AACACTCCTGCCATG-3′) and Act11R (5′-CTGCAAGGTCCAAACGCAGA-3′) for *ACT11*, and At2S2F (5′-GAGCCAGTTTGTGTTTGC-3′) and At2S2R (5′-TAAGGAGGGAAGAAAGGG-3′) for *At2S2*.
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34. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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36. We thank J. Moore for help with the mutagenesis, M. Koornneef for providing the parental tetraploid line, W. Herr, R. Martienssen, and B. Stillman for comments on the manuscript, and the Hernandez, Hengartner, and Stillman laboratories for space on sequencing gels. Special thanks go to V. Sundaresan and R. Martienssen for providing us access to their insertional mutagenesis system before publication and to S. Strome for allowing us to cite unpublished data. Supported by the Cold Spring Harbor Laboratory President's Council, the Robertson Research Foundation, and fellowships from the European Molecular Biology Organization, the Human Frontier Science Program, and the Janggen-Poehn Foundation to U.G.

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Cathepsin L: Critical Role in Ii Degradation and CD4 T Cell Selection in the Thymus

Terry Nakagawa,* Wera Roth,* Phillip Wong, Andrew Nelson, Andrew Farr, Jan Deussing, Jose A. Villadangos, Hidde Ploegh, Christoph Peters, Alexander Y. Rudensky†

Degradation of invariant chain (Ii) is a critical step in major histocompatibility complex class II–restricted antigen presentation. Cathepsin L was found to be necessary for Ii degradation in cortical thymic epithelial cells (cTECs), but not in bone marrow (BM)–derived antigen-presenting cells (APCs). Consequently, positive selection of $CD4^+$ T cells was reduced. Because different cysteine proteinases are responsible for specific Ii degradation steps in cTECs and BM-derived APCs, the proteolytic environment in cells mediating positive and negative selection may be distinct. The identification of a protease involved in class II presentation in a tissue-specific manner suggests a potential means of manipulating $CD4^+$ T cell responsiveness in vivo.

Major histocompatibility complex (MHC) class II molecules are assembled with the assistance of Ii in the endoplasmic reticulum (ER) and transported to an endocytic compartment where Ii undergoes rapid degradation by lysosomal proteinases (*1*). Peptides derived from amino acids 81 to 104 of Ii are termed CLIP (class II–associated Ii peptides), and remain bound in the class II peptide binding groove, until removed by the chaperone molecule H-2M (*1*). This allows peptides derived from the proteolytic degradation of foreign and self proteins (*2*) to then bind class II molecules and appear on the cell surface. Self peptide–class II complexes expressed on cTECs positively select maturing $CD4^+$ T cells, whereas those expressed by BM-derived APCs mediate negative selection (*3*).

Cathepsins are lysosomal endoproteinases that have been implicated in the MHC class II processing pathway by in vitro studies using purified enzymes or cathepsin inhibitors (*4*). Because of the apparent redundancy of these enzymes and the inherent difficulty of mimicking the complex proteolytic environment of endocytic compartments, the specific role of cathepsins in the class II presentation pathway in vivo remains unclear. Generation of cathepsin-deficient mice (*5*, *6*) provided a direct experimental approach toward the elucidation of their specific functions and targets.

Cathepsin L (CTSL)–deficient mice have periodic shedding of fur and abnormal skin morphology, which recapitulate the spontaneous mouse mutation *furless* (*6*). To assess the role of CTSL in the class II presentation pathway, we examined splenic APCs from $ctsl^{-/-}$ and littermate control mice (Fig. 1A). Flow cytometric analysis of these cells stained with monoclonal antibodies (mAbs) specific for several independent I-A^b epitopes, and for I-A^b bound to CLIP (*7*) and non-CLIP peptides (*8*), did not reveal any effects of the CTSL mutation on class II expression. Similarly, class II expression was not altered in splenic dendritic cells (sDCs) and thioglycollate-activated, interferon-γ–induced peritoneal macrophages (pMph) (*9*). In addition, splenocytes, sDCs and peritoneal Mph from $ctsl^{-/-}$, $ctsl^{+/-}$, and wild-type (WT) mice did not reveal significant differences in the amounts of Ii or SDS-stable and SDS-unstable I-A^b dimers (*9*). No differences were found in presentation by $ctsl^{-/-}$ and littermate control splenocytes or peritoneal macrophages of a broad range of foreign and self antigens, including hen egg lysozyme, ribonuclease A (RNase A), *Trypanosoma cruzi* SA85 major surface antigen, heat-killed *Chlamydia trachomatis*, immunoglobulin M (IgM), β_2-microglobulin (β_2-M), H-2M, and actin (Fig. 1B) (*9*). Thus, class II expression in peripheral APCs and their ability to present self and foreign antigens were not impaired in $ctsl^{-/-}$ mice. This finding, in conjunction with the observation of normal class II presentation in cathepsin B null ($ctsb^{-/-}$) mice (*9*), implied that another cysteine proteinase, perhaps cathepsin S (CTSS) (*10*), must be involved in class II processing in peripheral APCs.

To determine whether the CTSL deficiency affects $CD4^+$ T cell selection, we next analyzed thymocytes and splenocytes from $ctsl^{-/-}$, $ctsl^{+/-}$, and WT mice for CD4, CD8, and T cell antigen receptor (TCR) expression. Although the total cellularity of the lymphoid organs in all these mice was comparable, $ctsl^{-/-}$ mice had reduced numbers of $CD4^+$ T cells in the thymus and periphery (~60 to 80% reduction) (Fig. 2A). Consequently, $CD8^+$ T cells were relatively increased. In contrast, T cell subsets were normal in $ctsl^{+/-}$ littermates and in cathepsin B ($ctsb^{-/-}$) and D ($ctsd^{-/-}$) null mice (Fig. 3) (*9*). Purified $CD4^+$ T cells isolated from immunized $ctsl^{-/-}$ mice were

T. Nakagawa, P. Wong, A. Y. Rudensky, Howard Hughes Medical Institute and Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195, USA.
W. Roth, J. Deussing, C. Peters, Medizinische Molekularbiologie, Abteilung Heamatologie-Onkologie, Klinikum der Albert-Ludwigs-Universitat Freiburg, 79106 Freiburg, Germany.
A. Nelson and A. Farr, Department of Biological Structure and Department of Immunology, University of Washington School of Medicine, Seattle, WA 98195, USA.
J. A. Villadangos and H. Ploegh, Department of Pathology, Harvard Medical School, Boston, MA 02115, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed: E-mail: sasha@nucleus.immunol.washington.edu

able to mount an antigen-specific proliferative response to Eα52-68 peptide, a known I-A^b binder, although at a lower level than *ctsl*$^{+/-}$ mice (Fig. 2B). Furthermore, *ctsl*$^{-/-}$ CD4$^+$ T cells mounted a mixed lymphocyte reaction to irradiated allogeneic BALB/c splenocytes, whereas responses against syngeneic B6, *ctsl*$^{+/-}$, and *ctsl*$^{-/-}$ splenocytes were low (9). Thus, CD4$^+$ T cells from *ctsl*$^{-/-}$ mice appeared to be functional and not autoreactive.

Impaired CD4$^+$ T cell development in *ctsl*$^{-/-}$ mice may result from CTSL deficiency in the T cells themselves or in the cTECs involved in T cell selection. TECs are radiation-resistant, whereas BM-derived T lineage cells are radiation-sensitive. To further elucidate the mechanism of CD4$^+$ T cell deficiency, we constructed reciprocal radiation BM chimeras by transferring BM cells from *ctsl*$^{-/-}$ or Ly5-marked WT mice into lethally irradiated *ctsl*$^{-/-}$ or WT hosts (Fig. 3). As controls, *ctsb*$^{-/-}$ mice were analyzed in the same way. CD4$^+$ T cells developed normally in all the WT recipients; in contrast, the reciprocal transfer of the WT BM cells into *ctsl*$^{-/-}$ hosts resulted in decreased thymic and peripheral CD4$^+$ T cells, similar to control *ctsl*$^{-/-}$ → *ctsl*$^{-/-}$ chimeras. Thus, the lack of CTSL in cTECs, but not in BM-derived cells, is responsible for the deficient selection of CD4$^+$ T cells.

This defect may be due to a differential expression or function of CTSL in the thymus versus spleen. To address this question, we first performed active-site labeling of cysteine proteinases with benzyloxycarbonyl-[^{125}I]-Tyr-Ala-CN_2 with cells isolated from thymus, spleen, liver, and kidney. CTSL activity was detected in thymus, liver, and kidney, but not in the spleen (Fig. 4). Within the thymus, CTSL activity was found in cTECs and thymocytes, but not in BM-derived thymic DCs; whereas CTSB was ubiquitously expressed (Fig. 4). In contrast, CTSS activity was detected in thymic DCs (tDCs) but not cTECs. Lack of CTSL did not result in compensatory expression of CTSS (9). Finally, splenic DCs and B cells exhibit only CTSS, whereas activated pMph display both CTSS and CTSL activities (9). These findings indicate that CTSL and CTSS activities are differentially expressed in most BM-derived APC versus cTECs, whereas CTSB activity is abundant in both cell lineages.

Strong evidence for the importance of CTSL in class II processing in cTECs was provided by immunohistochemistry of thymic tissue sections (Fig. 5A). The I-A^b–specific mAb Y3P intensely stained medullary and cortical regions in WT and mutant mice. However, when thymic sections were stained with 30-2, which binds CLIP–I-A^b complexes, the cortex of littermate control thymi stained weakly, but cortical 30-2 staining of *ctsl*$^{-/-}$ thymi was increased. In contrast, medullary 30-2 labeling was not significantly changed in *ctsl*$^{-/-}$ thymi. The 30-2 mAb also recognizes I-A^b bound to larger intermediates of Ii degradation [the 21- to 24-kD leupeptin-induced peptide (LIP) and ~12-kD small LIP (SLIP) fragments] in addition to CLIP (*11*); such intracellular complexes may contribute to the overall increase in

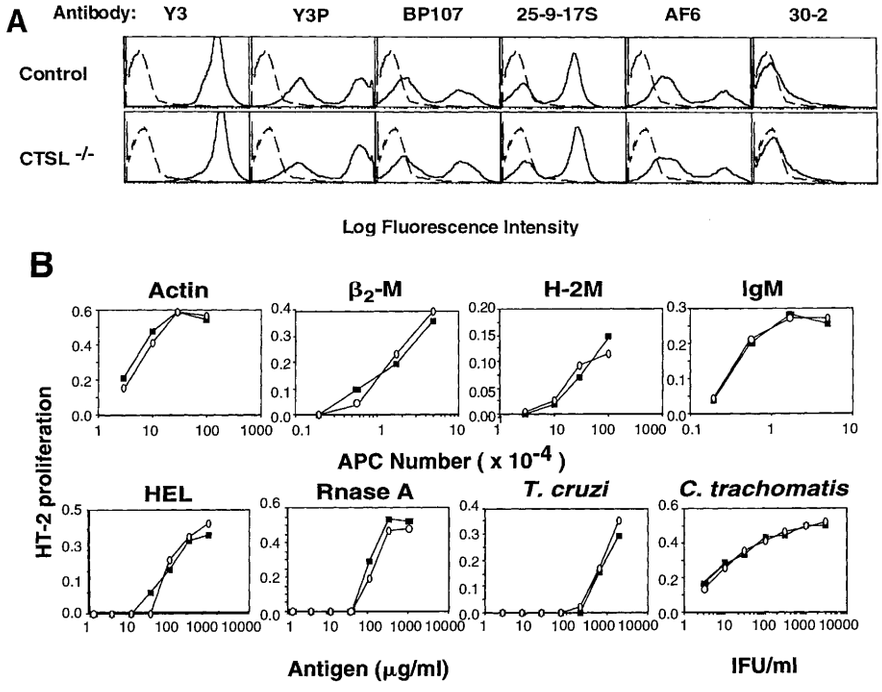


Fig. 1. Normal expression of surface MHC class II molecules and antigen-presenting function of *ctsl*$^{-/-}$ SCs. (**A**) Expression of class II molecules in control and mutant SCs (*18*). SCs from 7-week-old littermates were analyzed by flow cytometry after staining with the following biotinylated mAb antibodies (*19*): Y3P, AF6, 25-9-17S, BP107, 30-2 (solid line), or control mAb Y17 (dotted line). Results represent one of five identical experiments. (**B**) T cell hybrid responses to peptides derived from endogenous (actin, β_2-M, H-2M, and IgM) and exogenous (HEL, RNase A, heat-killed *C. trachomatis*, recombinant *T. cruzi* outer surface protein) protein antigens presented by SCs from control (open circle) and mutant (closed square) mice (*19*). *Chlamydia trachomatis* titration is shown as infectious units (IFU)/ml. IL-2 production was detected by the IL-2 indicator cell line HT-2 and measured by Alamar Blue colorimetric assay. Results are presented as mean arbitrary OD units (A_{570}-A_{600}). Standard deviations in all experiments were less than 10% of the mean. Results are shown for one out of three identical experiments.

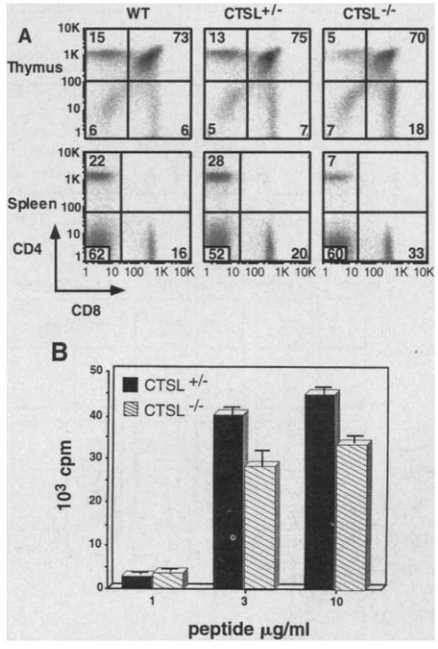


Fig. 2. Reduced number of functional CD4$^+$ T cells in CTSL-deficient mice. (**A**) Flow cytometric analyses of thymocytes and SCs from 7-week-old *ctsl*$^{-/-}$, *ctsl*$^{+/-}$, and WT mice stained for CD4, CD8, and TCRβ chain (*20*). Percentages of CD4$^+$, CD8$^-$, and CD4$^+$CD8$^+$ cells are indicated in each quadrant. The TCR expression levels were similar on corresponding mutant and control T cell subpopulations (*21*). Results of 1 of 10 identical experiments are shown. (**B**) In vitro proliferative responses of purified CD4$^+$ T cells from mutant and control mice to Eα52-68 peptide. Lymph node CD4$^+$ T cells isolated on day 9 after priming were cultured in the presence or absence of Eα52-68 peptide for 72 hours. Results are presented as mean counts per minute of [^{3}H]thymidine incorporation in triplicate cultures. A representative of three similar experiments is shown.

30-2 staining in $ctsl^{-/-}$ thymi. LIP and SLIP fragments contain the NH_2-terminal portion of Ii (*4*), thus, accumulation of these fragments or intact Ii would result in increased labeling with the In-1 mAb, specific for this region of Ii (*12*). Indeed, cortical In-1 staining was also increased in $ctsl^{-/-}$ versus control thymic tissue (Fig. 5A). Because H-2M regulates the amount of CLIP–I-A^b complexes (*8*), we also assessed H-2M expression in $ctsl^{-/-}$ and control thymi. H-2M was not changed in the $ctsl^{-/-}$ thymus nor was another class II–like molecule, H-2O, which interacts with H-2M (*9*, *13*).

To differentiate between the accumulation of intact Ii or its fragments in cTECs, we performed protein immunoblot analysis of thymic stroma from mutant and control mice, using the In-1 mAb (Fig. 5B). Both LIP and SLIP Ii fragments accumulated in $ctsl^{-/-}$ thymic stroma, whereas intact p31 and p41 Ii were comparable to control thymic stroma. In contrast, LIP and SLIP expression in splenic stroma or splenocytes was unchanged.

The administration of leupeptin to APCs in vitro results in the accumulation of class II–bound LIP and SLIP fragments as well as a decrease in surface class II (*4*). To assess surface expression of total I-A^b as well as CLIP–I-A^b and non-CLIP–I-A^b complexes, control and $ctsl^{-/-}$ cTECs were analyzed by three-color flow cytometry. Increased 30-2 staining in mutant cTECs was due either to elevated levels of CLIP or the potential escape of LIP or SLIP bound to I-A^b to the plasma membrane. Lower BP107 staining reflects a concomitant decrease in non-CLIP–I-A^b. Detection of total surface I-A^b by Y3P was only moderately decreased (Fig. 5C).

In H-$2M^{-/-}$ mice, most of the class II molecules contain CLIP, and many $CD4^+$ T cells are reactive to syngeneic WT APCs (*8*, *14*). This raised the possibility that expression of an altered self peptide–class II repertoire by $ctsl^{-/-}$ cTECs, but not by $ctsl^{-/-}$ BM-derived APCs, may lead to significant $CD4^+$ T cell deletion. If true, then it might be predicted that the lack of class II molecules on negatively selecting BM-derived cells would result in an increase in $CD4^+$ T cells selected by $ctsl^{-/-}$ cTECs. Indeed, in preliminary experiments, we observed an increase in the thymocyte $CD4^+/CD8^+$ ratio in class $II^{-/-} \rightarrow ctsl^{-/-}$ as compared to $ctsl^{-/-} \rightarrow ctsl^{-/-}$ chimeras (1.4 versus 0.66, respectively). Furthermore, analogous BM chimera experiments showed that three different I-A^b–restricted transgenic TCRs cannot be positively selected by both H-$2M^{-/-}$ and $ctsl^{-/-}$ thymic epithelium (*9*). Thus, altered specificity of positive selection seems to be responsible for the overall low numbers of $CD4^+$ T cells in $ctsl^{-/-}$ mice.

Our results suggest that the proteolytic environments of cTECs and BM-derived APCs, cells that mediate positive versus negative selection of $CD4^+$ T cells (*15*), respectively, are different. This difference

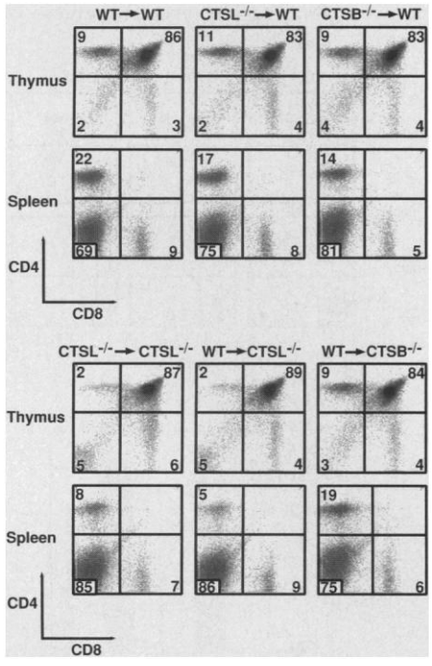
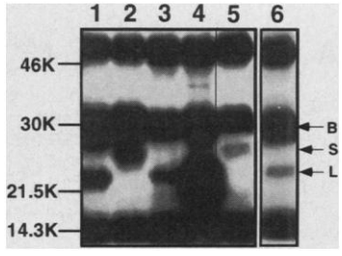


Fig. 3. Radioresistant thymic epithelial cells are responsible for the defect in development of $CD4^+$ T cells in $ctsl^{-/-}$ mice. Seven-week-old WT Ly5-congenic B6.SJL, $ctsb^{-/-}$, and $ctsl^{-/-}$ mice were used both as BM donors and recipients. Flow cytometric analyses of thymocytes and SCs from BM chimeras stained for CD4, CD8, and TCRβ 7 weeks after reconstitution were performed as described (*22*). Chimeric mice showed >90% replacement of host BM cells with cells of donor origin (*21*). Percentages of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ cells are indicated in each quadrant. The TCR expression levels were not different on corresponding mutant and control T cell subpopulations (*21*). Lower numbers of $CD8^+$ SCs in these chimeras versus nonirradiated $ctsl^{-/-}$ mice likely reflect a delayed kinetics of $CD8^+$ versus $CD4^+$ T cell repopulation in the periphery. Results are representative of one of four similar experiments.

Fig. 4. Expression of cysteine proteinases in WT mice. Analysis of cysteine proteinase activity in WT thymus (lane 1), spleen (lane 2), liver (lane 3), kidney (lane 4), tDCs (lane 5), and cTECs (lane 6). Cell suspensions were obtained by mechanical disruption and enzymatic digestion of corresponding tissues; tDCs and cTECs were purified as described (*23*). All cell populations were incubated with the active-site cysteine proteinase labeling reagent benzyloxycarbonyl-[^{125}I]-Tyr-Ala-CN_2 (*24*). Arrows indicate positions of cathepsin B, L, and S in the gel. Molecular size markers (left) are in daltons.



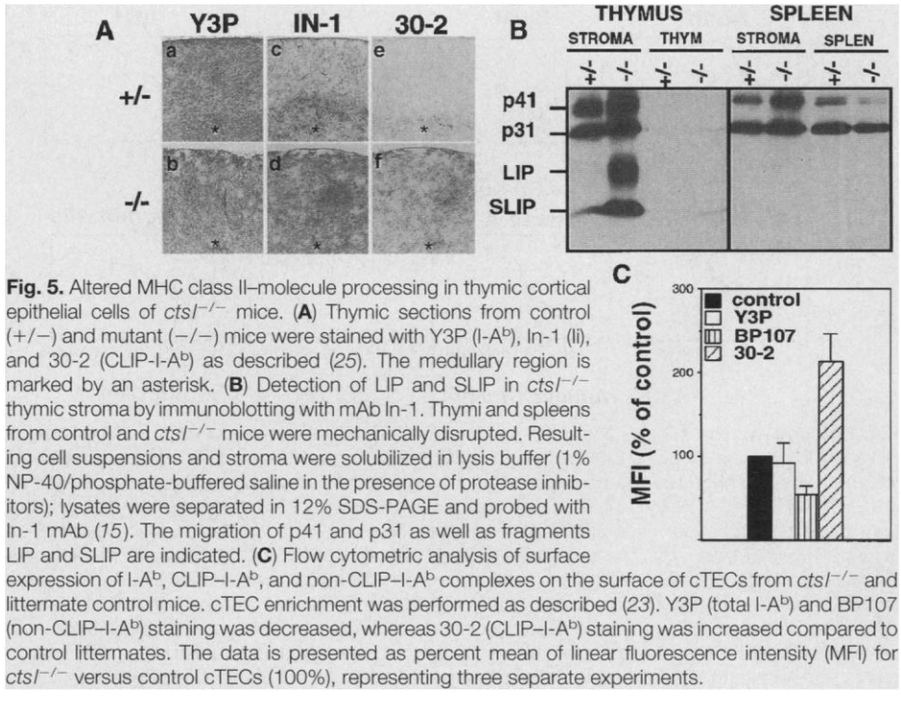


Fig. 5. Altered MHC class II–molecule processing in thymic cortical epithelial cells of $ctsl^{-/-}$ mice. (**A**) Thymic sections from control (+/−) and mutant (−/−) mice were stained with Y3P (I-A^b), In-1 (Ii), and 30-2 (CLIP-I-A^b) as described (*25*). The medullary region is marked by an asterisk. (**B**) Detection of LIP and SLIP in $ctsl^{-/-}$ thymic stroma by immunoblotting with mAb In-1. Thymi and spleens from control and $ctsl^{-/-}$ mice were mechanically disrupted. Resulting cell suspensions and stroma were solubilized in lysis buffer (1% NP-40/phosphate-buffered saline in the presence of protease inhibitors); lysates were separated in 12% SDS-PAGE and probed with In-1 mAb (*15*). The migration of p41 and p31 as well as fragments LIP and SLIP are indicated. (**C**) Flow cytometric analysis of surface expression of I-A^b, CLIP–I-A^b, and non-CLIP–I-A^b complexes on the surface of cTECs from $ctsl^{-/-}$ and littermate control mice. cTEC enrichment was performed as described (*23*). Y3P (total I-A^b) and BP107 (non-CLIP–I-A^b) staining was decreased, whereas 30-2 (CLIP–I-A^b) staining was increased compared to control littermates. The data is presented as percent mean of linear fluorescence intensity (MFI) for $ctsl^{-/-}$ versus control cTECs (100%), representing three separate experiments.

may provide a biochemical foundation for the hypothesis that distinct subsets of self peptides may be displayed by class II molecules on different cell types. Even if cathepsins S and L are not responsible for the endoproteolytic cleavage of specific antigenic target proteins, the differential expression of these enzymes may affect both the site and the rate of Ii degradation within the endocytic compartment of these cells. Drastic differences in expression of major self peptide–class II complexes observed in cTECs and BM-derived APCs (*16*) are consistent with this hypothesis.

Accumulation of LIP and SLIP in cTECs suggest that these fragments, but not intact Ii, are CTSL substrates in vivo. This result is consistent with the model of sequential Ii degradation (*4*, *10*). The nature of the proteinases involved in early stages of Ii degradation remains unclear, because we did not find effects of CTSB and CTSD deficiency in thymic and peripheral MHC class II presentation (9). An in vitro study of Ii cleavage with an inhibitor of CTSS activity suggested that SLIP or an even smaller fragment is cleaved by this cysteine proteinase in splenocytes (*10*). Our finding that CTSL activity is present in cTECs, whereas CTSS is expressed in BM-derived APCs, would explain why class II function is normal in *ctsl*$^{-/-}$ splenocytes. These observations, in conjunction with the normal class II presentation observed in *ctsb*$^{-/-}$ mice (9), implicates CTSS in class II processing in peripheral APCs.

In conclusion, our study shows that CTSL is specifically involved in class II processing, namely, in the late stages of Ii degradation, in cTECs. BM-derived APCs use another cysteine proteinase, most likely cathepsin S (*10*). This is the first identification of a specific protease involved in the class II presentation pathway in vivo in a tissue-specific fashion. Our findings suggest a potential means of manipulating the CD4$^+$ T cell repertoire and intervening in autoimmunity by targeting specific lysosomal enzymes.

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18. Animals used were C57BL/6 (B6), Ly5-congenic B6.SJL (H-2^b, Ly5.1) and were purchased from the Jackson Laboratory. *ctsl*$^{-/-}$ mice (6) and *ctsb*$^{-/-}$ mice (J. Deussing *et al.*, in preparation) are on a mixed B6/129/J background. *ctsl*$^{+/-}$ littermates showed normal phenotype and were used as controls.
19. Single-color analyses for MHC class I and II molecules were done on erythrocyte-depleted splenocytes (SCs) from control and mutant littermates. Cells were incubated on ice with biotinylated mAbs Y3 (anti-K^b; ATCC HB176), Y17 (anti-I-E$^{d/k}$; ATCC HB179), and I-A^b–recognizing mAbs Y3P (ATCC HB183), BP107.2.2 (ATCC TIB 154), 25-9-17S II (ATCC HB26), AF6-120.1.2 (ATCC HB163), and 30-2 (CLIP–I-A^b) (7), followed by streptavidin-Tricolor (Caltag). T cell hybrid assays: I-A^b–restricted T hybrids (0.5 × 10^5 to 1.0 × 10^5 per well) specific for IgM (77.1), actin (15.10), β_2M (4.1), and H-2Mα (33.7) were cultured in duplicates for 20 hours with variable numbers of control or mutant SCs. In parallel, 1 × 10^5 SCs were incubated with T hybrids specific for HEL (BO4), RNase (1BE6A1), *T. cruzi* SA85 (71.5) and *C. trachomatis* (116.3) and variable amounts of exogenous antigen. Interleukin-2 (IL-2) production was assessed using HT-2 proliferation as determined by the Alamar Blue colorimetric assay. The results are expressed as the difference in arbitrary optical density (OD) units at 570 nm versus 600 nm (A_{570}-A_{600}).
20. Thymocytes and SCs were stained with anti-CD4–phycoerythrin (PE), anti-CD8α–fluorescein isothiocyanate (FITC), and anti-TCRβ–biotin mAbs (Pharmingen) followed by streptavidin-Tricolor and analyzed by a FACScan flow cytometer (Becton-Dickinson).
21. T. Nakagawa, P. Wong, A. Y. Rudensky, data not shown.
22. P. Wong and A. Y. Rudensky, *J. Immunol.* **156**, 2133 (1996). Control and mutant mice were injected in the hind footpads with 10 μg of Eα52-68 peptide emulsified in complete Freund's adjuvant. Draining lymph nodes were collected 9 days later, and CD4$^+$ T cells were enriched to 80 to 90% purity with magnetic bead depletion with anti-CD8, anti-B220, and anti-I-A. CD4$^+$ T cells were cultured with indicated doses of Eα52-68 peptide with 5 × 10^5 irradiated (2000 rads) stimulator SC triplicate in 96-well plates. Proliferation was measured as [^{3}H]thymidine incorporation after pulsing with [^{3}H]thymidine (1 μCi per well) for the last 18 hours of a 3-day culture. Chimeric mice were generated by injecting 2 × 10^6 to 4 × 10^6 T cell-depleted BM cells into lethally irradiated (950 rads) recipients. Thymocytes and SCs were analyzed for CD4, CD8, and TCRβ expression 6 to 8 weeks after reconstitution. Reconstitution of recipients by donor BM was greater than 90% as assessed by staining with anti-Ly9.1 or anti-Ly5.1 mAb (Pharmingen).
23. A. G. Farr, D. J. Eisenhardt, S. K. Anderson, *Anat. Rec.* **216**, 85 (1986). Briefly, thymi from 3- to 4-week-old mice were dissociated with a collagenase/dispase/deoxyribonuclease cocktail (Boehringer Mannheim) and fractionated on a discontinuous Percoll gradient. cTEC-enriched low-density fractions were stained with mAbs 6C3 (BP-1, a cTEC marker) (*17*) and N418 (CD11c, a DC marker) in addition to the biotinylated class II–specific mAbs (Y17, Y3P, BP107, 30-2), followed by a secondary cocktail of PE–anti-rat IgG, FITC–anti-hamster IgG (Axell), and streptavidin-Tricolor. The 6C3$^+$N418$^-$ cTEC population was electronically gated and assessed for class II expression. In some experiments, cTEC-enriched fractions were sorted for 6C3$^+$N418$^-$ cTEC on a FACStar Plus cytometer. Cysteine proteinase active-site labeling: 1 × 10^5 to 5 × 10^5 cells were incubated for 2 hours at 37°C with the iodinated cysteine proteinase inhibitor benzyloxycarbonyl-[^{125}I]-Tyr-Ala-CN_2 (Fig. 4). Cells were washed, lysed, and analyzed by 12% SDS–polyacrylamide gel electrophoresis (PAGE).
24. R. W. Mason, D. Wilcox, P. Wikstrom, E. N. Shaw, *Biochem. J.* **257**, 125 (1989).
25. Details of immunohistochemical procedures have been described (*16*). The anti–class II mAb Y3P, Ii-specific mAb In-1 (*12*), and CLIP–I-A^b–specific 30-2 were biotinylated and used for staining. Sections were developed with 3,3′-diaminobenzidine without counterstaining. Control mAb Y17, specific for I-E^b, showed minimal background staining.

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