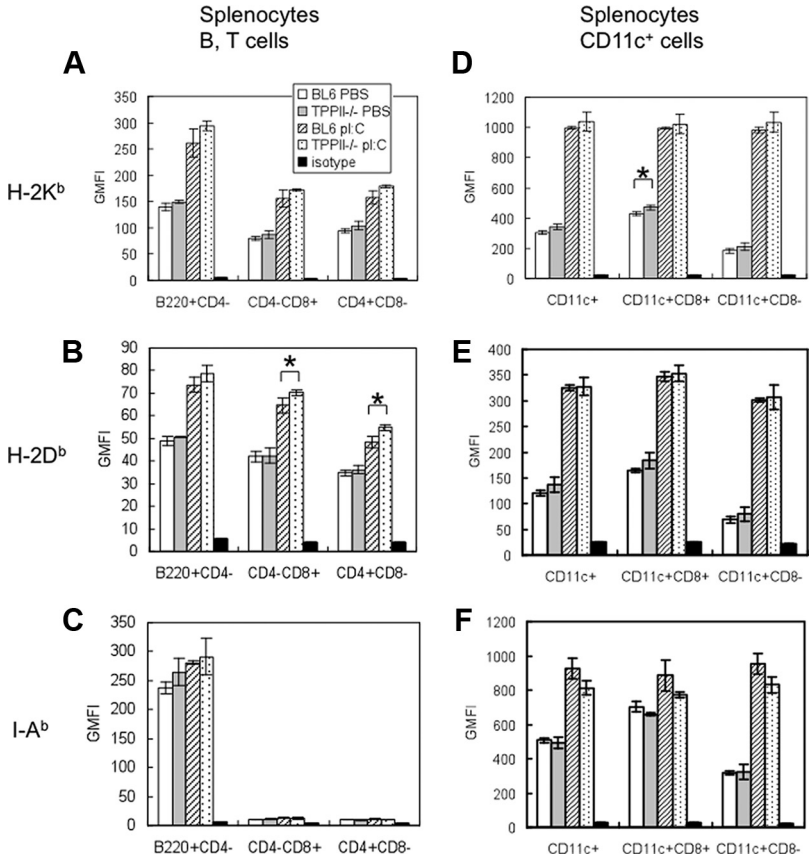

FIGURE 4. TPPII gene-trapped and WT splenocytes show differences in MHC class I, but not in MHC class II, levels. The mice were injected i.p. with PBS or poly(I:C) (pI:C). After 24 h, splenocytes were stained with appropriate Abs and analyzed by FACS. Each graph shows geometric mean fluorescence intensity (GMFI), which represents the expression levels of H-2K^b (*A* and *D*), H-2D^b (*B* and *E*), or I-A^b (*C* and *F*). The Student *t* test was used to determine statistical significance (*, $0.01 < p < 0.05$). Error bars represent SDs ($n = 3$). Data are representative of three independent experiments.

similarly contributes to trimming of long precursors in murine cells, we isolated multiple independent MEF lines from the progeny of TPPII$^{+/-}$ mouse crosses, thus generating homozygous gene-trapped, heterozygous, and WT MEFs. The homozygous TPPII gene-trapped and WT lines were indistinguishable in terms of their morphology and growth characteristics for at least 25 passages.

Since TPPII is a cytosolic peptidase, we examined the ability of the gene-trapped MEFs to generate presented peptides from precursors expressed in the cytosol. For this experiment, several independent MEF lines were transfected with plasmids expressing SIINFEKL (S8L) as ubiquitin fusion proteins, either as the mature epitope (SIINFEKL) or with N-terminal extensions of varying lengths as summarized in Table I. Cleavage by ubiquitin C-terminal hydrolases, which reside in the cytosol, releases the peptide of interest without an initiating methionine, thus generating peptides similar to those generated by the proteasome. Subsequent trimming of the N-terminal residues releases SIINFEKL (S8L), which, if presented on H-2K^b, can be detected by staining with the mAb 25.D1.16.

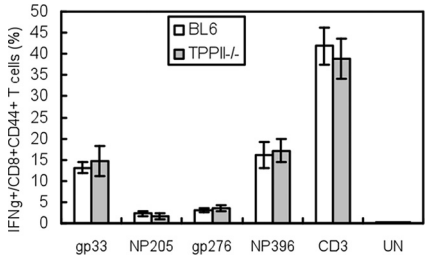

FIGURE 5. TPPII gene-trapped and WT mice respond similarly to LCMV infection in vivo. Splenocytes from TPPII$^{-/-}$ and BL6 mice infected with LCMV were harvested on day 8, and stimulated in vitro with peptides corresponding to LCMV epitopes for 5 h. Cells were then stained for intracellular IFN and analyzed by flow cytometry by gating on CD8$^+$CD44$^+$ cells. The graph represents average percentages of IFN-γ+ cells with SDs as error bars ($n = 6$). There was no significant difference between TPPII$^{-/-}$ and BL6 mice in their response to any of the four epitopes tested.

MEFs were transfected with various constructs and analyzed by flow cytometry by gating on cell populations expressing comparable amounts of GFP. Because absolute levels of surface H-2K^b were variable between different independent MEF lines, peptide presentation was normalized to the H-2K^b level of each cell. Presentations of mature S8L and of S8L with a 2-aa N-terminal extension were not statistically different between WT, heterozygous, and KO MEFs. In contrast, presentation of S8L with a 4- to 10-aa N-terminal extension was significantly reduced in the KO MEFs, compared with WT and heterozygous MEFs (Fig. 6*A*). These results are similar to (although less marked than) those previously observed in human HeLa cells (21) and indicate that TPPII contributes to the trimming of very long N-terminal extensions in some primary murine cells.

To further test the role of TPPII in trimming N-extended peptides in another primary cell type, we transduced bone marrow-derived DCs with lentiviruses expressing various S8L precursors. Lentivirus transduction does not affect DC maturation or Ag presentation (35). We constructed recombinant lentiviral vectors encoding S8L as a ubiquitin fusion protein, S8L with a 9-residue N-terminal extension as a ubiquitin fusion protein (N9-S8L), ubiquitin alone (as a control), or full-length OVA. Bone marrow-derived DC progenitors from WT or KO mice were transduced with each lentivirus on day 2 and stained with H-2K^b-S8L-specific Ab 25.D1.16 on day 7 by gating on GFP$^+$CD11c$^+$ cells. These cells presented the full-length OVA and S8L similarly. However,

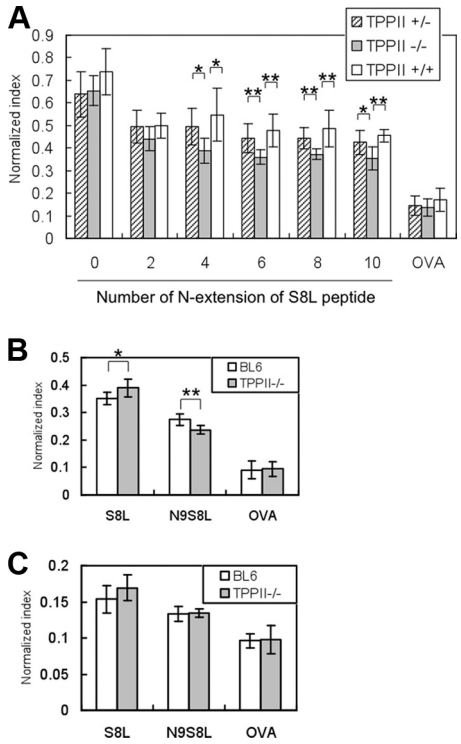

FIGURE 6. TPPII gene-trapped and WT embryonic fibroblasts, but not cultured DCs, respond differently to N-extended epitope precursors. *A*, TPPII$^{-/-}$, TPPII$^{+/-}$, or TPPII$^{+/+}$ MEFs were transfected with plasmids expressing, in the cytosol, the OVA H-2K^b-restricted epitope SIINFEKL (S8L) extended at the N-terminus by 0, 2, 4, 6, 8, or 10 aa as ubiquitin fusion constructs, or full-length OVA, and coexpressing GFP on the same mRNA. After 24 h the MEFs were stained with 25.D1.16 Ab. Cells were gated on similar levels of GFP expression. Data are normalized to expression level of H-2K^b in each MEF line. The Student *t* test was used to determine statistical significance (*, $0.01 < p < 0.05$; **, $p < 0.01$). Error bars represent SDs ($n = 9$ for TPPII$^{+/-}$, $n = 7$ for TPPII$^{-/-}$, $n = 4$ for TPPII$^{+/+}$). *B* and *C*, TPPII$^{-/-}$ or BL6 bone marrow-derived DCs were transduced with recombinant lentiviral vectors encoding S8L or N9S8L as ubiquitin fusion constructs, or full-length OVA, and then stained with 25.D1.16 Ab at 5 days posttransduction. Cells were gated on (*B*) high or (*C*) low levels of GFP expression. Data are normalized by expression level of H-2K^b in each DC line. The Student *t* test was used to determine statistical significance. Error bars represent SDs ($n = 6$).

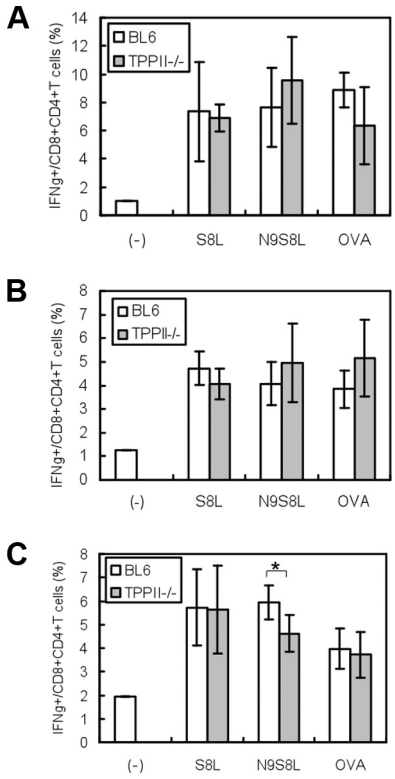

FIGURE 7. TPPII gene-trapped and WT mice respond similarly to N-extended epitope precursors in vivo. *A*, Mice were injected in the footpad with recombinant lentivirus encoding full-length OVA, ubiquitin alone (−), ubiquitin fused with SIINFEKL (S8L), or ubiquitin fused with S8L extended by 9 aa at the N terminus (N9S8L). *B*, Mice were injected in the footpad with recombinant vaccinia virus encoding the same constructs. *C*, Mice were injected i.p. with 1.25×10^6 PFU per mouse of recombinant vaccinia virus encoding the same constructs. Eight days later (*A*) or seven days later (*B* and *C*), lymphocytes from a draining lymph node (*A*) or splenocytes (*B* and *C*) were harvested and stimulated for 5 h with 5 μM of the appropriate epitope. Cells were then stained for intracellular IFN-γ and analyzed by flow cytometry by gating on CD8$^+$CD44$^+$ cells. The graphs represent average percentages of IFN-γ^+ cells with SDs as error bars ($n = 5$). Data are representative of two independent experiments. The Student *t* test was used to determine statistical significance (*, $0.01 < p < 0.05$). The difference in response to i.p. vaccinia-N9S8L seen between TPPII KO and WT mice in *C* ($p = 0.03$) was not seen in the repeat experiment ($p = 0.59$).

TPPII-deficient DCs also showed reduced presentation compared with WT cells of the N9-S8L construct when it was expressed at high levels (GFP^{high} cells) (Fig. 6*B*). Interestingly, the difference in N9-S8L presentation by WT and KO cells was not seen at more limiting Ag concentrations (GFP^{low} cells) (Fig. 6*C*), presumably because the alternate trimming mechanisms for long peptides only become limiting at the higher Ag dose.

Analysis of the role of TPPII in trimming long precursors in vivo

Since TPPII has some effect on the trimming of long precursor peptides in some cell types, we sought to examine its role in this process in animals. He et al. reported that s.c. injection of lentivirus resulted in direct transduction of skin-derived DCs and potent and prolonged Ag presentation (36). We injected recombinant lentiviruses into footpads of TPPII gene-trapped mice and C57BL/6 mice as described (36, 37). We used recombinant lentivirus harboring genes for the various S8L-derived peptides described above and for full-length OVA. In this system the magnitude of the CD8 T cell response was dependent on the amount of virus injected, and therefore we used limiting doses of viruses so as to be on a sensitive portion of the dose-response curve. On day 8 after infection, lymphocytes from the draining popliteal lymph node were stimulated in vitro with the S8L peptide, followed by staining for intracellular IFN-γ levels. The frequency of IFN-γ^+ CTLs to S8L and to the N-extended S8L precursor was not different between WT and TPPII-deficient mice (Fig. 7*A*), even at limiting doses of the virus (where responses were not maximal).

We next immunized mice with recombinant vaccinia viral vectors encoding the same set of genes as recombinant lentiviral vectors described above. We infected WT and gene-trapped mice either by footpad injection or i.p. injection with the various recombinant vaccinia viruses. On day 7, splenocytes were similarly stimulated in vitro with the S8L peptide, followed by staining for intracellular IFN-γ levels. No consistent, statistically significant difference in response to any of the constructs was seen, although a statistically significant decrease of IFN-γ^+ CTLs to N9S8L was observed in one of two i.p. injection experiments (Fig. 7, *B* and *C*).

Discussion

Immune surveillance for virally infected cells and tumors is dependent on cells processing their proteins into fragments and displaying a fraction of these peptides on MHC class I molecules on the cell surface. The magnitude of the CTL response to a particular peptide and the immunodominance hierarchy of responses are at least partly dependent on the numbers of MHC class I-peptide complexes at the cell surface. This in turn is influenced by several factors, such as the affinity of binding between the peptide and MHC class I allele, the number of protein precursors, and the frequency with which the peptide is generated or destroyed by the Ag-processing pathway. To understand and predict the specificity of responses, therefore, it is important to identify proteases that generate or destroy peptides that bind, or could bind, MHC class I molecules.

Most intracellular proteins are degraded by proteasomes, and proteasomes are essential for generating most MHC class I-presented epitopes (38). Experiments with model peptides and proteasome inhibitors have shown that the proteasome generally provides the only activity in cells that can make the proper cleavage to generate the C terminus of an MHC class I epitope (39–41). In contrast, peptides that have the correct C-terminal residue for binding MHC class I, but that are too long at the N terminus, can be trimmed by aminopeptidases into mature epitopes. Epitopes can also be destroyed if aminopeptidases or endopeptidases trim them below the minimum size needed for MHC class I binding (6). Therefore, several groups have pursued the question of whether trimming by aminopeptidases is normally important in Ag presentation, and if so which aminopeptidases contribute to this process (16, 42–44).

A number of aminopeptidases have been proposed to play a role in MHC class I Ag presentation, based on biochemical observations, experiments in tissue culture, or KO mice. ER aminopeptidase 1 (ERAP1) has been shown to play an important role in trimming peptides in the ER for Ag presentation and in the generation of CD8 T cell responses in mice in ways that increase or decrease responses to certain peptides (9–14). There is also strong evidence that peptide trimming by aminopeptidases in the cytosol contributes to Ag presentation (10, 39, 42, 43, 45). Although biochemical experiments with various aminopeptidases (including leucine aminopeptidase, bleomycin hydrolase, and puromycin-sensitive aminopeptidase) suggested they may have important functions in Ag processing (42, 43), mice lacking these aminopeptidases have shown at most very limited effects on Ag presentation and immune responses (25, 46, 47). To fully understand the contribution of various peptidases to Ag presentation, it is therefore important to generate and analyze peptide-deficient mice.

TPPII is an abundant intracellular peptidase that cleaves triplets of amino acids from the N terminus of peptides and has been reported to also act as an endoprotease (19). The physiological functions of TPPII have been unclear. Early studies suggested that TPPII might be able to compensate for the loss of proteasomes (48), although subsequent experiments did not support this possibility (49). In cell extracts, TPPII has been shown to help generate a few MHC class I-presented peptides, and studies with inhibitors have suggested a similar role in vivo (16, 19, 48).

A recent paper suggested that in fact TPPII may be essential for almost all MHC class I Ag presentation, and the authors proposed a model in which proteasomes in intact cells normally produce only long peptides (more than 16 aa long) and TPPII was needed to trim these long precursors; in this model, TPPII was an essential bridge between proteasome-generated peptides and degradation to amino acids by conventional peptidases (16). We have previously tested this model in cultured human cells by using siRNA to knock down TPPII levels (21). We found that, while TPPII was indeed important (although not essential) for converting long peptides to shorter forms, TPPII was not required for MHC class I Ag presentation in cultured cells: presentation of peptides from full-length proteins required proteasomes, but TPPII knockdown did not reduce (and if anything slightly increased) MHC class I peptide production. We tentatively concluded that relatively few very long precursor peptides are normally generated in vivo and therefore TPPII is not required for most Ag presentation. However, the possibility remained that TPPII is required for a natural immune response in intact animals (e.g., if the HeLa cells we tested are not representative of normal cells, or if the initiation of an immune response has different requirements for Ag presentation).

Mice containing a gene trap between exons 2 and 3 have a >90% reduction in the expression of TPPII mRNA and at least a >87.5% reduction in the expression of protein. They are viable, fertile, and are grossly normal in appearance and behavior; in particular, and in contrast to observations in TPPII KO mice (34), the gene-trapped TPPII-deficient mice did not show extramedullary hematopoiesis or splenitis. These differences may be because of differences in housing or in mouse background, or may be because of very low (although undetectable in our assays) levels of functional TPPII in our mice. The lack of chronic inflammation in these gene-trapped mice offers the ability to compare Ag presentation in the presence and near-absence of TPPII, without the confounding effects of inflammation on MHC class I Ag presentation.

Some cells (particularly thymocytes) from TPPII gene-trapped mice express a higher level of surface MHC class I than do those from C57BL/6 mice (Fig. 2). In contrast, the synthesis of heavy chains (measured by immunoprecipitation of metabolically labeled H-2K^b) and light chains (β_2-microglobulin) was not significantly different in thymocytes from WT vs TPPII-deficient mice (data not shown). Since MHC class I can only reach the cell surface when it is bound to an appropriate peptide, this suggests that the overall supply of presented peptides may be reduced by TPPII. In other words, the net effect of TPPII may be to destroy more peptides than it helps produce. It is possible that residual TPPII in the gene trap mice is contributing some function, and a more pronounced phenotype would be observed in the complete absence of this peptidase. However, our findings are similar to observations with TPPII KO mice (24).

In previous studies, the most important contribution of TPPII to Ag presentation was its ability to trim very long precursor peptides (16, 21). However, this activity has only been examined in cultured human tumor cells and not in primary cells or cells of murine origin. Therefore, we made minigene constructs to test the trimming of full-length protein, short and long antigenic precursors, and mature epitope in primary cells from gene-trapped mice. For these studies, we produced embryonic fibroblast cell lines (MEFs), transfected these cells with plasmids expressing various precursors of the H-2K^b-binding peptide SIINFEKL, and quantified presentation of H-2K^b-SIINFEKL as a measure of Ag processing. Presentation of SIINFEKL from short precursors tested was similar in WT, heterozygous, and homozygous gene-trapped cell lines, while presentation of SIINFEKL from precursors with 4- to 10-aa N-terminal extensions was significantly reduced by TPPII deficiency. A similar defect in processing a long precursor (N9-S8L) was observed in TPPII-deficient bone marrow-derived DCs under conditions of high Ag expression. These results are consistent with our previous findings with HeLa-Kb cells (21), although the extent of reduction in the MEFs was less marked than in the previous experiments, perhaps because of the variability between independent MEF cell lines, different species (human vs mouse), expression

level of TPPII, and/or presence of other aminopeptidases in the different cell types.

We also tested the effect of TPPII knockdown on the generation of an authentic antiviral immune response to viral proteins and antigenic precursors. We infected TPPII gene-trapped mice and C57BL/6 mice with LCMV, and after 8 days we analyzed the frequency of $CD8^+$ T cell responses to four LCMV MHC class I-restricted epitopes. Consistent with the recent findings (23, 24), we found no difference in the responses to any of the epitopes (Fig. 5). Additionally, we specifically tested the importance of TPPII on generation of MHC class I epitopes from precursors with long N-terminal extensions, using recombinant lentiviruses and vaccinia viruses expressing ubiquitin-fused minigenes. TPPII gene-trapped mice generated similar levels of CTL to these precursors as did WT mice, demonstrating that TPPII is not essential for generating immune responses to long N-extended precursors in vivo. We presume that this is because the reduction in presentation we observe in TPPII-deficient DCs is too small to affect responses in vivo. Alternatively, since we only see a defect in presentation at high Ag concentrations it is possible that such high amounts of Ag expression are not obtained in vivo. Finally, it is also possible that the variability in responses between individual mice could obscure the detection of small differences.

Taken together, we conclude that TPPII is not required for MHC class I Ag presentation in vivo. This conclusion is consistent with recent papers using cultured cells (23) or TPPII KO mice (24) and adds to the weight of evidence against the model proposed by Reits et al. (16). This could in principle be because TPPII is not required for trimming precursors with long N-terminal extensions, and/or because such precursors are not generated very often in cells.

A substantial amount of trimming of Ag precursor peptides occurs in the cytosol (10). Although a number of cytosolic aminopeptidase (LAP, BH, PSA, and TPPII) can trim such precursors in cell extracts, their elimination from cells and in KO mice does not inhibit peptide trimming in vivo (Refs.25, 46, 47 and this paper). This raises the possibility that there may be other cytosolic aminopeptidases that are important to Ag presentation and remain to be identified. Alternatively, since the known cytoplasmic aminopeptidases generally have broad substrate specificity, the enzymes' function may be sufficiently redundant so that the lack of one peptidase could be readily compensated for by other ones. Crossing of several kinds of KO mouse strains will help to define the functional redundancy or a unique role of aminopeptidases.

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Disclosures

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