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Puromycin-Sensitive Aminopeptidase Limits MHC Class I Presentation in Dendritic Cells but Does Not Affect CD8 T Cell Responses during Viral Infections¹

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Previous experiments using enzyme inhibitors, cell lysates, and purified enzyme have suggested that puromycin-sensitive aminopeptidase (PSA) plays a role in creating and destroying MHC class I-presented peptides although its precise contribution to these processes is unknown. To examine the importance of this enzyme in MHC class I Ag presentation, we have generated PSA-deficient mice and cell lines from these animals. PSA-deficient mice are smaller and do not reproduce as well as wild type mice. In addition, dendritic cells from PSA-deficient mice display more MHC class I molecules on the cell surface, suggesting that PSA normally limits Ag presentation by destroying certain peptides in these key APCs. Surprisingly, MHC class I levels are not altered on other PSA-deficient cells and the processing and presentation of peptide precursors in PSA-deficient fibroblasts is normal. Moreover, PSA-deficient mice have normal numbers of T cells in the periphery, and respond as well as wild type mice to eight epitopes from three viruses. These data indicate that PSA may play a role in limiting MHC class I Ag presentation in dendritic cells in vivo but that it is not essential for generating most MHC class I-presented peptides or for stimulating CTL responses to several Ags. *The Journal of Immunology*, 2008, 180: 1704–1712.

Major histocompatibility complex class I (MHC-I)³ complexes provide a means whereby CTLs can survey intracellular events and assure that pathogens and aberrant cells are recognized and eliminated. The TCR on CTL transiently binds to the MHC-I/peptide complex, and the CTL is activated when an appropriate agonist peptide is presented. Peptides that bind MHC-I molecules are derived from intracellular proteins that are generally degraded by the ubiquitin/proteasome pathway. In vitro proteasomal degradation of proteins generates peptides that are roughly 3–22 aa long (1, 2). Most peptides are further hydrolyzed by peptidases to generate amino acids that can be recycled into protein synthesis (3).

From the vast array of peptides generated by the proteasome, a small fraction bind to the TAP complex and are transported into the endoplasmic reticulum (ER) (4). Once inside the ER, peptides that are the proper size and sequence may bind MHC-I molecules.

Peptides that can stably bind MHC-I molecules are generally 8, 9, or 10 residues in length. Peptides of this length constitute roughly 15% of proteasomal products (2). Another 15 to 25% of peptides produced by the proteasome are longer peptides that can be further trimmed to generate 8–10mers (2). Various aminopeptidases have been shown to be capable of removing extra N-terminal residues (5). Because cells lack carboxypeptidase activities, extra flanking residues at the C terminus must be removed by the proteasome in order for the peptide to be presented (4, 6, 7).

There is substantial evidence that peptide trimming can occur in the ER, after TAP transport. In the absence of the ER aminopeptidase ERAP1, N-extended peptides in the ER fail to be trimmed and CTL responses to several viral and self Ags are reduced (8–12). Because TAP does not translocate peptides with a Pro residue at P2,3 (which are anchor residues in many MHC-I alleles), these are translocated by TAP as N-terminally extended peptides and trimmed to the correct size by ERAP in the ER (10, 13). However, peptide trimming can also occur in the cytoplasm. Peptide precursors that are too long to be efficiently transported by TAP (14) can be trimmed in the cytosol and presented by MHC-I (7). This suggested that N-terminal trimming activities in the cytosol could help generate MHC class I peptides from proteasomal products. This was further suggested from observations that peptides with N-terminal extensions can still be trimmed and presented in ERAP1-deficient cells as long as TAP-transport is functional (8, 9, 11, 12, 15, 16), which suggests that there are processing activities in the cytosol that contribute to the MHC-I peptide pool and can compensate for the lack of ERAP1.

A number of cytosolic aminopeptidases have been implicated in trimming antigenic peptide precursors. Biochemical studies have shown that purified tripeptidyl peptidase II (TPPII), leucine aminopeptidase (LAP), puromycin-sensitive aminopeptidase (PSA), and bleomycin hydrolase (BH) can all trim MHC-I peptide precursors. We have recently reported that although purified LAP can trim antigenic peptides in vitro, LAP is not essential for presentation of most MHC-I

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³ Abbreviations used in this paper: MHC-I, MHC class I; ER, endoplasmic reticulum; TPPII, tripeptidyl peptidase II; LAP, leucine aminopeptidase; PSA, puromycin-sensitive aminopeptidase; BH, bleomycin hydrolase; VSV, vesicular stomatitis virus; KO, knock out; WT, wild type; SVNP, Sendai virus nucleoprotein; DC, dendritic cell.

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peptides, and is not required for the presentation of a number of viral epitopes to CTLs (16). In intact cells, TPPII plays an important role in trimming very long precursors but not shorter ones, which can be hydrolyzed by other aminopeptidases (17, 18). Therefore, an important question is which other cytosolic aminopeptidases are involved in trimming peptides for MHC-I presentation and whether this modifies the MHC-I peptidome.

PSA (EC 3.4.11.14), also known as MP100 (19) or NPEPPS (20), is a 99kDa metalloprotease that was initially identified in 1981 (21). It is a highly conserved protein, sharing 98% identity between human and mouse (22), and contains a housekeeping gene structure that results in stable gene expression throughout the cell cycle (22). Its expression is not altered in cells treated with IFN (IFN- γ) (23, 24) unlike LAP (25) or ERAP1 (13). The protein is highly abundant, and is mainly cytoplasmic, although one membrane-associated form has been identified in the brain (26, 27). It has been suggested to play a role in Alzheimer's disease progression by generating β -amyloid peptide (28), although this has been disputed (19). In addition, PSA has been suggested to be involved in neuropeptide degradation (29–31), but this has also been questioned because PSA is mainly a cytoplasmic protein (26), and because the expression and distribution of neuropeptides in the brains of PSA-gene-trapped mice appears to be normal (32). Mitotic spindle formation and cell cycle regulation may also be affected by the proteolytic activity of PSA (33). A recent publication has suggested that PSA is the main enzyme responsible for poly-glutamine stretches in intracellular proteins, such as huntingtin, which in a mutated form, has been linked to a number of neurodegenerative diseases (34). Despite this and other reports mentioned above, much of the physiological role of PSA in various biological processes is unclear.

Several biochemical studies have suggested that PSA could play a role in MHC-I peptide processing. Purified PSA and cytosolic extracts containing PSA can both generate MHC-I epitopes from the N-extended precursors of the vesicular stomatitis virus (VSV) nucleoprotein epitope (NP52-59) (24), and RU1 peptide precursors (VPYGSFKHV) (35). Conversely, PSA efficiently degrades some antigenic peptides in cell lysates (23), suggesting that it may inhibit Ag presentation in some cases. PSA is sensitive to the aminopeptidase inhibitor AAF-CMK, which was shown in living cells to inhibit the presentation of VSV NP52-59 from extended precursors (24). However, AAF-CMK has also been used to inhibit TPPII (36), and it may inhibit other aminopeptidases as well that prefer cleavage after aromatic residues.

PSA-deficient mice were previously generated by gene-trapping methodology (37). These mice exhibit dwarfism, infertility in both males and females, increased anxiety, and impaired pain responses (37–39). Their ability to present peptides on MHC-I was not evaluated and the mice were not available for such experiments. Moreover, because the gene-trap approach disrupts the reading frame but does not eliminate any part of the genomic sequence, these mice may still express a PSA-lac Z fusion protein and contain residual puromycin-sensitive aminopeptidase activity (37). In effort to address the importance of PSA in MHC-I peptide generation in intact cells and mice, we used gene targeting technology to generate a new line of PSA-deficient mice that completely lack PSA. We report that PSA-deficient dendritic cells (DC) express significantly higher surface levels of MHC-I. In contrast, PSA-deficient fibroblasts and other cells of hemopoietic origin express normal levels of MHC-I and present model Ags as well as control cells. In infected PSA-deficient mice, CTL responses to a variety of viral epitopes are not decreased. These findings indicate that PSA does not normally play a dominant role in the generation of MHC-I peptides, nor is it essential for mounting a response to viral

epitopes during viral infections. Instead, PSA may normally limit the pool of presented peptides, at least in certain cell types like splenic DCs. It also appears to play an important nonimmunological role in maintaining normal growth rates, and may be important in some neurological functions as well.

Materials and Methods

Plasmids, primers, and PCR

A three-primer PCR protocol was used to screen for PSA-deficient mice. mPSA-(R2) 5'-GGAGCATTGAATACCTTCCAGAG-3' lies downstream of the knock-out (KO) insert in the genomic sequence, and therefore amplifies both the wild type (WT) and disrupted alleles. mPSA-KO(F1) 5'-TCATTCTCAGTATTGTTTGGCC-3' lies within the KO allele, and amplifies only the disrupted allele. mPSA-WT(F3) 5'-GTTGCATAAGTTCTGAAGCTGAGTCTG-3' lies within the WT allele and amplifies only the WT allele. Real-time PCR analysis of LAP expression levels in mouse embryonic fibroblasts (MEFs) was performed using mLAP-RT(F3) 5'-CTTAACAGGTGCCATGGATGTAG-3' and mLAP-RT(R3) 5'-ACATCAGCAAGCTGGCAATC-3'. BH expression was quantitated by real-time PCR using mBH-702F 5'-GGAACCTGGTACACAGTGG-3' with mBH-820R 5'-CAGGTGAAGGTCTCTGGTGG-3'. ERAP expression was quantitated using mERAP1-2215F 5'-GAGGATGCTCAGGAGCAGCTC-3' and mERAP1-2358R 5'-AACACAGCCAAGGTCACATCA-3'. Expression levels for all aminopeptidases were normalized using β -actin with primers that have been previously described (40).

Construction of plasmids expressing SIINFEKL precursors fused to the C terminus of ubiquitin, followed by an internal ribosome entry site and GFP is described elsewhere (18). The SIINFEKL precursors expressed by the various plasmids are summarized in Fig. 5.

Real-time PCR

mRNA was extracted from 2×10^5 cells using RNeasy kits (Qiagen) according to the manufacturer's instructions. cDNA was made using Superscript II (Invitrogen Life Technologies) according to the manufacturer's instructions. Finally, real-time PCR was performed using 2X iQ SYBR Green supermix (Bio-Rad) on a MyCycler machine (Bio-Rad).

Generation of PSA^{-/-} mice

The mouse PSA (NPEPPS) gene (GeneID: 19155) was deleted using VelociGene technology (41). In brief, a large targeting vector (BACvec) was constructed by bacterial homologous recombination in which 18.1 kb of the PSA gene (Exons 3–9) were replaced by a lacZ-neo cassette. A $129 \times$ C57BL/6 F1 ES cell line was electroporated with the BACvec, and subjected to G418 selection. Drug-resistant clones were screened for loss of one copy of the LAP gene by quantitative PCR using probes at either end of the deletion. Two independent targeted ES cell clones were microinjected into C57BL/6 blastocysts, which were implanted in C57BL/6 females to generate chimeras. Chimeras were bred back to C57BL/6 to generate F₁ heterozygote mice. A line derived from one of these clones was used for subsequent experiments.

Virus infection of mice

Mice were injected i.p. with 5×10^4 pfu/mouse of LCMV Armstrong (a gift from Dr. Ray Welsh, University of Massachusetts Medical School, Worcester, MA), or with 5×10^6 pfu/mouse of recombinant vaccinia (provided by Drs. Jon Yewdell and Jack Bennink, National Institutes of Health, Bethesda, MD). Recombinant vaccinia viruses included one containing chicken OVA (42) and one encoding the immunodominant epitope (FAPGNYPAL) from Sendai virus nucleoprotein (SVNP). Mice were infected i.v. with 5×10^6 pfu/mouse of VSV (a gift from Dr. Ray Welsh). Eight days (LCMV) or 7 days (Vac-OVA, Vac-SVNP, Vac-ER-SVNP, and VSV) postinfection, splenocytes were harvested and incubated for 5 h with the appropriate peptide (5 μ M for LCMV, Vac-OVA, and Vac-SVNP. 2 μ M for VSV), or with anti-CD3 ϵ (BD Biosciences) in the presence of GolgiPlug (BD Biosciences). Vaccinia peptides that were used to stimulate IFN- γ production were B8R (TSYKFESV) (43), S8L from OVA (SIINFEKL), Sendai NP324–332 (FAPGNYPAL), and P10 (STLNFNNL) (44). LCMV peptides included gp33 + 34 (KAVYNFATC), gp276 (SGVENPGGYCL), NP205 (YTVKYPNL), and NP396 (FQPQNGQFI) (45, 46). The VSV peptide (RGYVYQGL) was also used. All peptides were synthesized (Anaspec). Cells were then stained for CD8, CD44, and intracellular IFN- γ using commercial Abs (BD Biosciences), and analyzed by flow cytometry. Experiments involving injection of mice with poly-I:C were performed as previously described (16).

Cells and tissue culture

MEFs were generated from 12 to 14 day embryos and cultured at 37°C and 10% CO₂ in DMEM plus 20% FCS as described in Current Protocols. In brief, timed pregnancies were conducted by housing a PSA^{+/-} male with 2–3 PSA^{+/-} females for 2 days, after which, the males were removed, and the pregnant females were sacrificed 12 days later. Embryos were removed under sterile conditions and processed separately. Dark tissue (internal organs) and heads were removed, and the remaining tissue was homogenized by mincing. The minced tissue was incubated in 0.25% trypsin (Life Technologies) for 10 min at 37°C, vortexing vigorously every 5 min. The homogenized tissue and trypsin from each embryo was added to 20 ml of DMEM plus 20% FCS and incubated overnight in a T75 flask (Corning-Costar). When the cells grew to confluency, a fraction of cells were removed from each flask for the purposes of genotyping each MEF line. Cells were cultured in flasks, and transfections were performed in 6-well plates (Corning-Costar). For specific experiments, cells were incubated in the media described above with 50 U/ml murine IFN- γ (BD Biosciences).

Abs and flow cytometry

The mAb 25.D1.16 (anti-K^b+SIINFEKL) (47), AF6–88.5 (anti-K^b) (48), Y3 (anti-K^b) (49), M1/42 (anti-H2) (50), or H36.4.5 (anti-influenza HA) (a gift of W. Gerhard, The Wistar Institute, University of Pennsylvania, Philadelphia, PA) were used as primary Abs in staining MEFs for flow cytometry. After incubation in one of the primary Abs, the cells were washed with PBS and stained with donkey anti-mouse (or donkey anti-rat) F(ab')₂ fragments conjugated to Cy5 (Jackson ImmunoResearch). For staining cells isolated from spleen, AF6–88.5 (H-2K^b), KH95 (H-2D^b), AF6–120.1 (I-Ab), and 53–6.7 (CD8) Abs conjugated to a fluorophore were used according to the manufacturer's directions (BD Biosciences), as were PO3.1 (CD86) N418 (CD11c), RM4–5 (CD4), and RA3–6B2 (B220) Abs (eBioscience). The cells were then analyzed by flow cytometry on a FACSCalibur apparatus (Becton Dickinson) with FlowJo software (Tree Star).

Peptide injection and fluorescence measurement

Peptides were injected into cells and their half-life measured as previously described (4). The synthesis and sequences of the internally quenched peptides have been previously described in detail (17). Analysis of peptide degradation rates was performed as previously described (4).

Transfections

MEFs were transfected with various constructs using Fugene 6 (Roche) according to the manufacturer's instructions. To express N-extended SIINFEKL precursors, we constructed plasmids consisting of ubiquitin with SIINFEKL precursors fused to the C terminus. C-terminal ubiquitin hydrolases efficiently release peptides thus fused to ubiquitin (51). An internal ribosome entry site downstream of the ubiquitin-SIINFEKL fusion followed by GFP, was used for determining comparable levels of plasmid expression in cells.

Results

Generation of PSA-deficient mice

PSA-deficient mice were generated using VelociGene technology (41) resulting in a loss of exons 4–9 of the PSA genomic sequence (Fig. 1A). In total, ~18.1 kb was deleted. Presence of the neogene and loss of the PSA exons 4–9 was confirmed by PCR (Fig. 1B). This deletion includes exon 9, which encodes half of the enzymatic active site. The other half of the active site is encoded in exon 10, and no mRNA transcripts encoding exons 10 and 11 were detectable by real-time PCR (data not shown), suggesting that PSA mRNA transcripts in these mice do not contain exons after the neogene insertion. PSA deficiency did not result in a compensatory increase in the transcription of several other aminopeptidases (LAP, BH, or ERAP1), as determined by real-time PCR (data not shown).

The PSA-deficient mice were bred to homozygosity and were viable. We found some similarities as well as some differences in comparing our mice to the PSA-gene trapped mice that have been previously reported. Presumably these differences are due to the different gene disruption technologies (resulting in complete gene disruption vs fusion protein) or the different genetic backgrounds of the animals, because the gene-trapped mice are on the Balb/C background.

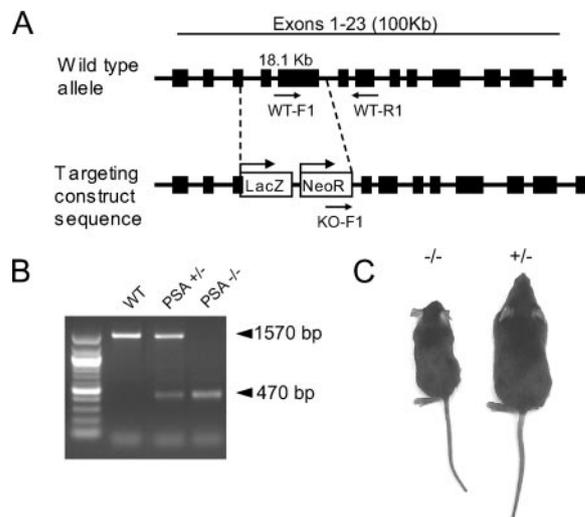


FIGURE 1. Generation of PSA-deficient mice. *A*, Genomic organization of the mouse PSA gene (upper) and structure of the targeting vector (lower). Exons 1–23 are shown as boxes with the coding regions in black. Exons 4–9 (a total of 18.1 kb) were deleted by homologous recombination. The active site of PSA is located in exons 9 and 10. The locations of the primer sequences used for PCR genotype analysis are shown with arrows. *B*, PCR genotype analysis of WT, heterozygote, and PSA-deficient littermates. Amplification of the WT allele results in a 1570 bp fragment, whereas the disrupted allele produced a 470 bp fragment. *C*, Comparison of PSA^{-/-} and PSA^{+/-} littermates at 4 wks of age.

The gene-trapped mice were reported to be infertile (38, 39). In contrast, our PSA-deficient mice can reproduce, but not at normal levels. The average litter size from homozygous KO breeding pairs was 3.27, whereas heterozygous breeding pairs had an average of 6.28 pups per litter. A total of 14.1% of the pups delivered by heterozygous breeding pairs were PSA-deficient, which would suggest that PSA-deficient mice are less viable as embryos. There was no significant mortality of pups between birth and weaning. Like the gene-trapped animals, PSA-deficient mice are smaller than controls at weaning and into adulthood (Fig. 1C), suggesting that PSA is required for normal growth.

The gene-trapped mice were reported to have some neurological abnormalities including reduced locomotor activity and behaviors associated with increased anxiety. Our PSA-deficient mice show grossly similar abnormalities. In contrast to the gene-trapped mice, which were reported to have no abnormalities in gross movement, we have noted that a number of the PSA-deficient mice develop a movement disorder of their hind limbs that affects their gait. This occurred with variable penetrance. These findings are of interest especially because PSA has been implicated in the degradation of poly-Q sequences that accumulate in certain neurological diseases (34). However, because this study is focusing on the role of PSA in Ag presentation and immune responses, we have not further analyzed the neurological phenotype.

DCs express more MHC-I in PSA-deficient mice

Elimination of PSA could in principle reduce peptide supply to MHC-I (if PSA predominately trims long peptides to mature epitopes), or increase peptide supply (if PSA preferentially degrades peptides to sizes smaller than the 8–10 aa in length needed to bind to MHC-I). MHC-I molecules must bind peptides to be released from the ER and therefore peptide supply is an important factor that influences the level of MHC-I molecules on the cell surface. We therefore evaluated the expression of MHC-I on lymphocytes in the PSA-deficient animals. Spleens and lymph nodes

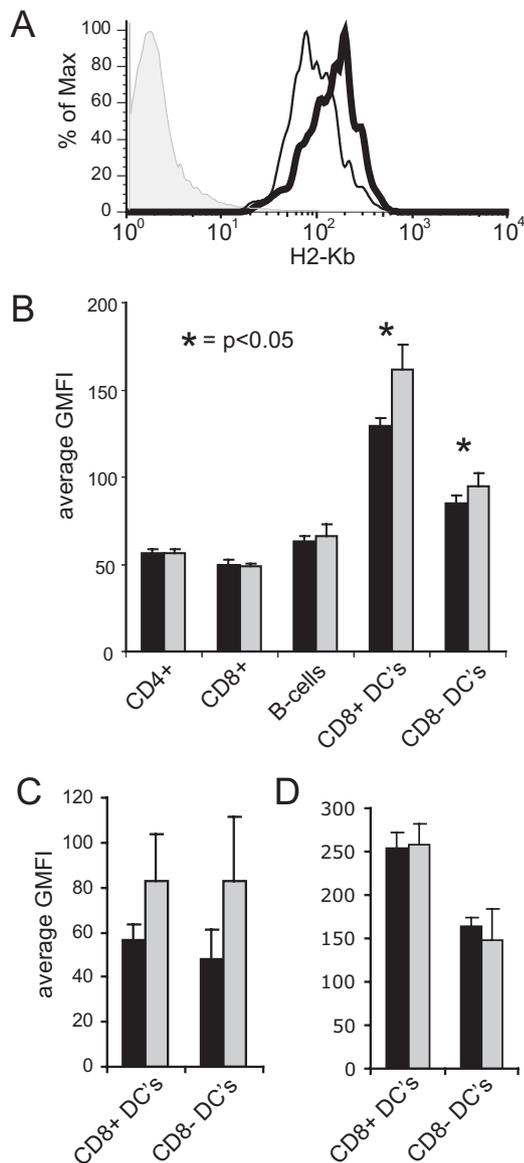


FIGURE 2. MHC-I expression on splenocytes from PSA^{-/-} and WT mice. Splenocytes were stained with appropriate Abs and analyzed by FACS. *A*, Histogram representing the difference in H-2K^b levels between WT (thin black line) and PSA-deficient (thick black line) CD11c⁺ DCs. *B*, CD4⁺ cells were identified based on a gate for B220⁻, CD8⁻, and CD4⁺ events. CD8⁺ cells were identified based on a gate for B220⁻, CD4⁺ and CD8⁺ events. B-cells were identified based on a gate for B220⁺, CD4⁻, CD8⁻ events. DCs were identified based on a gate for CD11c⁺ high cells (*C*) H-2D^b levels and (*D*) MHC class II (I-Ab) on CD8⁻ and CD8⁺ DCs, stained as described above (Black bars = WT, and gray bars = PSA^{-/-}). Student's *t* test assuming unequal variances was used to determine statistical significance (*, *p* < 0.05). Data represent five WT mice and three PSA^{-/-} mice in *A* and *B*. Data represent three WT mice and three PSA^{-/-} mice in *C* and *D*. Error bars in *B–D* represent SD within each genotype. All data are representative of three independent experiments.

were harvested from sex-matched PSA-deficient and WT control mice. After lysis of red-blood cells, splenocytes were counted and stained for MHC-I and a variety of other cell markers. There were no significant differences in surface levels of MHC-I on CD8⁺ T cells, CD4⁺ T cells, or B-cells (B220⁺, CD4⁻ and CD8⁻) isolated from spleen and lymph nodes of PSA^{-/-} and control mice (Fig. 2*B*). There was also no significant difference in MHC class I expression between these cells isolated from WT and PSA-deficient

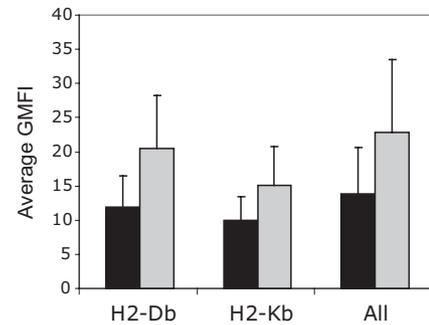


FIGURE 3. Characterization of PSA^{-/-} MEFs. Four independent lines from each genotype were stained for MHC-I with B8.14.8S (H-2D^b), Y3 (H-2K^b), or M142 (all) and analyzed by FACS. Bars represent the average Geometric Mean Fluorescence of the independent MEF lines (Black bars = WT, and gray bars = PSA^{-/-}). Error bars represent the SD within each group. Data are representative of at least three independent experiments.

mice treated with poly I:C, a potent type I IFN-inducer that increases MHC-I expression (data not shown). However, splenic CD11c⁺ DCs from PSA^{-/-} mice remarkably expressed up to 25% more MHC-I than WT cells (Fig. 2, *A–C*). This is based on gating on CD11c^{high} population in splenic cells, followed by separation of CD11c^{high} cells into CD8⁺ and CD8⁻ cells. The increase in MHC-I was reproducible and statistically significant (*p* < 0.05). In contrast, the expression of MHC class II molecules was equivalent on cells from control and PSA-deficient mice, including on DCs (Fig. 2*D*). Class I levels were not significantly increased on DCs grown in GM-CSF or on FLT3-ligand expanded cells (data not shown), suggesting that PSA deficiency might selectively affect DCs only in certain states.

To further extend this analysis to cells of nonhemopoietic origin, we generated multiple independent embryonic fibroblast (MEF) cell lines from PSA-deficient or control embryos as previously described (16). We similarly analyzed the expression of MHC-I molecules in these cells and found that presentation of H-2K^b, H-2D^b, and total MHC-I showed no statistically significant difference between the multiple independent PSA-deficient and WT MEFs (Fig. 3). Similarly, after stimulation by incubation with the IFN- γ , there was also no difference in MHC-I expression on these WT and PSA-deficient cells (data not shown). This result was similar to what was observed with T and B cells from spleen. However, there was variability in the levels of MHC-I between different independent cell lines and we cannot rule out that this might have obscured small but real differences in class I expression between the PSA⁺ and PSA⁻ cells (e.g., of the magnitude observed with DCs). In any case, these results clearly demonstrate that PSA is not essential for the generation of MHC-I-presented peptides in MEFs and conversely does not destroy enough peptides to detectably limit overall peptide supply.

The CD8⁺ T cell response to viral epitopes is not altered in PSA^{-/-} mice

To more specifically examine effects of PSA KO on presentation of MHC-I peptides and CD8 T cell responses, we analyzed the response of PSA^{-/-} mice to various viral infections.

Mice were infected i.p. with LCMV and analyzed 8 days later by harvesting spleens and incubating the splenocytes with gp33 + 34 (KAVYNFATC); gp276 (SGVENPGGYCL), which binds D^b; NP205 (YTVKYPNL), which binds K^b; or the D^b-binding NP396 (FQPQNGQFI) peptide in the presence of brefeldin A for 5 h. As a result, T cells that have previously been primed by their cognate Ag on MHC-I will produce IFN- γ . Cells that were not incubated

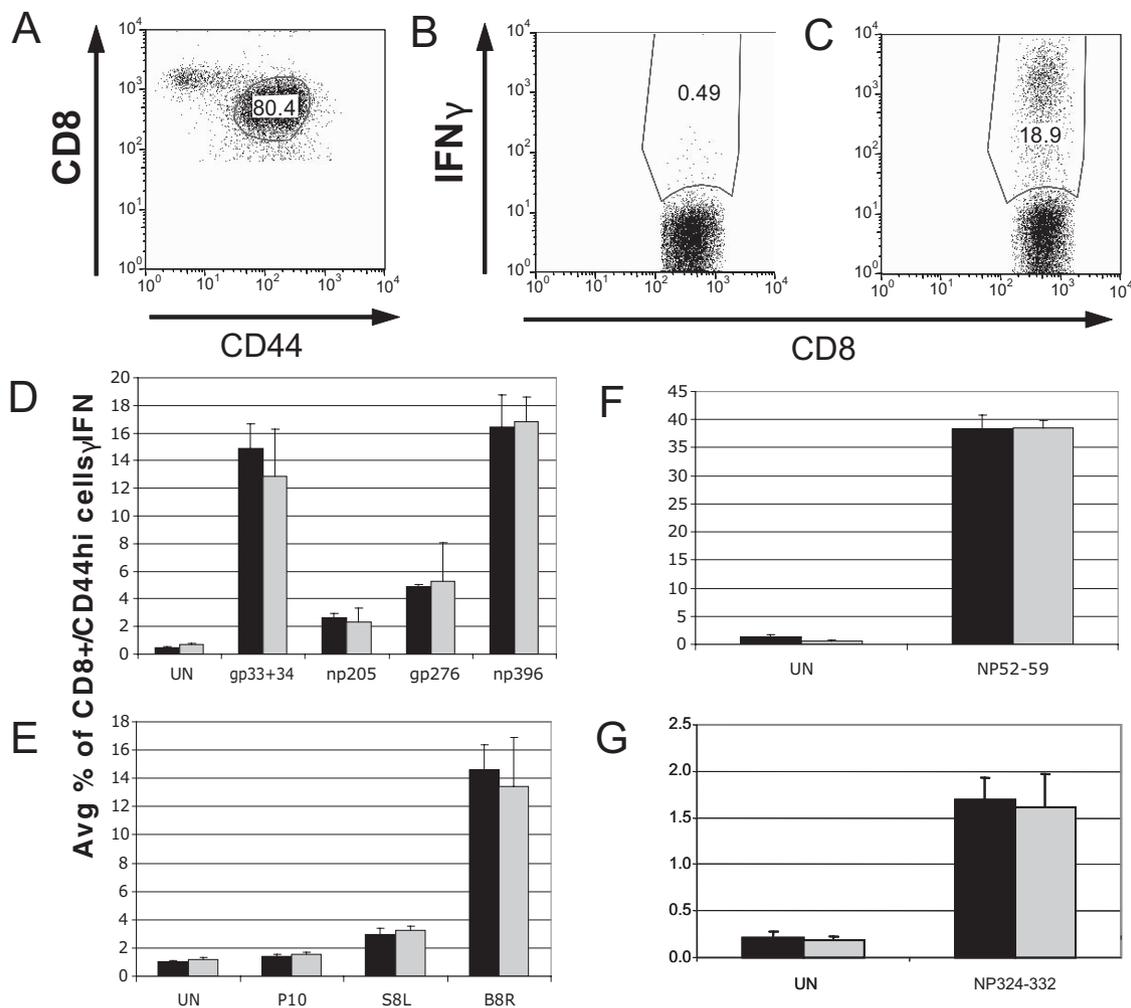


FIGURE 4. Intracellular IFN- γ staining of peptide-specific T cells. Spleen cells from PSA^{-/-} and WT mice infected with virus were harvested and stimulated in vitro with appropriate peptide for 5 h. Cells were then stained and analyzed by flow cytometry by gating on (A) CD8⁺, CD44^{high} cells. Cells were further gated according to IFN- γ expression, as in B (unstimulated cells) and C (cells stimulated with anti-CD3 Ab). D–G depict average percentages of IFN- γ ⁺ cells that respond to viral peptides (D) 8 days after infection with LCMV, (E) 7 days after infection with SIINFEKL-recombinant vaccinia, (F) 7 days after VSV infection, or (G) 7 days after infection with Sendai NP-recombinant vaccinia. Black bars = WT mice, and gray bars = PSA^{-/-} mice. Error bars represent the SD within each group ($n = 5$). Each graph (D–G) represents at least two independent experiments. There was no significant difference between PSA^{-/-} and WT mice in their response to any of the nine epitopes tested.

with peptide did not produce IFN- γ (Fig. 4B). Thus, we are able to measure how PSA affects the processing (and subsequent presentation) of MHC-I peptides from viral proteins. The percentage of CTLs that responded to each of the tested LCMV epitopes was equivalent between PSA-deficient and WT animals, suggesting that PSA is not essential for maintaining the well documented immunodominance hierarchies of these epitopes (Fig. 4D). It also indicates that PSA-deficient T cell repertoire is not skewed through thymic positive selection, at least for LCMV-specific cells.

To expand the number of epitopes that we examined, infections with recombinant vaccinia (encoding OVA) were performed via i.v. injection. After 7 days, splenocytes were treated as before and were incubated with B8R (TSYKFESV), which is K^b restricted (43); P10 (STLNFNLL) (44); or SIINFEKL peptide (derived from the recombinant OVA protein). In contrast to suggestions that PSA was important for the presentation of SIINFEKL from OVA protein (24), the observation that PSA-deficient mice can mount a normal response to SIINFEKL peptide implies that PSA is not essential for recognition of this peptide by CTLs (Fig. 4E).

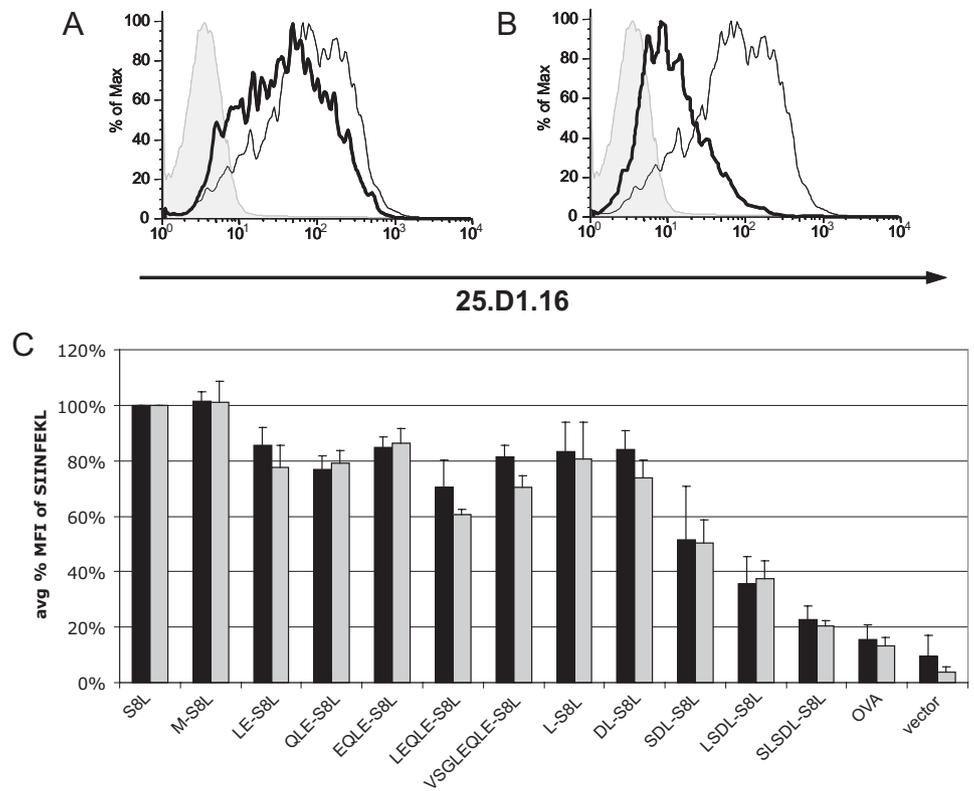
Because PSA was previously shown to be important for the generation and presentation of the VSV-NP peptide (RGYVYQGL)

(24), VSV was introduced into mice by i.v. injection. After 7 days, the T cell response was analyzed by incubation with the VSV-NP peptide followed by flow cytometry. CTLs from PSA-deficient and WT controls responded equally well to this peptide suggesting that PSA is not required for this epitope to be presented in vivo (Fig. 4F).

The SVNP epitope has also been suggested to be a substrate for PSA degradation that destroys this epitope in vitro and might limit Ag presentation (23). Therefore, presentation of this epitope (FAPGNYPAL) to CTLs from PSA-deficient and WT mice was compared after a 7-day infection. The IFN- γ production from WT and PSA-deficient CTLs was comparable, suggesting that PSA does not limit the presentation of this particular peptide to CTL during a viral infection in vivo (Fig. 4G).

PSA-deficient mice mounted comparable CTL responses to all the viral epitopes tested (a total of nine peptides), including the VSV-NP epitope and the SVNP epitope, which have been reported to be substrates for PSA (23, 24). These data indicate that PSA is not required for presentation of these viral peptides in vivo. Moreover, because the CTL responses were not increased in PSA-deficient mice, peptide destruction by PSA does not detectably reduce responses to

FIGURE 5. SIINFEKL presentation in PSA^{-/-} MEFs from peptide precursors. *A*, MEFs were transfected with pUG-SIINFEKL (thin black line), pUG-LEQLESIINFEKL (thick black line), or with empty vector alone (gray filled curve) and then stained with 25.D1.16 48 h later. Cells were gated based on similar levels of GFP expression. *B* compares presentation of SIINFEKL in cells transfected with the SIINFEKL construct (thin black line), the full-length OVA construct (thick black line), or vector (gray filled) after 48 h. *C* represents the average MFI of three independent lines of WT (black bars) and PSA-deficient (gray bars) MEFs that were transfected with the indicated construct as in *A* and *B*. Data are expressed as an average percentage of the MFI of the SIINFEKL construct. Error bars represent the SD between three different MEF lines per genotype. Data are representative of at least three independent experiments.



any of the epitopes tested. Therefore, although DCs have increased MHC-I levels (possibly because fewer peptides are being destroyed in the absence of PSA) this does not affect the ability of PSA^{-/-} mice to mount a normal CTL response to many viral epitopes.

PSA^{-/-} MEFs present peptide as well as WT MEFs

In contrast to primary DCs, B cells, and T cells, MEFs are readily transfectable and can be used to assay the presentation of different transfected antigenic constructs. To more closely examine the processing of specific peptide precursors in PSA^{-/-} cells, MEFs were transfected with a series of constructs encoding N-extended SIINFEKL precursors fused to the C terminus of ubiquitin (Fig. 5). When these constructs are translated in cells, ubiquitin C-terminal hydrolases cleave the C-terminal ubiquitin moiety and thereby generate the SIINFEKL precursor in the cytosol (51, 52). To be presented on MHC-I molecules, the resulting N-terminally extended SIINFEKL peptides must be trimmed by aminopeptidases.

The amino acid sequence that naturally appears upstream of SIINFEKL in OVA was used because it is well characterized and is known to be trimmed in live cells (7). Although the level of SIINFEKL presentation by MEFs varied from one ubiquitin construct to another (presumably due to the length or sequence of the precursor), the level of presentation by PSA-deficient and WT MEFs was similar, indicating that PSA is not required for efficient trimming of V, S, G, L, E, M, or Q (Fig. 5). PSA is also not required for trimming of SIINFEKL precursors ranging from 9 to 16 residues in length (Fig. 5). In addition, presentation of SIINFEKL from full length OVA was the same between the WT and KO cells (Fig. 5), suggesting that PSA is not involved or not critical for trimming N-extended peptide precursors generated by the proteasome.

To examine a sequence that has previously been shown to be trimmed by PSA in cell lysates (24), the sequence upstream of the VSV nucleoprotein epitope was used to replace the N-terminal sequence of SIINFEKL in the ubiquitin constructs (Fig. 5). Pre-

sentation from these VSV N-terminal constructs were the same between PSA^{-/-} and WT MEFs suggesting that other aminopeptidases can trim this sequence to generate SIINFEKL peptide (Fig. 5). However, there was a significant drop in SIINFEKL presentation when it was preceded by the VSV sequence as compared with the natural flanking sequence. This difference continued to increase as the N-terminal flanks were increased in size up to five residues (SLSDDL- vs LEQLE-), but was not due to PSA activity.

Lack of PSA has no effect on the rate of peptide trimming in cells

MHC-I surface expression is an indirect measure of the generation of antigenic peptides. It is possible that a contribution of PSA to this process could be missed if the trimming of peptides is not a

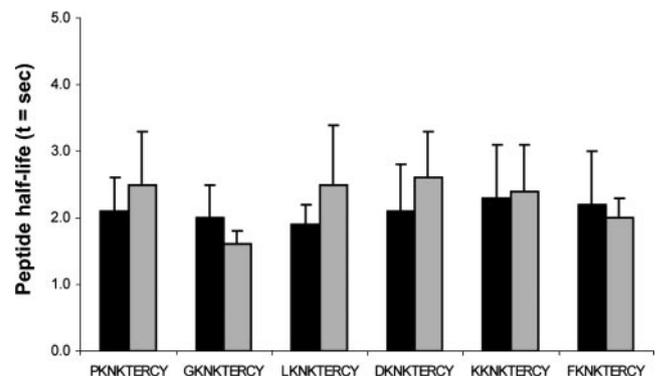


FIGURE 6. Half-life of microinjected peptides in PSA^{-/-} and WT MEFs. Peptides with different residues in the P1 position were microinjected into PSA^{-/-} or WT control MEFs. Half-life of peptide was determined by following the generation of fluorescence signal. Statistical significance was determined by Student's *t* test (two-tailed distribution assuming equal variances). No difference in half-life was detected in any of the tested peptides between the two cell lines.

rate-limiting step in the pathway. We therefore directly measured the rate of peptide degradation in control, and PSA-deficient cells by microinjection of peptides containing fluorescein and quencher adducts (4, 17). These substrates generate a fluorescent signal when aminopeptidases cleave and thereby separate the residues containing the fluorophore and quencher moieties. Peptides with different amino acids in the P1 position were microinjected into cells, and the fluorescence resulting from trimming the peptides was measured.

Although there was some variation in the rate of trimming of different peptide sequences, there was no statistically significant difference in the half-life of any of the tested peptides between the two cell lines (Fig. 6). These data demonstrate that PSA activity is not rate limiting for the trimming of these peptides in the cytosol, and that PSA is not essential for trimming P, K, or F *in vivo*, in addition to the other amino acids examined in previous assays.

Discussion

In living cells, proteasomes can generate peptides that are either too long or too short to bind to MHC-I molecules, as well as peptides that are the proper length for binding. The proteasome must generate the correct carboxy-terminus of the MHC-I peptide, but when a proteasome-derived peptide has extra residues at the amino terminus, aminopeptidases can remove N-terminal residues from the precursor to generate MHC-I-binding peptides (4, 6, 7). Because most aminopeptidases do not have a molecular ruler mechanism that tells them when to stop trimming, they can also destroy antigenic peptides by trimming them below the minimal size needed for binding to MHC-I molecules (24, 53). Consequently, >99% of peptides generated in the cytosol are degraded before they contact TAP (4). Some of the cytosolic aminopeptidases that perform these functions in cells have been tentatively identified. However, the precise role of many of these aminopeptidases in these processes has not yet been determined.

Several lines of evidence have implicated PSA as an aminopeptidase that is important for trimming antigenic peptides and peptide precursors during the process of Ag presentation. First, PSA and BH were identified as being important in trimming the VSV-NP epitope in cell lysates cleared of proteasomes by ultracentrifugation (24). Second, presentation to CTL of the VSV-NP epitope and SIINFEKL from OVA by living cells was inhibited by incubation of the cells with the aminopeptidase inhibitor AAF-CMK (24). Third, an analysis of cell lysates and purified enzymes suggested that PSA degrades the Sendai NP epitope faster than any other peptidase (23). Fourth, incubating purified recombinant PSA or proteasome-depleted cytosolic extracts with AAF-CMK or puromycin was shown to prevent the generation of the antigenic RU1 peptide (VPYGSFKHV) from N-extended peptide precursors, suggesting that PSA is involved in the generation of this peptide (35). Fifth, inhibition of PSA in cell extracts with puromycin can limit the trimming of some, but not all peptides (23), indicating that PSA may preferentially trim/degrade some peptide sequences. Together, these data implicated PSA in both destructive and constructive processes in MHC-I peptide generation. However, the inhibitors used in many of these studies were not monospecific for PSA and it was not certain whether the results from cellular extracts accurately model the *in vivo* situation.

The generation of mice genetically deficient in PSA allowed a definitive examination of the role of this peptidase in cells and animals. Interestingly, we did find an Ag presentation phenotype in the PSA-deficient mice. This was a highly reproducible and statistically significant increase in MHC-I levels on splenic DCs. Although a similar trend was observed in bone marrow-derived or FLT3-ligand expanded DCs, it was not statistically significant

(data not shown). This result indicates that PSA normally limits MHC-I levels on splenic DCs. A reduction in MHC-I molecules would be expected if PSA is involved in the net destruction of peptides resulting in a reduction in overall peptide supply. Such a phenotype would be consistent with earlier findings that PSA was the major peptidase that destroyed certain peptides in cell extracts. Although this is presumably the basis for the observed phenotype, it is difficult to prove because the magnitude of the increase in MHC-I expression in the PSA^{-/-} DCs is small.

Why are class I levels increased on PSA^{-/-} DC but not several other cell types? This might be because DCs are the cell type with the highest expression of MHC-I molecules and may therefore also be the cells in which the normal pool of peptides available for class I is most limiting. If PSA deletion slightly increases the intracellular peptide pool, it may be detected only in these cells and not in cells where peptide supply is less limiting for MHC-I loading and expression. Alternatively, perhaps PSA is more important in DC than in other cell types because of differences in the composition of proteases in different cells. Interestingly, the increase in MHC-I on DCs did not result in enhanced CD8 T cell responses to viral epitopes, which might suggest that the normal levels of MHC-I (or those induced during viral infection) was not rate limiting for responses, although it is possible that the specific responses we measured were to peptides not affected by the absence of PSA.

We did not find any evidence that PSA was involved in destroying peptides in other cell types. In MEFs, there was no increase in the presentation of SIINFEKL initially generated in the cytosol as an N-extended precursor or mature epitope. Moreover, *in vivo* CD8 T cell responses to nine different epitopes were not increased in PSA-deficient mice. Importantly, this latter analysis measured CTL responses to epitopes (RGYVYQGL and FAPGNYPAL) previously shown to be destroyed in cell lysates (albeit human) by PSA *in vitro*. We have previously shown that these *in vivo* assays can detect an increase in peptide supply in certain peptidase-deficient mice (12). Furthermore, the half-life of several peptides in the cytosol of PSA-deficient cells was directly measured and was not increased. In addition, MHC-I levels were not increased on T cells, B cells, or fibroblasts.

Surprisingly, we also found no evidence that PSA played a role in generating MHC-I-presented peptides. Overall levels of MHC-I were not decreased on any of the cells examined from PSA-deficient mice. In addition, the generation of an MHC-I-presented epitope from N-extended SIINFEKL precursors was not reduced in PSA-deficient fibroblasts. Similarly, there was also no reduction in the presentation of SIINFEKL constructs containing N-flanking residues from VSV that were previously shown to be preferentially trimmed by PSA. Although there was a clear difference in the presentation of SIINFEKL precursors preceded by VSV or natural OVA sequences, this difference was not affected by the presence or absence of PSA. It is likely to be due to the sequence of the flanking residues rather than the length because peptides of equal lengths generated different levels of SIINFEKL peptide.

We cannot exclude the possibility that PSA is critical for generating a subset of antigenic peptides that might differ in sequence from those examined herein. However, if that is the case, such peptides would presumably represent only a small subset of total peptides because overall levels of surface MHC-I (which are dependent on peptides) were not decreased on PSA-deficient cells.

These phenotypes are in contrast to what occurs in the absence of ERAP1, which causes a reduction of 10–25% in the surface levels of most MHC-I alleles (8, 9, 12). Such data suggest that ERAP1 is important for MHC-I peptide generation, but do not suggest that cytosolic enzymes are not contributing to this process.

There is strong evidence that precursor peptides are trimmed in the cytosol of cells. Some peptides require trimming to be transported by TAP into the ER (7), and we have recently reported that TPPII is important for the trimming of longer proteasomal products in living cells (18). Because these longer peptides are less efficiently transported by TAP, they are likely trimmed before transport, suggesting that cytosolic aminopeptidase activities are important for the processing of at least a subset of MHC-I peptides. Moreover, N-extended precursors generated in the cytosol but not ER are trimmed in cells lacking ERAPI (11).

If PSA is not essential to this process, what other aminopeptidase(s) carries out this function? It is possible that a single cytosolic aminopeptidase (such as TPPII) is responsible for peptide trimming. The finding that certain protease inhibitors can block Ag presentation might be consistent with this hypothesis, however the agents that have been used thus far are not highly specific and have been used by numerous groups to inhibit various aminopeptidases. Alternatively, it seems more likely that several cytosolic aminopeptidases can carry out this function. In this scenario, loss of a single peptidase might not impair the trimming process. This would explain why single peptidases such as PSA and LAP can trim or destroy peptides *in vitro* but their individual loss from mice does not dramatically affect peptide trimming. It is also possible that the loss of a single aminopeptidase such as PSA causes a compensatory increase in other peptidases; however, we have been unable to detect such changes in PSA-deficient mice, at least for the peptidases that we have measured. To try and examine the potential redundancy between PSA and LAP, we have attempted to breed double-deficient mice. However, we have been unable to generate viable offspring. This latter finding clearly indicates that on some level PSA and LAP must serve redundant functions.

Aminopeptidases are thought to play a role in a number of cellular functions (e.g., recycling proteasomal products to amino acids), and PSA has specifically been proposed to participate in a number of nonimmune functions such as neuropeptide degradation, β -amyloid precursor processing, reproduction, mental anxiety, cell cycle progression, and pain response. Although not a primary focus of this investigation, we find that PSA-deficient mice do have a number of nonimmune phenotypes. Some of these, such as dwarfism and altered behavior, have been previously reported in a PSA gene-trap mouse. However, we also observed some differences in the phenotype of our PSA-deficient mouse with the phenotypes reported for the PSA gene trap mouse. Specifically, the gene trap mouse was infertile while our PSA-deficient mouse can reproduce, albeit at reduced rates. Our mice delivered litters regularly when female KO mice were mated with WT or heterozygous males. PSA^{-/-} males were also capable of breeding although these breeding pairs produced litters less frequently. We have also observed a movement disorder developing in some of the mice that was not described in the gene trap mice. Future studies will use the PSA-deficient mice to explore the role of PSA in these nonimmune functions.

In summary, PSA is an important aminopeptidase for a number of physiological processes, but is not essential. PSA-deficient mice are viable, reproduce, can mount normal T cell responses to a number of viral epitopes during viral infections, and have normal amounts of lymphocytes. This demonstrates that although PSA has been shown *in vitro* to be an important enzyme for peptide trimming, other proteases may compensate for its loss in this role *in vivo*.

Disclosures

The authors have no financial conflict of interest.

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