Asparagine Endopeptidase Is Not Essential for Class II MHC Antigen Presentation but Is Required for Processing of Cathepsin L in Mice[1]

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Class II MHC molecules survey the endocytic compartments of APCs and present antigenic peptides to CD4 T cells. In this context, lysosomal proteases are essential not only for the generation of antigenic peptides but also for proteolysis of the invariant chain to allow the maturation of class II MHC molecules. Recent studies with protease inhibitors have implicated the asparagine endopeptidase (AEP) in class II MHC-restricted Ag presentation. We now report that AEP-deficient mice show no differences in processing of the invariant chain or maturation of class II MHC products compared with wild-type mice. In the absence of AEP, presentation to primary T cells of OVA and myelin oligodendrocyte glycoprotein, two Ags that contain asparagine residues within or in proximity to the relevant epitopes was unimpaired. Cathepsin (Cat) L, a lysosomal cysteine protease essential for the development to CD4 and NK T cells, fails to be processed into its mature two-chain form in AEP-deficient cells. Despite this, the numbers of CD4 and NK T cells are normal, showing that the single-chain form of Cat L is sufficient for its function in vivo. We conclude that AEP is essential for processing of Cat L but not for class II MHC-restricted Ag presentation. *The Journal of Immunology,* **2005, 174: 7066–7074.**

Through display of antigenic peptides, class II MHC products are involved in T cell recognition, not only to allow elaboration of a functional CD4 T cell repertoire, but also for the initiation and effector phases of a CD4 T cell response. Ag presentation by class II MHC molecules is controlled by proteolysis minimally at three levels. First, the trafficking and maturation of class II MHC products involves a chaperone, the invariant chain (Ii)[3], whose destruction by proteolysis is essential to liberate the peptide-binding pocket of the class II MHC products. Second, the generation of antigenic peptides requires proteolysis of the polypeptides that contain the requisite peptide epitope(s). By the same logic, presentation of peptide epitopes is prevented if proteolysis destroys these peptides before interaction with class II MHC products. Third, the maturation of lysosomal proteases involved in Ag presentation from their inactive zymogens into active proteases requires proteolysis.

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[3] Abbreviations used in this paper: Ii, invariant chain; CLIP, class II Ii peptide; Cat, cathepsin; AEP, asparagine endopeptidase; BMDC, bone marrow-derived dendritic cell; MBP, myelin basic protein; OT-II, OVA-specific TCR; MOG, myelin oligodendrocyte glycoprotein; AEPi, AEP inhibitor; AMC, 7-amido-4-methyl coumarin; LAMP-1, lysosome-associated membrane protein 1.

Studies with protease inhibitors and protease-deficient mice have demonstrated that the degradation of Ii occurs in stepwise fashion (reviewed in Refs. 1–3). Studies with broad spectrum cysteine protease inhibitors such as leupeptin suggested the involvement of both cysteine and noncysteine proteases (4–6). The final cleavage of Ii generates the class II Ii peptide (CLIP) bound to class II MHC molecules from the larger Ii precursor, p10, in a reaction catalyzed by cysteine proteases of the cathepsin (Cat) family. The proteases involved can be cell type specific. For example, Cat S mediates the final step in the conversion of Ii in B cells and dendritic cells (7–10). In Cat L-deficient mice, this cleavage is impaired in cortical thymic epithelial cells (11). Cat F can compensate for loss of Cat L and Cat S in macrophages (12). The initial proteolytic steps in the degradation of Ii are less well understood. Asparagine (Asn) endopeptidase (AEP) has been considered an attractive candidate because a peptide-based inhibitor of AEP delayed early events in degradation of Ii in human B cell lines and monocyte-derived dendritic cells (13). Furthermore, the expression of AEP has been reported in other APCs, such as murine B cell lines, splenocytes, murine thymic dendritic cells, and bone marrow-derived dendritic cells (BMDCs) (14–16).

AEP, also called mammalian legumain, is a cysteine protease that hydrolyzes substrates C-terminal of Asn residues (17, 18). It shares homology with a family of proteases that includes caspases and separase (19) but not with the papain-fold lysosomal proteases. AEP is insensitive to leupeptin and E-64 (20, 21) and therefore may be responsible for Ii intermediates generated in the presence of leupeptin (3, 13). In addition to its role in Ii processing, AEP may be involved in proteolysis of Ags relevant for class II MHC presentation. For example, degradation of tetanus toxin requires AEP (21), whereas generation of the myelin basic protein (MBP) immunodominant epitope, potentially presented by HLA-DR2, is destroyed by AEP (14). These experiments primarily used purified AEP to attack the respective purified Ags in vitro or relied on

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peptide-based inhibitors to block AEP activity. The demonstration that a purified enzyme can generate class II MHC-restricted epitopes or cleave Ii in vitro cannot simply be extended to its involvement in these processes in living cells. The use of pharmacological inhibitors further entails the unavoidable risk of off-target inhibition. A genetic disruption of the AEP gene would in principle allow a less ambiguous demonstration of AEP involvement in class II MHC biosynthesis and in Ag presentation.

AEP-deficient mice exhibit a defect in maturation of Cat B, Cat H, and Cat L in kidney cells (15). These lysosomal proteases reach endocytic compartments as proforms or zymogens. It is here that the propeptide is removed proteolytically. The resulting single-chain form is then cleaved into the two-chain form (22). AEP mediates the latter cleavage event in kidney cells (15). Identification of Cats as substrates for AEP in APCs represents an additional step that might control protease activity and hence Ag presentation.

In the present study, we investigate class II MHC Ag processing and presentation in AEP-deficient mice. We find no abnormalities in class II MHC biosynthesis and maturation in primary APCs from AEP-deficient mice compared with wild type. Such anomalies are readily seen in animals deficient for Cat S (9, 10). Moreover, the presentation to primary T cells of two protein Ags occurs with similar efficacy in AEP-deficient and wild-type APCs. Although the processing of Cat L is blocked in AEP mutant mice, the number of CD4 and NK T cells, although dependent on a functional Cat L gene, are normal. We suggest that AEP is not essential for Ag presentation, at least in mice, and that the Cat L single-chain form suffices to function in generation of CD4 and NK T cells.

Materials and Methods

Mice and cell preparation

C57BL/6J and class II MHC-deficient mice were obtained from The Jackson Laboratory. Generation of class II MHC-, Cat B-, Cat L-, Cat S-, and AEP-deficient mice has been described previously (9, 15, 23–25). Cat L- and Cat B-deficient mice were backcrossed to the C57BL/6J background for five to six generations. Cat S-deficient animals on the C57BL/6J background were a kind gift from R. Riese (Brigham and Women's Hospital, Boston, MA). Transgenic mice that express an OVA-specific TCR (OT-II) have been described previously (26). Myelin oligodendrocyte glycoprotein (MOG)-specific transgenic 2D2 mice (27) were a kind gift from V. Kuchroo (Harvard Medical School, Boston, MA). Animals were bred in a pathogen-free environment in accordance with Harvard Medical School guidelines.

Single-cell suspensions from spleen, lymph node, liver, and thymus were generated by mechanical disruption of the tissues. RBCs were eliminated by hypotonic lysis. Mononuclear cells were enriched from liver over a Percoll gradient as described previously (28, 29). Bone marrow cells were harvested by flushing the femur and tibia of wild-type, AEP-deficient, or class II MHC-deficient mice using a 25-gauge needle. To generate BMDCs, bone marrow cells were plated at 1–2 $\times$ 10^6 cells/well (12-well plate) in complete DMEM (10% FCS) supplemented with 1 ng/ml IL-4 (Roche) and 10 ng/ml GM-CSF (PeproTech). Fresh media was replenished on days 2 and day 4. Immature BMDCs were generated after 5–6 days of culture.

Immunostaining and fluorescent confocal microscopy

Five-day-old BMDCs on coverslips were fixed in 3.7% paraformaldehyde in PBS for 10 min at room temperature. For costaining with transferrin, cells were incubated with Alexa594-conjugated transferrin (Molecular Probes) in serum-free media for 15 min at 37°C before fixation. Dendritic cells were then permeabilized for 10 min with 0.05% saponin in PBS (PBS-S) and incubated with 5% BSA in PBS-S for 30 min at room temperature. The cells were stained with rat mAb M5/114.15.2 (BD Biosciences) or hamster mAb N22 for class II MHC (I-A), rabbit polyclonal Ab JV11 for Ii, and rat mAb 1D4B for lysosome-associated membrane protein 1 (LAMP-1) for 1 h at room temperature, followed by incubation with Alexa488- or Alexa594-conjugated secondary Abs for 30 min at room temperature. After extensive washing, coverslips were mounted on glass slides using SlowFade Light Antifade kit (Molecular Probes). The cells were imaged with a CFI Plan Apo $\times$100 oil objective lens using a Nikon TE2000U inverted fluorescent microscope equipped with a PerkinElmer Ultra*VIEW* LCI spinning disk confocal head, a Kr-Ar laser, and a Hamamatsu orca ER-cooled charge-coupled device camera (12 bit) in the Nikon Imaging Center at Harvard University.

Pulse-chase and immunoprecipitation

Pulse-chase experiments were conducted as described previously (8). Briefly, single-cell suspensions were maintained in methionine-free medium for 1 h. Where indicated, leupeptin (Roche), pepstatin A (Sigma-Aldrich), or MV026630 (AEP inhibitor (AEPi)), synthesized as previously described (30), was added to final concentrations as indicated at 10 min before onset of the pulse and throughout the reminder of the experiment. Splenocytes were pulsed with 0.5 mCi of [^{35}S]methionine and BMDCs with 1 mCi of [^{35}S]methionine for 30 min and chased for the times indicated. At the end of the chase, the cells were washed with PBS and lysed in buffer A (50 mM Tris (pH 7.4), 5 mM $MgCl_2$, and 0.5% Nonidet P-40 with complete protease inhibitor mix (Roche)). After preclearing with normal rabbit and mouse serum together with staphylococcus aureus protein A, class II MHC molecules were immunoprecipitated with N22 Ab. Samples were either boiled in sample buffer or simply dissolved in sample buffer at room temperature to examine SDS-stable dimer formation. Where indicated, reimmunoprecipitation for Ii was conducted after dissociation of the complex in PBS/1% SDS by boiling and subsequent dilution of the SDS. Ii was immunoprecipitated using the polyclonal Ab JV5 recognizing the N terminus of Ii (31).

Protease assays and immunoblot

Postnuclear lysates from BMDCs (20 μg of protein, determined by BCA assay; Pierce) were incubated with 100 μM protease substrates (AEP: Z-Ala-Ala-Asn-AMC, Cat B/L: Z-Phe-Arg-AMC, Cat B: Z-Arg-Arg-AMC, Cat S: Boc-Val-Leu-Lys-AMC, Cat H: H-Arg-AMC, BACHEM) for 1 h at 37°C. The following buffers were used for AEP (20 mM citric acid, 60 mM Na_2HPO_4, 0.1% CHAPS (pH 5.5), 1 mM EDTA, and 1 mM DTT), Cat B/L and Cat B (50 mM Na_2HPO_4 (pH 6.25), 1 mM EDTA, and 1 mM DTT), Cat H (100 mM Bis-Tris propane, 1% polyethylene glycol 8000 (pH 7.5), 1 mM EDTA, and 1 mM DTT), and Cat S (100 mM Na_2HPO_4, 100 mM NaCl (pH 6.5), 1 mM EDTA, and 1 mM DTT). DTT was added immediately prior for all assays. Reactions were performed in black-walled 96-well plates (Nunc) in a total volume of 200 μl. Release of 7-amido-4-methyl coumarin (AMC) was measured at 460 nm with a Wallac fluorescence plate reader. For the evaluation of AEPi in cells, BMDCs were treated with AEPi (20 μM) or DMSO vehicle for 1 h at 37°C before preparation of postnuclear lysates.

For active site labeling, cells were lysed in buffer B (50 mM sodium acetate (pH 5), 5 mM $MgCl_2$, and 0.5% Nonidet P-40) on ice. After removal of cell debris and nuclei by centrifugation, 25 μg of protein were labeled with 5 μM DCG-04 in 50 μl of buffer B for 1 h at 37°C. For immunoprecipitation of Cat B and Cat L, 100 μg of protein were labeled with 10 μM DCG-04 in 100 μl of buffer B for 1 h at 37°C. The reaction was stopped by adding SDS to a final concentration of 1% and boiled. Rabbit anti-Cat L antiserum and staphylococcus aureus protein A was used to immunoprecipitate Cat L. For analysis of DCG-04 labeling, HRP-coupled streptavidin (Amersham Biosciences) was used. The synthesis of DCG-04 has been described previously (32). The polyclonal anti-gp96 antiserum was raised in our lab according to standard procedures. The polyclonal antiserum against Cat L was a kind gift from A. Erickson (University of North Carolina, Chapel Hill, NC).

For analysis by immunoblotting, cells were lysed in buffer A. Samples were analyzed by SDS-PAGE and immunoblot according to standard procedures. The secondary anti-rabbit Ab coupled with HRP was obtained from Southern Biotechnology Associates.

Cytofluorimetry

Single cell suspensions were stained on ice with the indicated Abs conjugated to FITC, PE, APC, PerCP, or biotinylated. Biotinylated Abs were detected by fluorophore-conjugated streptavidin (BD Pharmingen). Stained cells were acquired on a FACSCalibur followed by analysis using CellQuest software. Where indicated, dead cells were labeled with TO-PRO3 (Molecular Probes) for subsequent exclusion from analysis. The Abs used for FACS analysis, anti-I-A^b (AF6-120.1), -TCRβ (H57-597), -CD4 (RM4-5), -CD8α (53-6.7), -CD11c (HL3), -CD19 (1D3), -CD69 (H1.2F3), -Vβ5.1,5.2 (MR9-4), and -Vβ11 (RR3-15), were obtained from BD Pharmingen. CD1d tetramers were generated as previously described (29, 33), using α-galactosylceramide synthesized in our laboratory, as will be described elsewhere.

Ag presentation

BMDCs day 5 of culture were pulsed with either OVA (Sigma-Aldrich) or MOG for 6 h and washed to remove excess Ag. Recombinant MOG protein was expressed and purified as described previously (34). Transgenic OT-II or 2D2 CD4 T cells were harvested from the peripheral lymph nodes of TCR-transgenic mice. Lymph node cells were depleted of class II-positive cells by negative selection using anti-mouse class II MHC-magnetic microbeads (Miltenyi Biotec). Depletion of class II MHC-expressing cells was confirmed by flow cytometry. Three to 9×10^5 class II MHC-negative lymph node cells were added to Ag-pulsed BMDCs and incubated overnight. Cells present in suspension were harvested in PBS supplemented with 5% FCS/0.02% sodium azide and analyzed by flow cytometry. OT-II T cells were detected as CD4- and Vβ5-expressing cells. 2D2 T cells were detected as CD4- and Vβ11-expressing cells. T cell activation was assessed as the percentage of transgenic CD4 T cells expressing the early activation molecule, CD69, in response to Ag presented by class II MHC.

Results

Class II MHC molecules display normal localization in AEP-deficient cells

Alterations in protease activity of APCs can affect the steady-state distribution of class II MHC by influencing degradation of Ii, as readily seen by incubation of cells with leupeptin (4, 5, 35). Reportedly, AEP colocalizes with class II MHC molecules in endocytic compartments of BMDCs (16) and can use Ii as a substrate (13). Thus, we examined the role of AEP on distribution of class II MHC molecules in murine APCs derived from AEP-deficient mice and compared them to wild-type mice. By cytofluorimetry, surface expression of class II MHC molecules on splenic B cells ($CD19^+$), dendritic cells ($CD11c^+$), and BMDCs were comparable to wild type (Fig. 1*A*). The low class II MHC-expressing BMDC population most likely represents immature dendritic cells because LPS treatment led to disappearance of this population (data not shown). Confocal microscopy of BMDCs showed that in immature dendritic cells, class II MHC molecules localize to late endocytic compartments in both AEP-deficient and wild-type mice, as judged by colocalization with the late endosomal/lysosomal marker, LAMP-1, but not the early endosomal marker transferrin (Fig. 1, *B* and *C*). Therefore, neither cell surface expression nor overall intracellular localization of class II MHC molecules was obviously perturbed by the lack of AEP.

AEP has been reported to be involved in the cleavage of Ii C-terminal to CLIP (13). Cleavage of human Ii was proposed to occur at Asn residues 132 and 155 (3). In mice, cleavage at Asn residues 105, 147, 153, or 159 might result in the Ii remnants p10 or p22 by release of a C-terminal Ii fragment (Fig. 2*A*). Inhibition of this cleavage event could lead to a prolonged association of this C-terminal Ii fragment with the class II MHC molecule. To examine whether the lack of AEP alters the colocalization of the class II molecules with the relevant portion of Ii, we used a polyclonal anti-Ii serum raised against an epitope encoded in the C-terminal region of Ii (Fig. 2*A*; Ref. 31). We observed similar partial colocalization of class II MHC molecules with this region of the Ii in AEP-deficient and wild-type BMDCs (Fig. 1*D*). In addition, no change in localization of the N-terminal portion of Ii was observed (data not shown). We conclude that the steady-state distribution of class II MHC is not affected by the loss of AEP.

Processing of Ii proceeds normally in primary AEP-deficient cells

We next examined the maturation of class II MHC molecules to explore the possibility of more transient or subtle defects caused by the AEP deficiency. AEP was ascribed a role in generation of SDS-stable dimers (13), a hallmark of peptide-loaded class II MHC molecules (36). To address whether AEP plays a role in the generation of such complexes in murine dendritic cells, we used BMDCs to conduct pulse-chase experiments for class II MHC maturation. Radiolabeled class II MHC molecules were immunoprecipitated with N22, an Ab that recognizes properly folded class II MHC molecules. Peptide-loaded SDS-stable class II MHC molecules ($\alpha\beta$p) could be detected in wild-type and AEP-deficient BMDCs as early as after 1 h of chase (Fig. 2*B*, *left panels*). The role of AEP in class II MHC maturation has been assessed in human B cell lines by using the AEP-specific inhibitor MV026630 (AEPi, see Fig. 5*B*) (13). To address whether treatment with AEPi might have a differential effect on class II MHC maturation from the genetic disruption of AEP activity, we conducted pulse-chase experiments in the presence of AEPi. Addition of AEPi during the pulse-chase also did not influence formation of SDS-stable dimers in BMDCs when compared with control cells (Fig. 2*B*, *right panels*).

To examine whether other cysteine proteases might compensate for the loss of AEP, we analyzed the maturation of class II MHC molecules in BMDCs treated with leupeptin, a broad spectrum cysteine protease inhibitor. Addition of leupeptin led to accumulation of Ii remnants (p22, p18, and p10) in wild-type BMDCs that are also present in similar quantities in AEP-deficient BMDCs (Fig. 2*C*, *left panels*). BMDCs treated with leupeptin and AEPi or

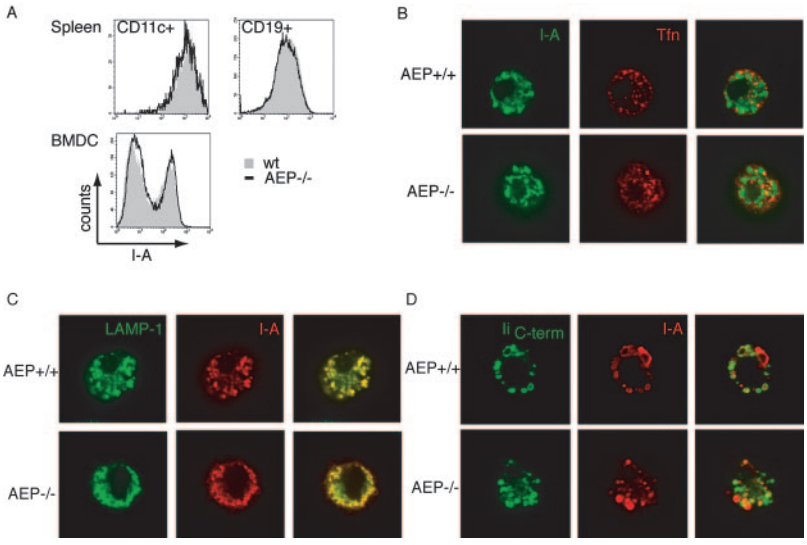

FIGURE 1. Intracellular distribution of class II MHC molecules is normal in $AEP^{-/-}$ mice. *A*, FACS analysis of class II MHC (I-A) surface expression on splenic dendritic cells ($CD11c^+$, *upper left panel*) and B cells ($CD19^+$, *upper right panel*) or BMDCs (*lower panel*). B cells were gated on a lymphocyte gate, whereas dendritic cells were gated on larger $CD11c^+$ cells based on forward and side scatter. The dendritic cells could be divided into populations with high and low class II MHC surface expression, most likely a reflection of their maturation and activation status. *B–D*, Confocal microscopy analysis of colocalization of class II MHC molecules with transferrin (Tfn), LAMP-1, or the luminal domain (COOH terminus) of Ii.

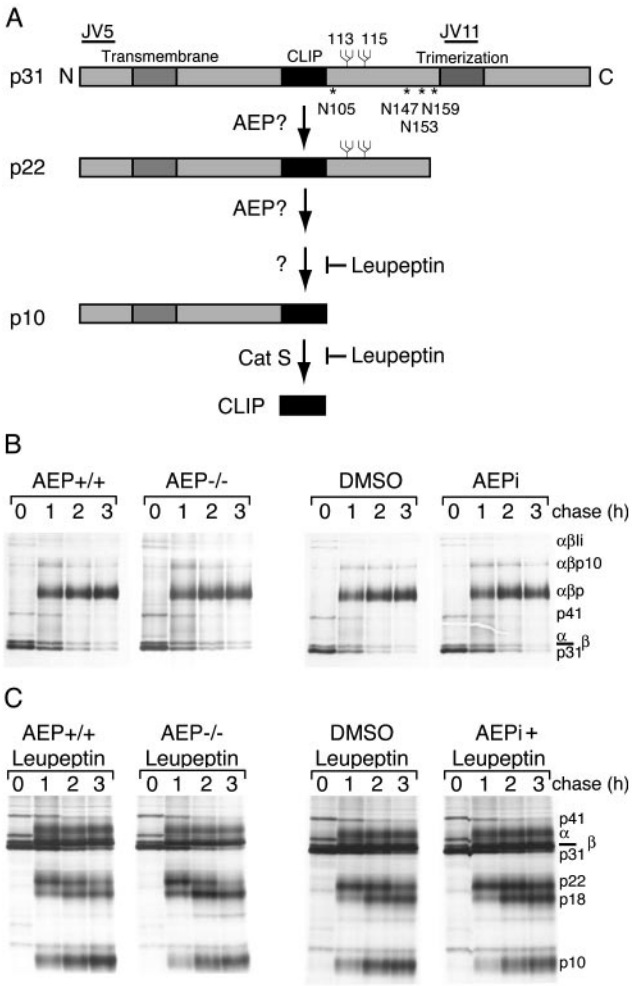

FIGURE 2. AEP is not essential for maturation of class II MHC molecules in BMDCs. *A*, Schematic overview of the Ii processing. Full-length Ii (p31) and its degradation products p22, p10, and CLIP are depicted. Epitopes for the anti-Ii antisera JV5 and JV11 are indicated. Conversion of p31 to CLIP can be blocked at different stages by the inhibitor leupeptin. Processing of the Ii intermediate p10 to CLIP is mediated by the leupeptin-sensitive protease Cat S in B cells and dendritic cells. Proteases involved in earlier cleavage events remain to be identified. Asn residues (N105, N147, N153, and N159) that might be a recognized by AEP are depicted. Asn residues 113 and 115 act as N-linked glycosylation sites. *B* and *C*, Radiolabeled pulse-chase experiments on BMDCs with a 30-min pulse and indicated chase times followed by recovery of class II MHC molecules and associated proteins by immunoprecipitation with N22. *B*, Generation of SDS-stable complexes is analyzed in wild-type ($AEP^{+/+}$) and AEP-deficient ($AEP^{-/-}$) BMDCs (*left panels*) or in BMDCs treated with 40 μM AEPi or control amount of DMSO (*right panels*). The samples were loaded under mildly denaturing conditions (nonboiled). *C*, Generation of Ii remnants was analyzed in presence of 1 mM leupeptin present during the pulse-chase. Samples were loaded after boiling.

leupeptin and DMSO afforded similar Ii intermediates associated with class II MHC (Fig. 2*C*, *right panels*). Neither the genetic disruption nor the chemical inhibition of AEP indicated a significant role for this enzyme in the class II MHC maturation in BMDCs, even upon inhibition of leupeptin-sensitive proteases. In contrast, inactivation of the Cat S gene or inhibition of Cat S activity results in accumulation of the p10 Ii intermediate in primary APCs (8–10).

A role for AEP in processing of Ii as well as in the generation of SDS-stable dimers was reported for human B cell lines (13). Thus, we used murine splenocytes as a source of primary B cells to conduct pulse-chase experiments for class II MHC maturation. As shown in Fig. 3*A*, the amount of Ii-associated class II MHC complexes ($\alpha\beta$Ii) decreased with time equally in both wild-type and AEP-deficient splenocytes, giving rise to SDS-stable peptide class II MHC complexes. After 1 h of chase, SDS-stable peptide class II MHC complexes and the p10 Ii intermediate were readily detectable in both cases. Therefore, the loss of AEP alone does not block class II MHC maturation in murine B cells. Similarly, the treatment of splenocytes with AEPi did not block the formation of SDS-stable dimers when compared with DMSO vehicle-treated cells (Fig. 3*B*). Furthermore, the Ii intermediates p22, p18, and p10 were readily detectable in both wild-type and AEP-deficient splenocytes treated with leupeptin (Fig. 3*C*). Therefore, the generation of these intermediates is not dependent on AEP and leupeptin-sensitive proteases. We performed reimmunoprecipitation experiments with the JV5 antiserum, directed against the cytoplasmic N-terminal portion of Ii, to unambiguously identify the Ii fragments indicated in the figure (data not shown).

To examine whether aspartyl proteases might compensate for the loss of AEP in the early steps of Ii processing, we conducted pulse-chase experiments in the presence of the broad spectrum aspartyl protease inhibitor pepstatin A together with leupeptin. The generation of the p22, p18, and p10 Ii fragments was reduced to the same extent in AEP-deficient and in wild-type splenocytes treated with leupeptin and pepstatin A (Fig. 3*C*). Maturation of class II MHC α subunits into complex-type oligosaccharide-bearing species was identical for cells treated with leupeptin alone or cells treated with both leupeptin and pepstatin A (Fig. 3*C*). Therefore, the effects of pepstatin A on Ii processing are not simply due to interference with intracellular transport of class II MHC products before arrival in endocytic compartments. Thus, even when leupeptin and pepstatin A-sensitive proteases were inhibited, the lack of AEP did not yield a block at any stage of Ii processing compared with wild type.

To test whether AEPi affects class II MHC maturation in murine splenocytes in the absence of leupeptin-sensitive protease activities, we conducted pulse-chase experiments in the presence of leupeptin combined with AEPi. SDS-stable dimers were observed after 1 h of chase in control cells (Fig. 3*D*). As expected, the generation of peptide-loaded SDS-stable dimers was blocked by leupeptin alone (4, 35, 37), as well as in the presence of leupeptin combined with AEPi (Fig. 3*D*). Moreover, the accumulation of Ii fragments p10, p18, and p22 was indistinguishable for leupeptin-treated cells with or without added AEPi (Fig. 3*D*). Taken together, our data indicate that AEP is dispensable for processing of Ii and maturation of class II MHC molecules into peptide-loaded complexes in murine BMDCs and splenocytes.

AEP is dispensable for Ag presentation of OVA and MOG

Class II MHC Ag presentation requires processing of proteins into antigenic peptides, whose generation or abundance might be altered by the loss of AEP. Indeed, AEP has been implicated in epitope generation from tetanus toxin and in the destruction of an immunodominant epitope from MBP (14, 21). To assess the role of AEP in class II MHC Ag presentation, we used OVA and MOG as protein Ags for which Ag-specific primary CD4 T cells were readily available from OT-II (26)- and 2D2 (27)-transgenic mice, respectively. The core epitope recognized by OT-II T cells (OVA 329-337: AAHAEI*N*EA, (38)) contains an Asn (OVA: Asn^{335}), whereas the peptide relevant for 2D2 recognition (MOG 38-51) (39) lies in close proximity to an Asn (MOG 38-55: GWYRSPFSRVVHLYr**n**gk). We considered several possibilities. The OVA

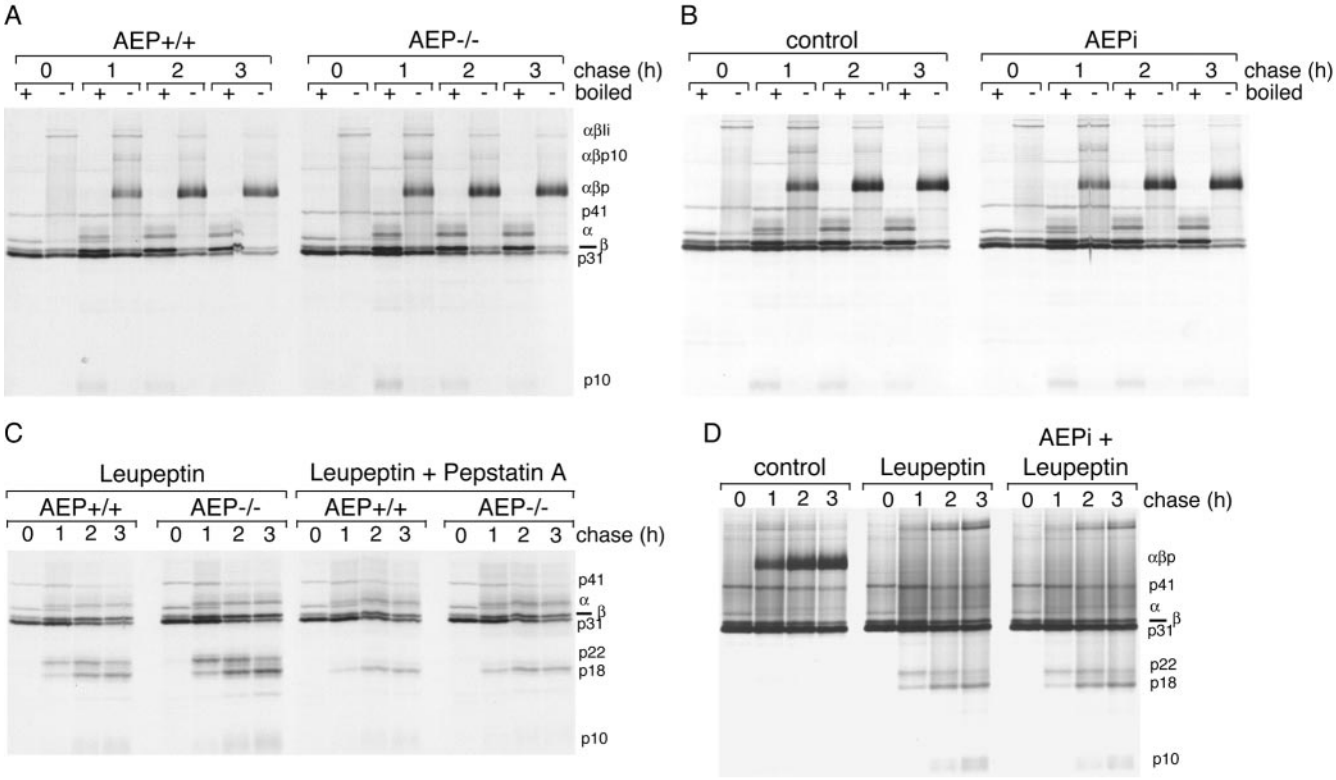

FIGURE 3. AEP is not essential for generation of Ii intermediates in splenocytes. *A–D*, Radiolabeled pulse-chase experiments on splenocytes with a 30-min pulse and indicated chase times. AEP-deficient ($AEP^{-/-}$) or wild-type control ($AEP^{+/+}$) cells (*A*) or wild-type splenocytes (*B*) treated with 40 μM AEPi or control amounts of DMSO were analyzed for SDS-stable dimer formation. Class II MHC molecules and associated proteins were recovered by immunoprecipitation with N22 and loaded under denaturing (boiled) or mildly denaturing (nonboiled) condition. *C*, Processing of class II MHC-associated Ii was examined in the presence of 1 mM leupeptin and 10 μM pepstatin A. The samples were loaded under denaturing conditions. p31, its splice variant p41, and the degradation intermediates p22, p18, and p10 are indicated. SDS-stable class II MHC complexes are found as class II MHC subunits α and β in association with full-length Ii ($\alpha\beta$Ii), p10 ($\alpha\beta$p10), or peptide ($\alpha\beta$p). *D*, Pulse-chase experiment of protease inhibitor treated wild-type splenocytes. A total of 1 mM leupeptin and 40 μM AEPi was used where indicated. Control cells were treated with DMSO.

epitope might be (partially) destroyed by AEP activity, and consequently, the absence of AEP might improve its presentation. If an initial cleavage of OVA by AEP, somewhere outside the epitope presented, were required to facilitate subsequent excision of the epitope by other proteases, then the AEP deficiency might impair presentation. It is not possible to choose a priori between these two possibilities. Although the MOG epitope lacks an Asn residue, an Asn residue is present in close proximity to the epitope proper. Excision of the MOG epitope might be favored by a prior cleavage involving AEP or be compromised if the hierarchy of cleavages that generates the MOG epitope would critically depend on the action of AEP.

BMDCs from AEP-deficient and wild-type mice were challenged with OVA or MOG protein and evaluated for class II MHC Ag presentation by activation of OT-II or 2D2 T cells, respectively. Class II MHC presentation of OVA and MOG by AEP-deficient BMDCs was comparable to that of wild-type BMDCs, as assessed by up-regulation of the early T cell activation marker CD69 (Fig. 4). Presentation of OVA or MOG protein by BMDCs derived from class II MHC-deficient mice yielded values for CD4 T cell activation of $<9\%$, even at the highest Ag concentrations (Fig. 4 and data not shown), confirming specificity and class II MHC dependency of the assay. Although AEP might be involved in processing of some Ags as shown in human B cell lines (14, 21), it is not essential for the generation of the peptide epitopes examined here.

AEP deficiency leads to absence of the two-chain form of Cat L

Endocytic proteases are synthesized as zymogens, which are then activated by proteolytic processing to their mature forms. Therefore, regulation of this maturation might have consequences for the class II MHC Ag presentation pathway. To examine whether AEP deficiency might alter cysteine protease activity in APCs, we used fluorogenic substrates selective for the different proteases. BMDC lysates from AEP-deficient mice failed to cleave a fluorogenic substrate selective for AEP (Fig. 5*A*), demonstrating that AEP is the predominant activity capable of hydrolyzing this substrate. In contrast, we did not find notable differences in the hydrolysis of Cat B/L-, Cat B-, or S-specific substrates between AEP-deficient and wild-type BMDC lysates. A modest reduction in the hydrolysis of a Cat H-selective substrate was observed in AEP-deficient BMDCs. The activity of the AEP-specific substrate was reduced in lysates from AEPi-treated BMDCs, whereas the activity toward the CatB/L-specific substrate was not affected (Fig. 5*C*). This confirms the previously reported (13) potent and selective activity of AEPi in intact cells.

In previous work, we had identified the active forms of Cats in BMDC lysates by taking advantage of their reactivity with the cysteine-protease mechanism-based probe, DCG-04 (40). Using DCG-04 (Fig. 5*D*), we determined the activity profile of cysteine proteases in AEP-deficient and wild-type BMDC lysates. A polypeptide species, previously identified as Cat L

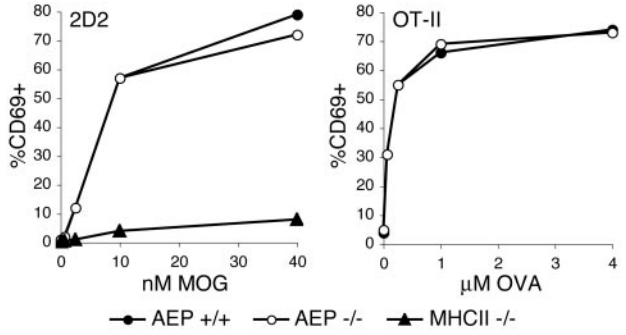

FIGURE 4. Normal Ag presentation of OVA and MOG to T cells. AEP-deficient BMDCs present MOG (*left panel*) and OVA (*right panel*) protein to Ag-specific CD4 T cells. AEP-deficient ($AEP^{-/-}$), wild-type ($AEP^{+/+}$), and class II MHC-deficient ($MHC\text{-}II^{-/-}$) BMDCs were pulsed with the indicated concentrations of proteins for 6 h. Following washing, the pulsed BMDCs were incubated together with class II MHC-depleted CD4 T cell preparations overnight. CD4 T cell activation was assessed by flow cytometry as the up-regulation of the early activation marker CD69 on CD4-positive Vβ5 (OT-II) or Vβ11 (2D2) cells.

(40), was absent in AEP-deficient compared with wild-type BMDCs (Fig. 5*E*). Instead, a polypeptide of higher molecular mass was present in AEP-deficient mice. In the kidney, AEP-deficient mice exhibit a defect in the generation of the Cat L two-chain form (Fig. 5*F*; Ref. 15). To determine whether a similar defect occurs in APCs, BMDC lysates labeled with DCG-04 were immunoprecipitated for Cat L. Both the Cat L single-chain and two-chain form were labeled with DCG-04 in wild-type and Cat B-deficient lysates (Fig. 5*G*). However, BMDC lysates from AEP-deficient mice contained only the single-chain and not the two-chain form. These data show a defect in processing of Cat L in AEP-deficient BMDCs and reveal the Cat L single-chain form as enzymatically active based on its labeling with the mechanism-based probe DCG-04.

To examine whether the generation of the mature Cat L two-chain form was blocked only in AEP-deficient mice, we performed Cat L immunoblots on samples from other available protease-deficient mice. Whereas the two-chain form of Cat L was present in wild-type, heterozygous, Cat S-deficient, and Cat B-deficient animals, only the single-chain and the pro-Cat L form could be detected in AEP-deficient animals (Fig. 5*H*). BMDC lysate from a Cat L-deficient mouse is included to demonstrate the specificity of the anti-Cat L sera. In addition, we analyzed lysates generated from spleen and thymus for expression of different Cat L isoforms. The amount of the Cat L single-chain form in AEP-deficient lysates was increased compared with wild-type and Cat B-deficient animals, whereas the Cat L two-chain form was undetectable essentially in AEP-deficient mice (Fig. 5*I*). Interestingly, the pro-Cat L isoform was increased markedly in thymic extracts of AEP-deficient mice.

Generation of CD4 and NK T cells is independent of AEP

The presence of Cat L is essential for the normal numbers of CD4 as well as NK T cells (11, 41). If Cat L function was compromised in AEP-deficient mice, as inferred from the inability to process the single-chain form, this deficiency might impair CD4 T cell generation. However, we observe normal CD4 and CD8 single-positive T cell numbers in the thymus of AEP-deficient mice in comparison to wild-type and heterozygous controls (Fig. 6*A*, *upper panel*; Table I). In addition, peripheral CD4 and CD8 T cell numbers were also normal in AEP-deficient mice (Fig. 6*A*, *middle* and *lower panels*; Table I). Wild-type, AEP-deficient, and Cat L-deficient mice were also analyzed for generation of NK T cells by staining with α-galactosylceramide-loaded CD1d tetramers (33). Although NK T cells were generated efficiently in the thymus of AEP-deficient and wild-type mice, a severe defect was observed in Cat L-deficient mice (Ref. 41; Fig. 6*B*, *upper panel*). Moreover, the percentage of NK T cells in peripheral organs such as spleen and liver in AEP-deficient and wild-type was comparable but strongly diminished in Cat L-deficient mice (Fig. 6*B*, *middle* and *lower panels*). CD4 as well as NK T cells are generated in normal numbers in AEP-deficient mice, suggesting that AEP interferes neither directly nor indirectly with the generation of these cell populations, despite the inability to generate the two-chain form of Cat L.

Discussion

The identification of the specific protease(s) involved in class II MHC Ag presentation may provide targets for therapeutic intervention. In this regard, AEP, a cysteine protease with a unique and, apparently nonredundant (Fig. 5), substrate specificity has attracted considerable attention. Initially, the role of AEP in Ag presentation was inferred from the ability of purified AEP to execute the early cleavages of tetanus toxin Ag (21). Furthermore, destruction of an immunodominant MBP epitope relevant for multiple sclerosis suggested the potential for intervention by inhibition of AEP activity (14). More recently, the use of a cell-permeable AEPi has suggested the involvement of AEP in class II MHC maturation through proteolysis of Ii (13). Proteolysis of Ii might influence timing and location of peptide loading onto class II MHC molecules and therefore may affect initiation of an immune response. We used AEP-deficient mice and AEPi to address the role of this cysteine protease on class II MHC maturation and Ag processing in primary APCs.

The distribution and maturation of class II MHC molecules are normal in AEP-deficient mice. As measured by flow cytometry, the loss of AEP did not adversely affect the amount of cell surface class II MHC molecules. Intracellular distribution of class II MHC molecules and Ii were also identical for AEP-deficient and wild-type mice. In the absence of AEP activity, neither formation of SDS-stable dimers nor the processing of Ii intermediates was impaired in murine BMDCs and splenocytes. Even in the presence of broad spectrum cysteine and aspartyl protease inhibitors, the AEP deficiency did not reveal significant differences in class II MHC maturation, ruling out compensatory effects of leupeptin- and pepstatin A-sensitive proteases. Furthermore, pharmacological inhibition of AEP with the cell-permeable inhibitor AEPi (13, 30) afforded no differences in class II MHC maturation. The reduction in leupeptin-induced Ii intermediates by additional treatment with aspartyl protease inhibitors has been described previously for human B-lymphoblastoid cells (42) and demonstrates that additional proteases in the processing of Ii remain to be identified. However, from our data, we conclude that AEP is not essential for class II maturation or proteolysis of Ii in murine BMDCs and splenocytes, where the most abundant class II MHC-positive cells are B cells.

Table I. *Total cell numbers (mean ± SD) calculated from living cells for indicated cell populations*

	Spleen		Thymus	
	CD4	*CD8*	*CD4SP*	*CD8SP*
Wild type $n = 8–9$	9.1 (± 2.0)	5.8 (± 1.2)	5.4 (± 3.8)	1.9 (± 1.4)
$AEP^{-/-}$ $n = 10$	12.3 (± 4.3)	7.3 (± 2.8)	8.1 (± 2.6)	2.5 (± 0.8)

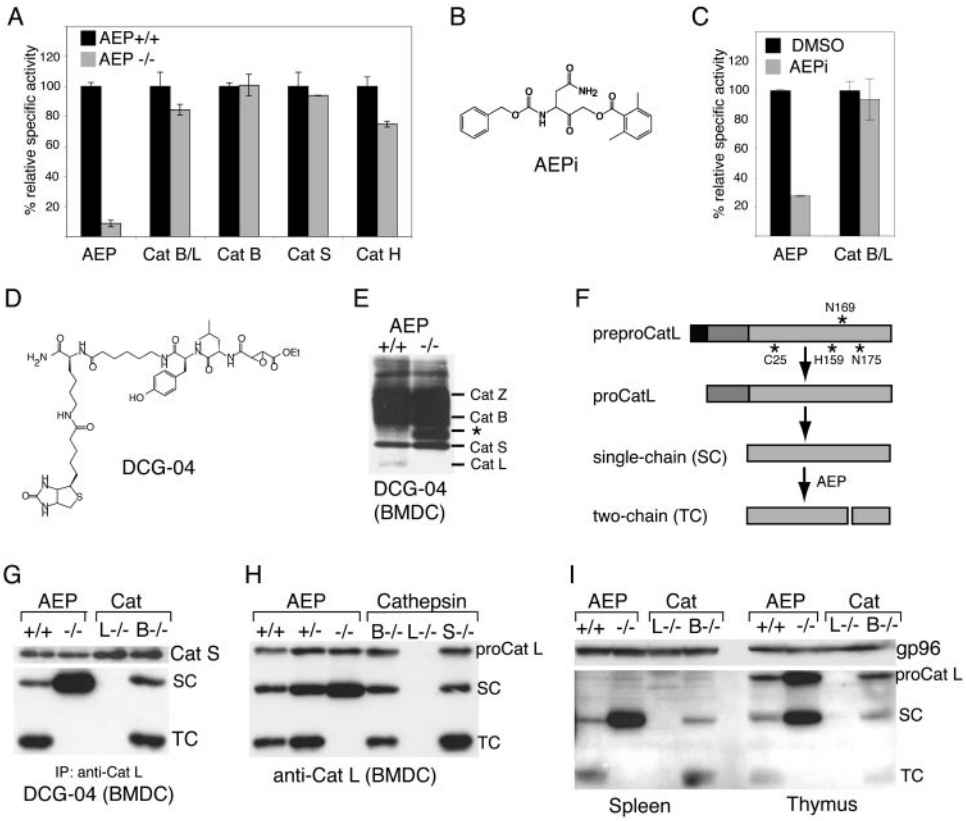

FIGURE 5. Generation of the mature Cat L two-chain form is abrogated in AEP-deficient mice. *A*, Postnuclear lysates of BMDCs from wild-type ($AEP^{+/+}$) and AEP-deficient ($AEP^{-/-}$) mice were assayed for protease activity with AMC substrates for AEP (Z-Ala-Ala-Asn-AMC), Cat B/L (Z-Phe-Arg-AMC), Cat B (Z-Arg-Arg-AMC), Cat S (Boc-Val-Leu-Lys-AMC), and Cat H (H-Arg-AMC). Bars represent the average of duplicate experiments. Error bars represent high and low values. The residual hydrolysis of Z-Ala-Ala-Asn-AMC by AEP-deficient BMDC lysates can be abrogated by E-64 (data not shown). *B*, Chemical structure of AEPi. *C*, Postnuclear lysates of wild-type BMDCs treated with 20 μM AEPi or corresponding volume of DMSO were analyzed for protease activity with AMC substrates for AEP or Cat B/L. *D*, Chemical structure of the active site-directed probe DCG-04. *E*, Analysis of BMDC postnuclear lysate for protease content reactive to the mechanism-based inhibitor DCG-04. The different polypeptide species are labeled based on previous work (40). An additional prominent DCG-04-reactive polypeptide present in AEP-deficient mice is indicated (*). *F*, Schematic overview of Cat L maturation (as reviewed in Refs. 22, 52). Cat L is synthesized as a preproenzyme, and the signal peptide is removed during the entry into the endoplasmic reticulum. Pro-Cat L is processed in an autocatalytically Cat D- or metalloprotease-dependent manner to its single-chain (SC) form. The murine two-chain (TC) form is generated by cleaving the SC form at Asn^{169} (N169). The amino acid residues Cys^{25} (C25), His^{159} (H159), and Asn^{175} (N175) are the basis for the catalytic activity of the enzyme. The amino acid positions are indicated (*) and are based on papain numbering. *G*, DCG-04-reactive Cat L was analyzed by immunoprecipitation (IP) for Cat L from DCG-04-labeled BMDC lysate and detection of the probe. Equal protein amounts were verified by comparable DCG-04-reactive Cat S in the input material. *H*, Immunoblot analysis of BMDCs for Cat L. Pro-Cat L and Cat L SC and Cat L TC forms are indicated. *I*, Immunoblot analysis of lysate from spleen and thymus for Cat L. Equal levels of gp96, as detected with a rabbit antiserum against gp96, served as a loading control.

To evaluate the role of AEP in Ag presentation, we used two protein Ags, OVA and MOG, for which Ag-specific, TCR-transgenic T cells are readily available. The OVA core epitope (OVA 329-339: VHAAHAEI*N*EA) recognized by OT-II T cells in the context of $I\text{-}A^b$ molecules contains an Asn at position 335 (38). This situation is similar to that seen for the HLA-DR2-associated MBP epitope reportedly destroyed by AEP (14). Although the Asn^{53} of the MOG protein is not in the core epitope (MOG 40-48) (43) and lies in a region not required for optimal 2D2 T cell activation (MOG 38-55: GWYRSPFSRVVHLYr**n**gk) (39), it might be important in generation of the recognized peptide from a whole protein because it is located close to the relevant epitope. OVA and MOG were presented efficiently on class II MHC molecules ($I\text{-}A^b$) by AEP-deficient and wild-type BMDCs, indicating that AEP has neither a positive nor a negative effect on the presentation of these epitopes. The presence of an Asn in a given T cell epitope does not necessarily render it susceptible to destruction by AEP. This is the case for the OVA class II MHC epitope examined. In addition, although AEP may cleave Asn residues distant from the peptide epitope and consequently alter recognition by T cells, this is not the case for the MOG class II MHC epitope. Therefore, AEP does not critically determine the MHC class II presentation of the protein Ags used in this study.

Collectively, our experiments suggest AEP is not essential for class II MHC maturation and Ag presentation per se in the mouse. This is contrary to earlier reports on AEP in human B cell lines, which suggested that AEP was crucial for class II MHC maturation (13) and presentation of exogenous Ags, such as tetanus toxin Ag (21) and MBP (14). These discrepancies may be explained by different protease activities among tissues or among the similar tissues from different species (44). Additionally, the previous studies were performed in B cell lines that might have different protease activity compared primary B cells used in this study. For example, Cat G instead of AEP was identified as a protease that controls MBP processing in human peripheral blood B lymphocytes (45). Compared with EBV-transformed human B cell lines, peripheral blood B lymphocytes exhibit 10-fold lower AEP activity and lower RNA levels, which may explain the observed differences for MBP

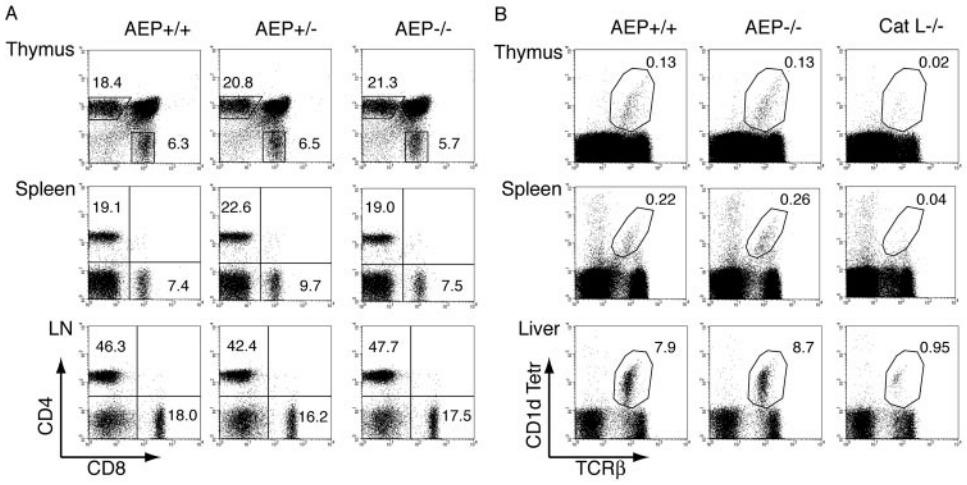

FIGURE 6. Normal CD4 T cell and NK T cell population in AEP-deficient mice. *A*, FACS analysis of CD4 and CD8 population in thymus (*upper panel*), spleen (*middle panel*), and inguinal lymph node (*lower panel*). Gated lymphocytes from AEP$^{-/-}$ and control heterozygous or wild-type animals were distinguished by their CD4 and CD8 surface expression. *B*, NK T cells from thymus (*upper panel*), spleen (*middle panel*), and liver (*lower panel*) were detected by their TCR expression together with reactivity to α-galactosylceramide-loaded CD1d tetramers. Lymphocytes from AEP$^{-/-}$, Cat L$^{-/-}$, and wild-type control mice were analyzed after exclusion of dead cells by TOPRO3 staining.

processing. The processing of protein Ags used in this study (OVA and MOG) may be less dependent on AEP compared with tetanus toxin (21) and MBP (14), despite the presence of an Asn in or in close proximity to the peptide epitope. Furthermore, the redundancy among lysosomal proteases involved in class II MHC Ag presentation may compensate for the AEP deficiency in murine APCs.

Although AEP is not essential for the class II MHC maturation and Ag presentation, we find a unique role for AEP in the processing of Cat L in a variety of murine tissues. Active site labeling experiments with the cysteine protease mechanism-based probe DCG-04 demonstrated a block in Cat L processing from the single-chain to the two-chain form in AEP-deficient BMDC lysates. Immunoblots of Cat L in splenic and thymic cell extracts from AEP-deficient mice confirm that the two-chain form fails to be generated in these tissues. The pattern of maturation of Cat L from the zymogen (pro-Cat L) to the mature single-chain and two-chain form is well established (22, 46), and the ratio between the Cat L single-chain and two-chain form differs among tissues, as reported for skin, liver, and kidney (47). It is less clear whether this conversion regulates Cat L activity, but the present results suggest that the single-chain form can carry out the essential functions of Cat L. In wild-type BMDCs, both the single-chain and the two-chain form of Cat L are active, as judged by their reactivity toward DCG-04. Although the loss of AEP activity resulted in up-regulation of lysosomal protease activity and inefficient processing of Cat B, Cat H, and Cat L in kidney homogenates (15), the use of fluorogenic peptide substrates selective for these proteases revealed no dramatic changes in their protease activities in BMDCs in vitro. The in vitro experiments with fluorogenic substrates and mechanism-based probes demonstrate comparable levels of Cat L activity in AEP-deficient and wild-type BMDCs. Although indicative of enzymatic activity, these assays do not necessarily allow an assessment of Cat L functionality in vivo.

To address the consequence of inefficient Cat L processing by AEP in vivo, we determined the numbers of CD4 and NK T cells in AEP-deficient and wild-type mice, given that Cat L is critical for normal CD4 and NK T cell development (11, 41, 48). Neither blockage of Cat L maturation by AEP deficiency nor the absence of AEP itself affects overall numbers of CD4 and NK T cells. We conclude that AEP is not essential for the generation of these cell populations and therefore that the Cat L single-chain form must suffice to carry out these function(s) of Cat L. It has been suggested that proteolytic activity of Cat L can shape the peptide repertoire bound to class II MHC products and by this means influence the selection process of CD4 T cells (48). An assessment of the proposed mechanism of action is not straightforward because Cat L can be secreted from cells and has elastase activity (49, 50), can be taken up by thymocytes (41), and might be involved in transcriptional control through its presence in the nucleus (51). Cat L-deficient mice exhibit periodic hair loss (25), a trait that has not been observed for AEP-deficient mice. Unless Cat L is alternatively processed in different tissues, the Cat L single-chain form is functionally sufficient.

In summary, our experiments with AEP-deficient mice and a cell-permeable AEP inhibitor demonstrate that AEP is dispensable for class II MHC maturation in murine BMDCs and splenocytes. Although AEP may determine the outcome of tetanus toxin and MBP Ag processing in human cells, the generation of relevant peptides from OVA and MOG is not blocked by loss of AEP activity, highlighting the selectivity of lysosomal proteases for protein degradation. Although AEP is not essential for proteolysis of Ii, we find that the generation of the Cat L two-chain form is impaired in AEP-deficient mice. Taking the role of AEP in the processing of Cat L as an example of a rather selective cleavage, one could argue by analogy that the generation of some antigenic peptides may strictly require AEP activity as well. Therefore, AEP-deficient mice should serve as an important immunological tool for identification and characterization of these Ags and the epitopes derived from them.

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Disclosures

The authors have no financial conflict of interest.

References

1. Villadangos, J. A., and H. L. Ploegh. 2000. Proteolysis in MHC class II antigen presentation: who's in charge? *Immunity* 12: 233–239.
2. Honey, K., and A. Y. Rudensky. 2003. Lysosomal cysteine proteases regulate antigen presentation. *Nat. Rev. Immunol.* 3: 472–482.
3. Watts, C. 2004. The exogenous pathway for antigen presentation on major histocompatibility complex class II and CD1 molecules. *Nat. Immunol.* 5: 685–692.
4. Amigorena, S., P. Webster, J. Drake, J. Newcomb, P. Cresswell, and I. Mellman. 1995. Invariant chain cleavage and peptide loading in major histocompatibility complex class II vesicles. *J. Exp. Med.* 181: 1729–1741.

5. Blum, J. S., and P. Cresswell. 1988. Role for intracellular proteases in the processing and transport of class II HLA antigens. *Proc. Natl. Acad. Sci. USA* 85: 3975–3979.
6. Nguyen, Q. V., W. Knapp, and R. E. Humphreys. 1989. Inhibition by leupeptin and antipain of the intracellular proteolysis of Ii. *Hum. Immunol.* 24: 153–163.
7. Riese, R. J., P. R. Wolf, D. Bromme, L. R. Natkin, J. A. Villadangos, H. L. Ploegh, and H. A. Chapman. 1996. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. *Immunity* 4: 357–366.
8. Villadangos, J. A., R. J. Riese, C. Peters, H. A. Chapman, and H. L. Ploegh. 1997. Degradation of mouse invariant chain: roles of cathepsins S and D and the influence of major histocompatibility complex polymorphism. [Published erratum appears in 1997 *J. Exp. Med.* 186:5945.] *J. Exp. Med.* 186: 549–560.
9. Shi, G. P., J. A. Villadangos, G. Dranoff, C. Small, L. Gu, K. J. Haley, R. Riese, H. L. Ploegh, and H. A. Chapman. 1999. Cathepsin S required for normal MHC class II peptide loading and germinal center development. *Immunity* 10: 197–206.
10. Nakagawa, T. Y., W. H. Brissette, P. D. Lira, R. J. Griffiths, N. Petrushova, J. Stock, J. D. McNeish, S. E. Eastman, E. D. Howard, S. R. Clarke, et al. 1999. Impaired invariant chain degradation and antigen presentation and diminished collagen-induced arthritis in cathepsin S null mice. *Immunity* 10: 207–217.
11. Nakagawa, T., W. Roth, P. Wong, A. Nelson, A. Farr, J. Deussing, J. A. Villadangos, H. Ploegh, C. Peters, and A. Y. Rudensky. 1998. Cathepsin L: critical role in Ii degradation and CD4 T cell selection in the thymus. *Science* 280: 450–453.
12. Shi, G. P., R. A. Bryant, R. Riese, S. Verhelst, C. Driessen, Z. Li, D. Bromme, H. L. Ploegh, and H. A. Chapman. 2000. Role for cathepsin F in invariant chain processing and major histocompatibility complex class II peptide loading by macrophages. *J. Exp. Med.* 191: 1177–1186.
13. Manoury, B., D. Mazzeo, D. N. Li, J. Billson, K. Loak, P. Benaroch, and C. Watts. 2003. Asparagine endopeptidase can initiate the removal of the MHC class II invariant chain chaperone. *Immunity* 18: 489–498.
14. Manoury, B., D. Mazzeo, L. Fugger, N. Viner, M. Ponsford, H. Streeter, G. Mazza, D. C. Wraith, and C. Watts. 2002. Destructive processing by asparagine endopeptidase limits presentation of a dominant T cell epitope in MBP. *Nat. Immunol.* 3: 169–174.
15. Shirahama-Noda, K., A. Yamamoto, K. Sugihara, N. Hashimoto, M. Asano, M. Nishimura, and I. Hara-Nishimura. 2003. Biosynthetic processing of cathepsins and lysosomal degradation are abolished in asparaginyl endopeptidase-deficient mice. *J. Biol. Chem.* 278: 33194–33199.
16. Trombetta, E. S., M. Ebersold, W. Garrett, M. Pypaert, and I. Mellman. 2003. Activation of lysosomal function during dendritic cell maturation. *Science* 299: 1400–1403.
17. Hara-Hishimura, I., Y. Takeuchi, K. Inoue, and M. Nishimura. 1993. Vesicle transport and processing of the precursor to 2S albumin in pumpkin. *Plant J.* 4: 793–800.
18. Dando, P. M., M. Fortunato, L. Smith, C. G. Knight, J. E. McKendrick, and A. J. Barrett. 1999. Pig kidney legumain: an asparaginyl endopeptidase with restricted specificity. *Biochem. J.* 339(Pt. 3): 743–749.
19. Chen, J. M., N. D. Rawlings, R. A. Stevens, and A. J. Barrett. 1998. Identification of the active site of legumain links it to caspases, clostripain and gingipains in a new clan of cysteine endopeptidases. *FEBS Lett.* 441: 361–365.
20. Chen, J. M., P. M. Dando, N. D. Rawlings, M. A. Brown, N. E. Young, R. A. Stevens, E. Hewitt, C. Watts, and A. J. Barrett. 1997. Cloning, isolation, and characterization of mammalian legumain, an asparaginyl endopeptidase. *J. Biol. Chem.* 272: 8090–8098.
21. Manoury, B., E. W. Hewitt, N. Morrice, P. M. Dando, A. J. Barrett, and C. Watts. 1998. An asparaginyl endopeptidase processes a microbial antigen for class II MHC presentation. *Nature* 396: 695–699.
22. Erickson, A. H. 1989. Biosynthesis of lysosomal endopeptidases. *J. Cell. Biochem.* 40: 31–41.
23. Madsen, L., N. Labrecque, J. Engberg, A. Dierich, A. Svejgaard, C. Benoist, D. Mathis, and L. Fugger. 1999. Mice lacking all conventional MHC class II genes. *Proc. Natl. Acad. Sci. USA* 96: 10338–10343.
24. Deussing, J., W. Roth, P. Saftig, C. Peters, H. L. Ploegh, and J. A. Villadangos. 1998. Cathepsins B and D are dispensable for major histocompatibility complex class II-mediated antigen presentation. *Proc. Natl. Acad. Sci. USA* 95: 4516–4521.
25. Roth, W., J. Deussing, V. A. Botchkarev, M. Pauly-Evers, P. Saftig, A. Hafner, P. Schmidt, W. Schmahl, J. Scherer, I. Anton-Lamprecht, et al. 2000. Cathepsin L deficiency as molecular defect of furless: hyperproliferation of keratinocytes and pertubation of hair follicle cycling. *FASEB J.* 14: 2075–2086.
26. Barnden, M. J., J. Allison, W. R. Heath, and F. R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based α- and β-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol.* 76: 34–40.
27. Bettelli, E., M. Pagany, H. L. Weiner, C. Linington, R. A. Sobel, and V. K. Kuchroo. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J. Exp. Med.* 197: 1073–1081.
28. Matsuda, J. L., L. Gapin, S. Sidobre, W. C. Kieper, J. T. Tan, R. Ceredig, C. D. Surh, and M. Kronenberg. 2002. Homeostasis of Vα 14i NKT cells. *Nat. Immunol.* 3: 966–974.
29. Schmidt-Supprian, M., J. Tian, E. P. Grant, M. Pasparakis, R. Maehr, H. Ovaa, H. L. Ploegh, A. J. Coyle, and K. Rajewsky. 2004. Differential dependence of $CD4^+CD25^+$ regulatory and natural killer-like T cells on signals leading to NF-κB activation. *Proc. Natl. Acad. Sci. USA* 101: 4566–4571.
30. Loak, K., D. N. Li, B. Manoury, J. Billson, F. Morton, E. Hewitt, and C. Watts. 2003. Novel cell-permeable acyloxymethylketone inhibitors of asparaginyl endopeptidase. *Biol. Chem.* 384: 1239–1246.
31. Driessen, C., R. A. Bryant, A. M. Lennon-Dumenil, J. A. Villadangos, P. W. Bryant, G. P. Shi, H. A. Chapman, and H. L. Ploegh. 1999. Cathepsin S controls the trafficking and maturation of MHC class II molecules in dendritic cells. *J. Cell Biol.* 147: 775–790.
32. Greenbaum, D., K. F. Medzihradszky, A. Burlingame, and M. Bogyo. 2000. Epoxide electrophiles as activity-dependent cysteine protease profiling and discovery tools. *Chem. Biol.* 7: 569–581.
33. Benlagha, K., A. Weiss, A. Beavis, L. Teyton, and A. Bendelac. 2000. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J. Exp. Med.* 191: 1895–1903.
34. Amor, S., N. Groome, C. Linington, M. M. Morris, K. Dornmair, M. V. Gardinier, J. M. Matthieu, and D. Baker. 1994. Identification of epitopes of myelin oligodendrocyte glycoprotein for the induction of experimental allergic encephalomyelitis in SJL and Biozzi AB/H mice. *J. Immunol.* 153: 4349–4356.
35. Neefjes, J. J., and H. L. Ploegh. 1992. Inhibition of endosomal proteolytic activity by leupeptin blocks surface expression of MHC class II molecules and their conversion to SDS resistance $\alpha\beta$ heterodimers in endosomes. *EMBO J.* 11: 411–416.
36. Germain, R. N., and L. R. Hendrix. 1991. MHC class II structure, occupancy and surface expression determined by post-endoplasmic reticulum antigen binding. *Nature* 353: 134–139.
37. Brachet, V., G. Raposo, S. Amigorena, and I. Mellman. 1997. Ii chain controls the transport of major histocompatibility complex class II molecules to and from lysosomes. *J. Cell Biol.* 137: 51–65.
38. Robertson, J. M., P. E. Jensen, and B. D. Evavold. 2000. DO11.10 and OT-II T cells recognize a C-terminal ovalbumin 323-339 epitope. *J. Immunol.* 164: 4706–4712.
39. Petersen, T. R., E. Bettelli, J. Sidney, A. Sette, V. Kuchroo, and B. T. Backstrom. 2004. Characterization of MHC- and TCR-binding residues of the myelin oligodendrocyte glycoprotein 38–51 peptide. *Eur. J. Immunol.* 34: 165–173.
40. Lennon-Dumenil, A. M., A. H. Bakker, R. Maehr, E. Fiebiger, H. S. Overkleeft, M. Rosemblatt, H. L. Ploegh, and C. Lagaudriere-Gesbert. 2002. Analysis of protease activity in live antigen-presenting cells shows regulation of the phagosomal proteolytic contents during dendritic cell activation. *J. Exp. Med.* 196: 529–540.
41. Honey, K., K. Benlagha, C. Beers, K. Forbush, L. Teyton, M. J. Kleijmeer, A. Y. Rudensky, and A. Bendelac. 2002. Thymocyte expression of cathepsin L is essential for NKT cell development. *Nat. Immunol.* 3: 1069–1074.
42. Maric, M. A., M. D. Taylor, and J. S. Blum. 1994. Endosomal aspartic proteinases are required for invariant-chain processing. *Proc. Natl. Acad. Sci. USA* 91: 2171–2175.
43. Mendel Kerlero de Rosbo, N., and A. Ben-Nun. 1996. Delineation of the minimal encephalitogenic epitope within the immunodominant region of myelin oligodendrocyte glycoprotein: diverse Vβ gene usage by T cells recognizing the core epitope encephalitogenic for T cell receptor Vβb and T cell receptor Vβa H-2^b mice. *Eur. J. Immunol.* 26: 2470–2479.
44. Turk, V., B. Turk, and D. Turk. 2001. Lysosomal cysteine proteases: facts and opportunities. *EMBO J.* 20: 4629–4633.
45. Burster, T., A. Beck, E. Tolosa, V. Marin-Esteban, O. Rotzschke, K. Falk, A. Lautwein, M. Reich, J. Brandenburg, G. Schwarz, et al. 2004. Cathepsin G, and not the asparagine-specific endoprotease, controls the processing of myelin basic protein in lysosomes from human B lymphocytes. *J. Immunol.* 172: 5495–5503.
46. Ishidoh, K., and E. Kominami. 2002. Processing and activation of lysosomal proteinases. *Biol. Chem.* 383: 1827–1831.
47. Ishidoh, K., T. C. Saido, S. Kawashima, M. Hirose, S. Watanabe, N. Sato, and E. Kominami. 1998. Multiple processing of procathepsin L to cathepsin L in vivo. *Biochem. Biophys. Res. Commun.* 252: 202–207.
48. Honey, K., T. Nakagawa, C. Peters, and A. Rudensky. 2002. Cathepsin L regulates $CD4^+$ T cell selection independently of its effect on invariant chain: a role in the generation of positively selecting peptide ligands. *J. Exp. Med.* 195: 1349–1358.
49. Felbor, U., L. Dreier, R. A. Bryant, H. L. Ploegh, B. R. Olsen, and W. Mothes. 2000. Secreted cathepsin L generates endostatin from collagen XVIII. *EMBO J.* 19: 1187–1194.
50. Fiebiger, E., R. Maehr, J. Villadangos, E. Weber, A. Erickson, E. Bikoff, H. L. Ploegh, and A. M. Lennon-Dumenil. 2002. Invariant chain controls the activity of extracellular cathepsin L. *J. Exp. Med.* 196: 1263–1269.
51. Goulet, B., A. Baruch, N. S. Moon, M. Poirier, L. L. Sansregret, A. Erickson, M. Bogyo, and A. Nepveu. 2004. A cathepsin L isoform that is devoid of a signal peptide localizes to the nucleus in S phase and processes the CDP/Cux transcription factor. *Mol. Cell* 14: 207–219.
52. Barrett, A. J., N. D. Rawlings, and J. F. Woessner. 1998. *Handbook of Proteolytic Enzymes*. Academic Press, London.