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Cathepsin S Required for Normal MHC Class II Peptide Loading and Germinal Center Development

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Summary

Major histocompatibility complex (MHC) class II molecules acquire antigenic peptides after degradation of the invariant chain (Ii), an MHC class II–associated protein that otherwise blocks peptide binding. Antigen-presenting cells of mice that lack the protease cathepsin S fail to process Ii beyond a 10 kDa fragment, resulting in delayed peptide loading and accumulation of cell surface MHC class II/10 kDa Ii complexes. Although cathepsin S–deficient mice have normal numbers of B and T cells and normal IgE responses, they show markedly impaired antibody class switching to IgG2a and IgG3. These results indicate cathepsin S is a major Ii-processing enzyme in splenocytes and dendritic cells. Its role in humoral immunity critically depends on how antigens access the immune system.

Introduction

The major histocompatibility complex (MHC) class II–associated invariant chain (Ii) is a type II transmembrane protein that complexes with newly synthesized MHC class II α/β heterodimers and directs their trafficking through the endosomal compartments of antigen-presenting cells (APC) (Cresswell, 1994; Germain, 1994; Wolf and Ploegh, 1995; Watts, 1997). The luminal domain of Ii organizes MHC class II dimers into nonameric complexes and prevents premature association of MHC class II molecules with endogenous polypeptides. Within endosomal/lysosomal compartments, Ii undergoes stepwise proteolytic degradation of its luminal domain to yield progressively smaller fragments of Ii that remain associated with the peptide-binding groove of MHC class II dimers. The smallest of these are a set of ~3 kDa peptides termed CLIP (*cl*ass II–associated *i*nvariant chain *p*eptides) (Ghosh et al., 1995). Within endosomal compartments, CLIP rapidly dissociates from MHC class II dimers in the presence of DM, a MHC class II–like molecule (Sherman et al., 1995; Sloan et al., 1995; Fung-Leung et al., 1996; Miyazaki et al., 1996). Thus, endosomal conversion of Ii to CLIP generates the substrate for DM and allows loading and subsequent surface expression of MHC class II molecules with peptides generated from endocytosed protein.

Although numerous lysosomal proteases have been implicated in Ii proteolytic processing (Cresswell, 1994; Germain, 1994; Wolf and Ploegh, 1995; Watts, 1997), recent studies have shown that cathepsin S, a papain-type cysteine protease expressed in most if not all APC, is capable of generating CLIP from Ii in association with MHC class II dimers. Further, selective inhibition of cathepsin S in human B cell lines and murine splenocytes impairs Ii processing, CLIP formation, and MHC class II peptide loading in vitro (Riese et al., 1996, 1998; Villadangos et al., 1997; Pierre and Mellman, 1998). To establish the role of cathepsin S in Ii processing and MHC class II–restricted antigen presentation in vivo, we have targeted the murine cathepsin S gene by homologous recombination and characterized MHC class II function in the resulting mutant mice.

Results and Discussion

Targeting of Mouse Cathepsin S Gene

Mouse cathepsin S gene exon 5, which contains the active site cysteine of murine cathepsin S, was deleted by recombination with a targeting vector (Figure 1A) in ES 129 cells. Mice homozygous for mutant cathepsin S were generated in an MHC class II I-A^b background (Figure 1B). Cathespin S $-/-$ ($catS^{-/-}$) mice have normal fertility and appear normal up to 6 months in microisolators. Splenocytes and purified dendritic cells from $catS^{-/-}$ mice contained no detectable cathepsin S protein by immunoblotting (Figure 1C) or by active site labeling with [^{125}I]Z-tyr-ala-diazomethylketone, an irreversible inhibitor of cysteine proteases (Riese et al., 1998) (data not shown). Other cathepsins were comparably expressed in $catS^{+/+}$ and $catS^{-/-}$ mice as judged by immunoprecipitation of cathepsin D and immunoblotting and active site labeling of the cysteine proteases cathepsins B and L in extracts of spleen and lung (data not shown). FACS analyses of thymic, lymph node, and splenic lymphocytes from 1–2 month old animals revealed normal ratios of $B220^+$ B cells and $CD4^+$ and $CD8^+$ T cells and equivalent expression of the differentiation markers CD44 and CD69 on lymph node T cells. Purified splenic T cells from $catS^{-/-}$ and $catS^{+/+}$ mice proliferated equally in response to allogeneic stimulation (data not shown). We conclude that $catS^{-/-}$ mice do not show major developmental abnormalities in their lymphocytes.

Analysis of MHC Class II and Ii Processing

In splenocytes from normal mice, MHC class II–associated Ii was almost fully degraded and replaced with peptides within hours of synthesis (Figure 2A). In contrast, MHC class II complexes in splenocytes from $catS^{-/-}$ mice retained an ~10 kDa fragment of Ii (Ii p10),

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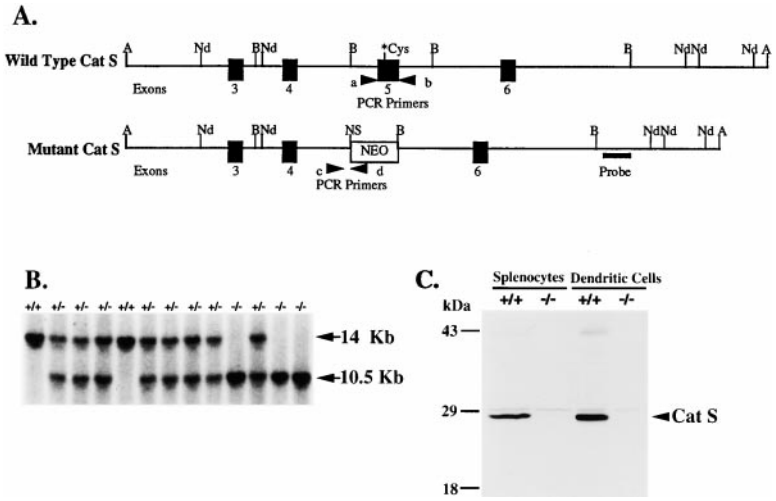


Figure 1. Generation of Mice with Cathepsin S Deficiency

(A) Scheme of the targeted murine cathepsin S gene after homologous recombination. "*Cys" denotes the active site cysteine critical for enzyme activity that is deleted along with all of exon 5 in the mutant gene. A, Apa1; Nd, Nde1; B, BamH1; N, Not 1; S, Sal 1.

(B) Southern blot showing the expected sizes of +/+ (14 kb) and −/− (10.5 kb) cathepsin S gene fragments when hybridized with the external probe denoted in (A). Most of the mice were subsequently tested by polymerase chain reactions using primer pairs "ab" and "cd," as shown in the scheme, which give 150 bp (wt) and 170 bp (mt) products, respectively.

(C) Western blot of lysates of splenocytes and dendritic cells obtained from +/+ and −/− mice showing the absence of immunoreactive cathepsin S in the −/− mice. Similar results were seen when intact splenocytes were labeled with the irreversible inhibitor of cysteine proteases, Z-tyr-ala-CHN2 (Riese et al., 1996). Levels of the other major cysteine protease in splenocytes identified by this inhibitor, cathepsin B, were unaffected by the mutation in cathepsin S.

the identity of which was confirmed by immunoprecipitation with anti-Ii antibodies (data not shown). Prior studies of Ii processing have shown that Ii p10 is comprised of the region of Ii extending from its N terminus through the C terminus of CLIP (Villandangos et al., 1997). Accumulation of MHC class II/Ii p10 complexes coincided with a near total blockade of MHC class II peptide loading as revealed by a failure to acquire SDS resistance (Figure 2A). Indistinguishable results were observed with unfractionated lymph node cells from cathepsin S–deficient mice and with dendritic cells purified from spleens of cathepsin S–deficient mice exposed 9–14 days to the cytokine flt-3 known to promote proliferation and maturation of dendritic cells in vivo (Maraskovsky et al., 1996; Shurin et al., 1997) (data not shown).

Surprisingly, steady-state surface expression of MHC class II molecules on splenocytes of *catS*$^{-/-}$ mice was not reduced significantly. Both FACS analysis (Figure 2B) and direct surface labeling of splenocytes followed by immunoprecipitation of MHC class II complexes (Figure 2C) showed surface expression of α/β heterodimers approximately 75% (range 50%–100% in five different experiments) that of normal littermates. Surface MHC class II complexes from splenocytes of *catS*$^{-/-}$ mice consisted of SDS-stable MHC class II/peptide complexes and the complexes of MHC class II containing Ii p10 (Figure 2C); the latter are absent from *catS*$^{+/+}$ mice.

To assess the rate of appearance of newly synthesized MHC class II complexes on cell surfaces, splenocytes were pulse labeled for 1.5 hr with [^{35}S]methionine and surface biotinylated 3 or 20 hr later. MHC class II complexes were immunoprecipitated and the biotinylated fraction reprecipitated with avidin-conjugated agarose beads. Whereas newly synthesized MHC class II molecules of *catS*$^{+/+}$ mice appear on the cell surface within 3 hr, no such complexes were seen in *catS*$^{-/-}$ mice, which instead show surface MHC class II dimers in association with Ii p10 after 20 hr (Figure 2D). In mixed lymphocyte reactions, proliferation of I-A^{d} splenocytes in response to stimulation with splenocytes of *catS*$^{-/-}$ I-A^{b} mice was reduced ~50% compared to stimulation with cells from +/+ littermate controls, consistent with a significant fraction of surface I-A^{b} of *catS*$^{-/-}$ mice being occupied by Ii p10 (data not shown). Nonetheless, while peptide loading and surface expression of newly synthesized MHC class II heterodimers in *catS*$^{-/-}$ mice are markedly delayed, and many retain Ii p10, a large fraction ultimately acquire peptide and are displayed on the cell surface (Figure 2C).

Immunostaining revealed cathepsin S immunoreactivity localized to medullary regions of thymus (data not shown). To explore the possible role of cathepsin S in Ii processing in thymus, both extracts of thymocytes and thymic stroma were immunoblotted with antibodies against Ii and MHC class II–α/β dimers and the accumulation of processing intermediates compared with that in spleens of *catS*$^{-/-}$ mice. As expected, steady-state levels of both Ii-p10 and SDS stable α/β/Ii-p10 complexes were markedly increased in spleens of cathepsin S–deficient mice (Figure 3). In contrast, little Ii-p10 and no stable α/β/Ii-p10 complexes were detectable in thymic stroma or thymocytes of *catS*$^{+/+}$ or *catS*$^{-/-}$ mice. This contrasts with the marked accumulation of Ii-p10 in thymic stroma of cathepsin L–deficient mice (Nakagawa et al., 1998) and indicate that cathepsin S has only a minor role in Ii processing in thymus. This conclusion is consistent with the normal levels of CD4^{+} T cells in spleen and lymph nodes and the lack of autoreactivity seen in *catS*$^{-/-}$ animals.

Antigen Presentation In Vitro

To examine the influence of altered MHC class II processing in mutant mice on antigen presentation, established T cell hybridomas derived from *catS*$^{+/+}$ I-A^{b} mice that recognize either an ovalbumin (OVA) peptide (323–339) or a hen egg lysozyme (HEL) peptide (74–88) were exposed to OVA or HEL protein and APC from normal and mutant mice in vitro. Splenocytes and purified, mostly mature dendritic cells (high surface I-A^{b}) from normal mice induced IL-2 secretion in response to either

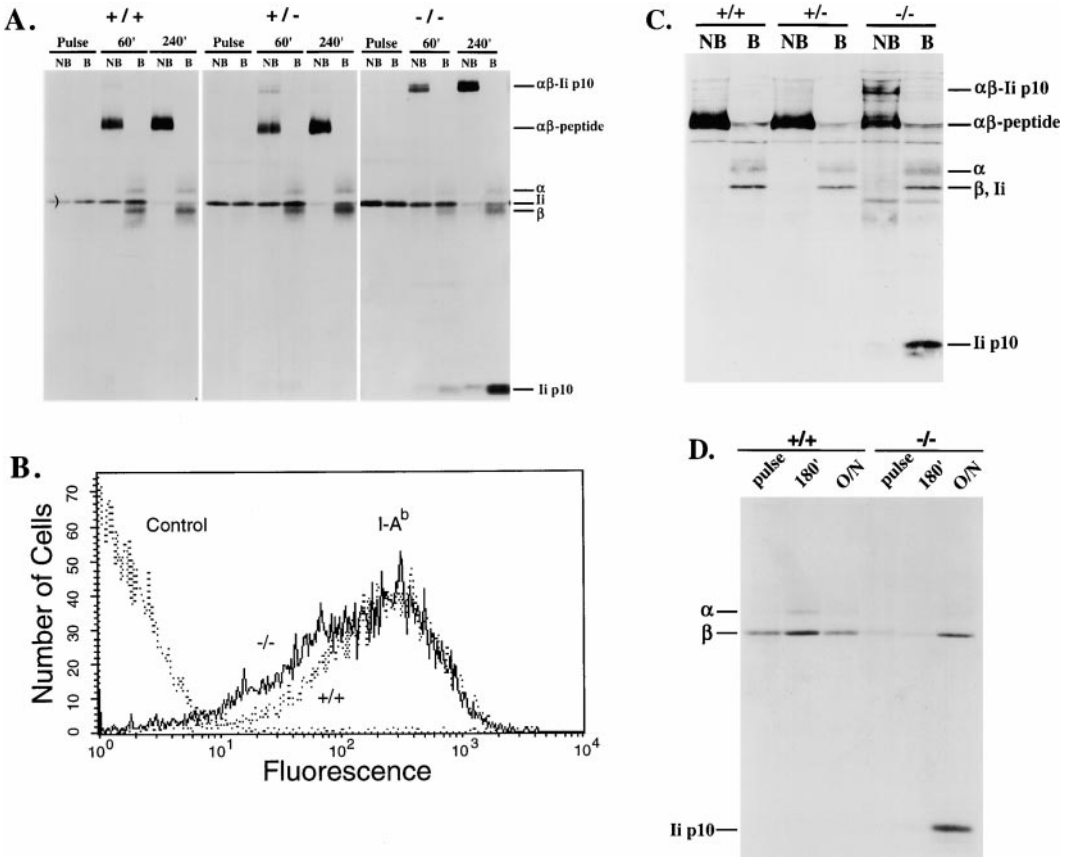


Figure 2. Cathepsin S–Deficient Mice Have Altered Processing and Surface Expression of MHC Class II Molecules

(A) Immunoprecipitates of MHC class II complexes from freshly isolated splenocytes following a 30 min pulse label with [^{35}S]methionine and 1 and 4 hr chases in unlabeled medium were analyzed without (NB) or after (B) boiling by SDS-PAGE. No SDS-stable peptide/MHC class II complexes are generated in $-/-$ mice, and instead MHC class II dimers remain associated with a Ii fragment (α/β Ii p10), which dissociates upon boiling of the complexes.

(B) FACs analysis of surface MHC class II molecules on splenocytes of $+/+$ and $-/-$ mice.

(C and D) Immunoprecipitation of MHC class II complexes following cell surface biotinylation at 4°C of isolated splenocytes. In (C), cells were biotinylated without metabolic labeling. In (D), cells were biotinylated 1.5, 3, and 20 hr after pulse labeling, and then MHC class II complexes were immunoprecipitated, boiled, and reprecipitated with avidin beads before autoradiography to reveal surface MHC class II molecules. Only splenocytes of $-/-$ mice show accumulation of surface α/β Ii p10 under steady-state conditions (C). In these mice, cell surface expression of MHC class II dimers is markedly delayed following their synthesis and most such dimers appear to contain Ii p10 after 20 hr (D).

antigen in a dose-dependent manner (Figures 4A–4C). Splenocytes and dendritic cells from *catS*$^{-/-}$ mice failed to process and present OVA. In separate studies, we have determined that inhibition of cathepsin S by a selective protease inhibitor does not block generation of peptide 323–339 in murine splenocytes but does block presentation to the same hybridoma (Riese et al., 1998). In contrast, APC from *catS*$^{-/-}$ mice presented the HEL epitope better than APC from *catS*$^{+/+}$ mice (Figure 4D). Splenocytes of *catS*$^{+/+}$ and *catS*$^{-/-}$ mice cocultured with the corresponding peptide epitopes ($OVA_{323–339}$ and $HEL_{74–88}$) stimulated comparable IL-2 production by the responding hybridomas (Figure 4). Thus, *catS*$^{-/-}$ APC have the intrinsic capacity to generate and even present certain antigenic epitopes despite retained Ii p10 in MHC class II dimers. This observation is consistent with both enhancing and inhibiting effects of Ii on MHC class II peptide loading (Peterson et al., 1992; Momburg et al., 1993; Bodmer et al., 1994) and raised the possibility that, in mutant mice, antigen presentation in vivo either could be unaffected or selectively impaired depending on the antigen examined.

Immunization in Freund's Adjuvant

Mice were immunized in footpads with a mixture of OVA and HEL in complete Freund's adjuvant (CFA). Ipsilateral- and contralateral-draining lymph nodes were examined 9 days later. Primary B cell follicles within the contralateral lymph nodes of *catS*$^{-/-}$ mice were consistently smaller than those of their *catS*$^{+/+}$ littermates (Figures 5A and 5E). Ipsilateral lymph nodes obtained from both immunized *catS*$^{+/+}$ and *catS*$^{-/-}$ mice were enlarged to a similar degree (3- to 5-fold) over the contralateral nodes. Microscopic examination (Figures 5B and 5F) revealed the expected numerous germinal centers in *catS*$^{+/+}$ mice but the near complete absence of germinal centers in *catS*$^{-/-}$ mice, suggesting failure of local B cell activation and proliferation in these mice. Tissue sections were stained with the lectin peanut agglutinin (PNA), which binds to immature, proliferating B cells within germinal

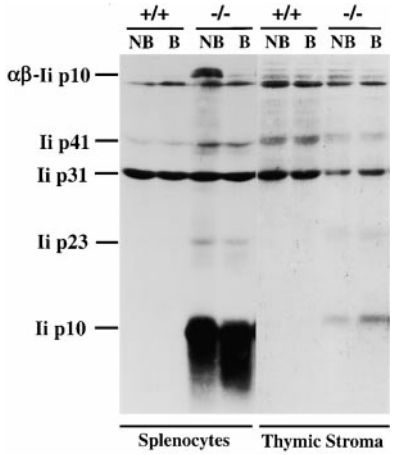


Figure 3. Invariant Chain Accumulates in Spleen but Not Thymus of Cathepsin S–Deficient Mice

Equal amounts of protein extracts of splenocytes and thymic stroma from $+/+$ and $-/-$ mice were immunoblotted for Ii (J5V, recognizing the Ii N terminus). The higher M_r species seen in the splenic extracts of $-/-$ mice was confirmed as MHC class II/Ii complexes by immunoblotting the same filter with class II antibodies. Extracts of thymocytes were immunoblotted similarly but showed no specific staining for Ii.

centers. PNA stained central follicular areas in *catS*$^{+/+}$ mice but showed much weaker or no organized staining in lymph node follicles of *catS*$^{-/-}$ mice (Figures 5C and 5G). Activated B cells within germinal centers undergo a process of somatic hypermutation of immunoglobulin (Ig) genes, leading to antigen-driven selection of high-affinity antibodies at these sites. Cells which produce Ig that bind antigen weakly or not at all become apoptotic (Pulendran et al., 1995; Shokat and Goodnow, 1995; Kelsoe, 1996; Liu and Arpin, 1997). As assessed by assays for increased DNA strand breaks, lymph nodes from the *catS*$^{-/-}$ mice were virtually devoid of organized apoptotic activity (Figure 5H), whereas clusters of apoptotic cells were widespread in the draining nodes of immunized *catS*$^{+/+}$ mice (Figure 5D). Thus, lymphocyte activation and proliferation in the context of antigenic stimulation in vivo is markedly depressed in the absence of cathepsin S. This interpretation is supported by results of functional assays of antigen presentation using unfractionated lymph node cells from the immunized mice. Proliferation of T lymphocytes obtained directly from immunized *catS*$^{-/-}$ mice were depressed following restimulation with antigens in vitro (Figure 5I), indicating defective in vivo presentation and sensitization.

Immunoglobulin Isotype Switching Analysis

Activated B cells not only undergo somatic hypermutation and selection within germinal centers, but they also undergo isotype switching from IgM to IgG1 as well as other isotypes depending on the cytokine milieu in which activation occurs (Kelsoe, 1996; Shokat and Goodnow, 1995). Because lymph node germinal center development is defective in *catS*$^{-/-}$ mice, we assessed the profile of Ig isotypes produced by normal and mutant mice in response to immunization. Animals were given a mixture of OVA and HEL admixed in CFA by peritoneal injection, boosted 7 days later, and antibody titers determined at 2 weeks. Whereas *catS*$^{+/+}$ mice develop IgM and the expected range of IgG isotypes in response to either antigen (Figure 6), *catS*$^{-/-}$ mice show IgM responses comparable to *catS*$^{+/+}$ mice but strongly diminished IgG responses (Figures 6A and 6B). Markedly deficient ($<$ 20% control) IgG 2a and IgG 3 responses to either antigen were observed in *catS*$^{-/-}$ mice. The lack of an IgG response could not be explained by a general defect in B cell proliferation, as splenocytes obtained from *catS*$^{+/+}$ and *catS*$^{-/-}$ mice proliferated equally in response to 1–10 μg/ml endotoxin (data not shown).

To explore the influence of time and antigen dose on the Ig response, *catS*$^{+/+}$ and *catS*$^{-/-}$ mice were immunized with 15 or 100 μg OVA in adjuvant, boosted, and the antibody titers followed over 4 weeks. The OVA-specific IgG1 and IgG2a antibody titers were comparable in response to 15 or 100 μg of immunogen (Figures 7A and 7B). The IgG2a response to either dose remained profoundly depressed for at least 4 weeks in the *catS*$^{-/-}$ mice. Similar results were seen for IgG3 (data not shown). However, the influence of cathepsin S deficiency on the ova-specific IgG1 response was antigen-dose dependent. At 3 and 4 weeks, the differences in OVA-specific IgG1 between *catS*$^{+/+}$ and *catS*$^{-/-}$ mice were minimized by increasing the initial immunogen from 15 to 100 μg (Figure 7B). This finding is consistent with impaired peptide loading of MHC class II molecules as the underlying APC defect in cathepsin S–deficient mice (Figure 2). When peptide loading is inefficient, an increase in antigen dose should favor antigen presentation, minimizing differences in the ensuing antibody response compared with that of normal mice.

Because of the marked defect in Ig class switching to IgG2a and IgG3, we also explored the ability of the cathepsin S–deficient mice to mount an IgE response. Mice (five in each group) were immunized i.p. with OVA (10 μg) admixed with alum, boosted (8 μg), and then exposed to OVA by nebulization 3 days prior to measurements of blood IgE and lung eosinophilia. This protocol is known to stimulate a vigorous IgE response in mice (Brewer et al., 1998; Taborda et al., 1998). Total IgE increased from 59 ($\pm$ 51) to 301 ($\pm$ 137) ng/ml in *catS*$^{+/+}$ mice and 32 ($\pm$ 29) to 300 ($\pm$ 107) ng/ml in *catS*$^{-/-}$ mice. Further, $>$50% of the bronchial lavage cells in ova-nebulized mice were eosinophils and this also was not different between *catS*$^{+/+}$ and *catS*$^{-/-}$ mice. Thus, in this model of humoral immunity, cathepsin S–deficient mice show no defect, confirming that the mice have no intrinsic defect in class switching and indicating that the influence of cathepsin S on humoral immunity is critically dependent on the milieu and manner in which presentation occurs. This conclusion may account at least in part for the apparent normal number and morphology of germinal centers in hilar nodes and spleens of *catS*$^{+/+}$ and *catS*$^{-/-}$ mice.

Conclusions

Cathepsin S activity is essential for the full range of normal MHC class II–dependent immunity. The mechanism by which cathepsin S promotes immunity is likely explained by its critical influence on the rate of peptide loading of MHC class II molecules (Figure 2A). As antigenic peptides are continuously being generated and

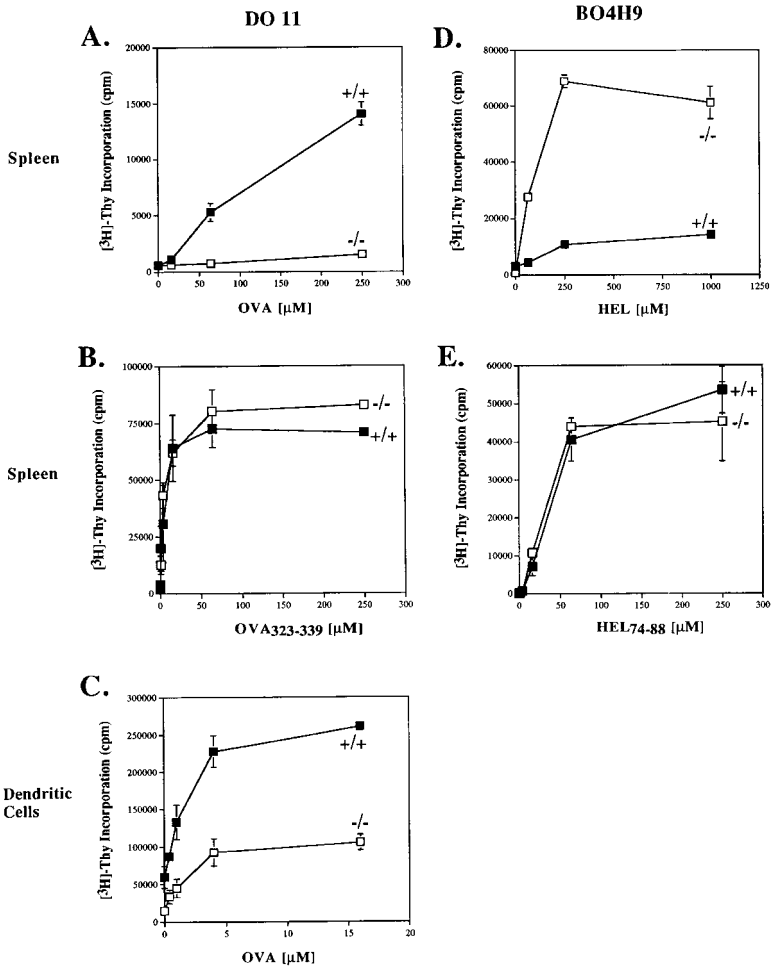
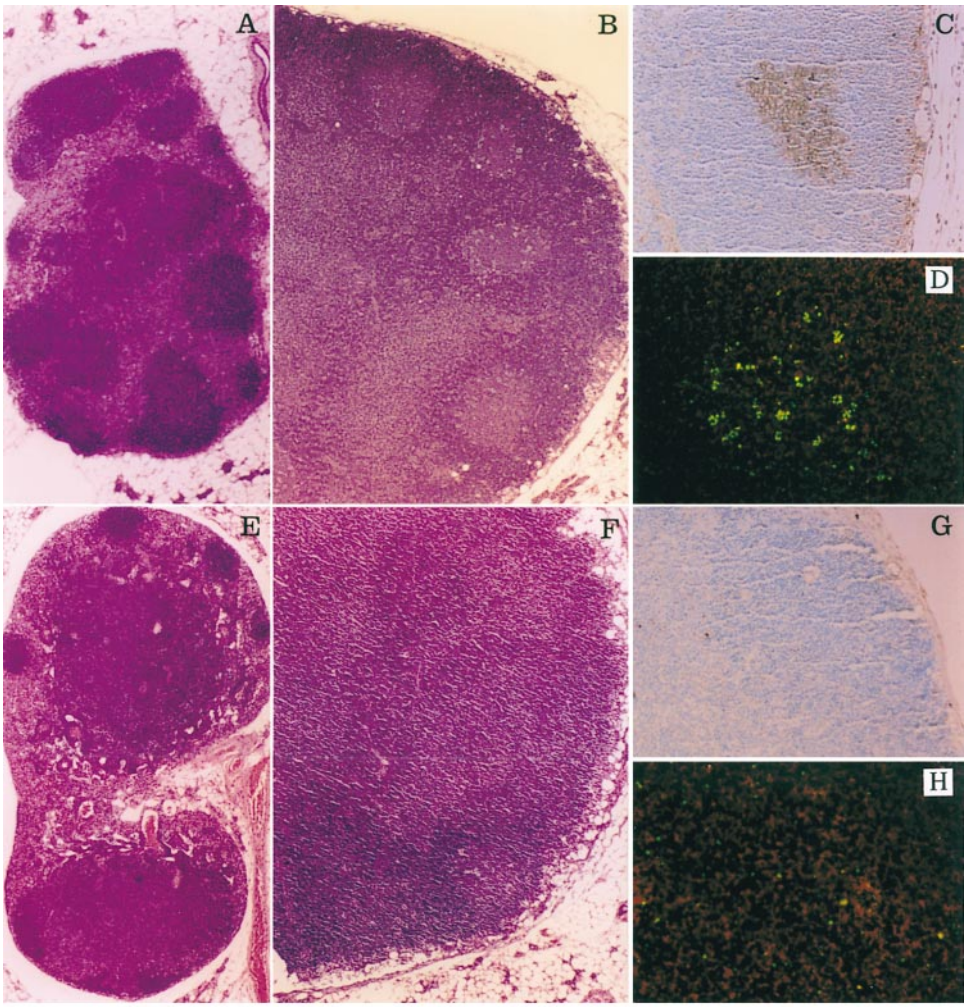


Figure 4. Cathepsin S–Deficient Mice Have Altered Antigen Presentation In Vitro

The graphs illustrate relative IL-2 release by T cell hybridomas (as revealed by [^{3}H]thymidine incorporation by the IL-2 dependent cell line HT2) in response to coculture with splenocytes (A, B, D, and E) or purified splenic dendritic cells (C) from *catS*$^{+/+}$ (closed squares) and *catS*$^{-/-}$ (open squares) mice at the indicated concentrations of OVA (A and C) and HEL (D). The IL-2 responses of the same hybridomas to $OVA_{323–339}$ (B) and $HEL_{74–88}$ (E) peptides at the indicated concentrations are shown for comparison.

degraded within endosomal/lysosomal compartments, efficient capture and presentation of antigens requires MHC class II dimers competent for peptide loading in the right place at the right time. In the absence of cathepsin S, the timing of these events is altered. Peptide loading by MHC class II/Ii p10 is slow and likely limited to those peptide epitopes that are resistant to rapid degradation and capable of being loaded in the presence of retained Ii p10. Trafficking of MHC class II/Ii p10 within APC may also be altered (Brachet et al., 1997). That MHC class II peptide loading nonetheless occurs in this setting is indicated both by the ability of cathepsin S–deficient splenocytes to present an HEL-derived peptide to a T cell hybridoma (Figure 3) and by the presence of surface MHC class II/peptide complexes on splenocytes of *catS*$^{-/-}$ mice (Figure 2B). This may explain why we observed neither major defects in T cell development nor increased autoreactivity in mixed lymphocyte reactions between *catS*$^{+/+}$ and *catS*$^{-/-}$ mice, as only few MHC class II/peptide complexes are required for normal maturation of thymocytes (Bevan, 1997; Fukui et al., 1997; Surh et al., 1997). Even so, in the absence of efficient antigen presentation, the immune responses to antigens administered in Freund's adjuvant were clearly truncated, and antibody isotypes indicative of class switching, as well as germinal center reactions that are sites of class switching, developed weakly or not at all.

The dichotomy between Th2- and Th1-like immune responses in cathepsin S–deficient mice is at present unexplained. A reasonable possibility is that an additional protease, such as cathepsin L, substitutes for cathepsin S in APC in the lung where the TH2-like response examined is largely based. This could explain our recent observations that in vivo administration of an irreversible cysteine protease inhibitor, highly selective but not exclusive for cathepsin S, abrogates IgE responses and lung eosinophilia in the same OVA model of pulmonary hypersensitivity (Riese et al., 1998). Cathepsin L has been implicated in Ii processing in the cortical epithelium of thymus (Nakagawa et al., 1998) and this or another unrecognized enzyme may substitute for cathepsin S in the lung. However, there is at present no evidence for an additional protease and purified dendritic cells express little or no cathepsin L (G.-P. S., unpublished data; Pierre and Mellman, 1998). An alternative possibility is that alterations in the quantity and quality of peptides loaded onto MHC class II dimers in the presence of retained Ii-p10 favor a Th2-like response. Prior in vitro observations indicate that surface peptide/MHC class II densities and peptide affinity for class II molecules influence the pattern of cytokine release by responding T cells (Hosken et al., 1995; Pfeiffer et al., 1995; Schountz et al., 1996; Arps et al., 1998; DiMolfetto et al., 1998). This possibility is favored



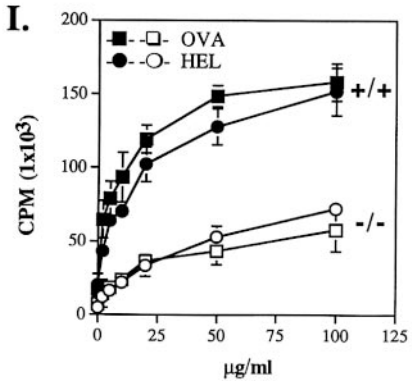


Figure 5. Defective Germinal Center Development, B Cell Selection, and Antigen-Presenting Activity in Lymph Nodes of Cathepsin S–Deficient Mice

Sections of contralateral (A and E) and ipsilateral (B and F) femoral lymph nodes obtained from mice immunized 9 days earlier with OVA and HEL in complete Freund's adjuvant and stained with hematoxylin and eosin (50× magnification), peanut agglutinin ([C and G] 100×), or probed for DNA strand breaks by dUTP-nick labeling ([D and H] 100×). (A–D) show sections from +/+ mice and (E–H) show sections from −/− mice. (I) Polyclonal proliferative responses after 48 hr cocultures of unfractionated lymph node cells from *catS*$^{+/+}$ (closed symbols) or *catS*$^{-/-}$ (open symbols) with OVA (square) and HEL (circle) at the indicated concentrations.

by the difference in the capacity of cathepsin S–deficient mice to mount IgG2a/IgG3 and IgE responses. Although the molecular basis for the selective influences of cathepsin S activity on humoral immunity will require further study, our observations establish cathepsin S as the major Ii processing enzyme in B cells and dendritic

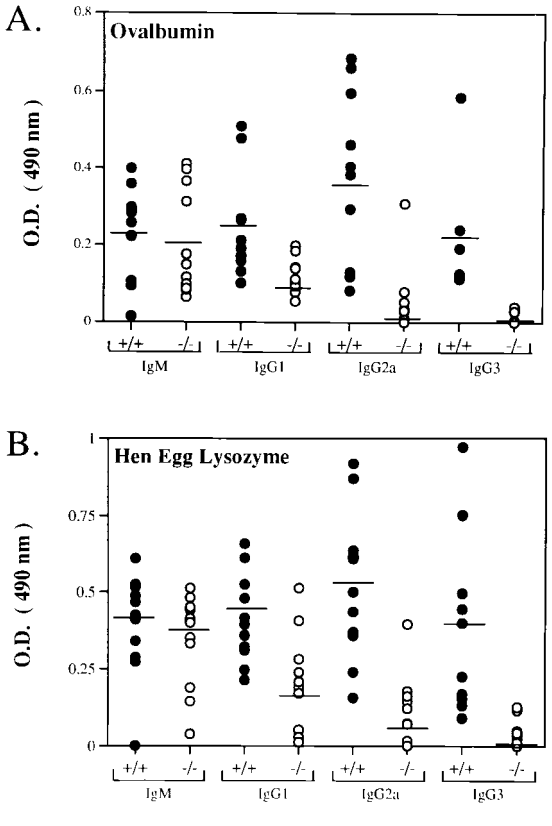


Figure 6. Selective Inhibition of IgG Isotype Responses Following Immunization of Cathepsin S–Deficient Mice

Antigen-specific antibodies were determined by ELISA assays of serial dilutions of serum obtained from each of 11 +/+ and −/− mice 14 days after immunization with a mixture of OVA and HEL and boosting on day 7. For each isotype a dilution range in which optical density (OD) was proportional to dilution was determined, and then antisera of *catS*$^{+/+}$ and *catS*$^{-/-}$ mice were compared at the same dilution. Data are expressed as change in OD between pre- and postimmune serum for each mouse. Antibody titers of IgG1 were reduced 5-fold in *catS*$^{-/-}$ mice; IgG2a and IgG3 of *catS*$^{-/-}$ mice were all reduced ≥10-fold with no titer measurable in several *catS*$^{-/-}$ mice ($p < .005$, Mann-Whitney, for both antigens and all isotypes tested when compared to that of +/+ littermate controls).

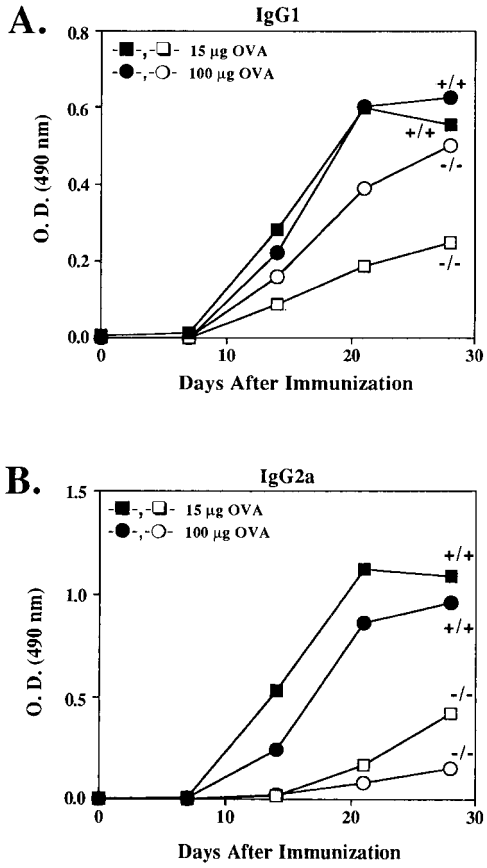


Figure 7. Time Course of IgG Isotype Response to Ovalbumin in Normal and Cathepsin S–Deficient Mice

Mice were immunized with 15 or 100 μg OVA, sera collected at weekly intervals, and IgG isotype titres determined by ELISA as described in Experimental Procedures. Data are shown as relative OD at a 1:10,000 dilution of sera for IgG1 and 1:100 dilution for IgG2a. Comparison of titres between *catS*$^{+/+}$ and *catS*$^{-/-}$ for IgG2a indicated >500-fold reduction in IgG2a titre in the *catS*$^{-/-}$ mice at 2–4 weeks at either dose of immunogen.

cells and demonstrate that the immune response can be strikingly altered in vivo by the activity of a single protease.

Experimental Procedures

Construction of Cathepsin S Targeting Vector

A murine cathepsin S cDNA fragment (500 bp) containing both cysteine and asparagine active sites was generated by reverse transcription and polymerase chain reaction (RT–PCR) using mouse lung RNA and human cathepsin S primers as described (Shi et al., 1992). A P1 clone containing 40 kb of mouse genomic DNA fragment was isolated using the 500 bp mouse cDNA probe (Genome Systems). A 2.3 kb DNA fragment containing exon 4 and a 6 kb fragment containing exon 6 were isolated from a 15 kb ApaI fragment derived from the original P1 clone. Both fragments were confirmed by Southern blot and DNA sequence analysis and subcloned into BamHI sites before and after a neomycin resistant gene (*neo*) of the pPNT targeting vector (Dranoff et al., 1994). The *neo* cassette replaced the active cysteine site within exon 5 (Shi et al., 1994).

Generation of Chimeric Mice

The cathepsin S targeting vector was linearized with NotI digestion and used to transfect D3 ES cells (129/SJ), followed by selections with both 300 μg/ml G418 and 2 μM gangcylovir. The positive ES cell clones, as confirmed by Southern blot analysis, were used to inject 3.5-day-old blastocysts. Germline transmission into C57BL/6 mice was assessed by Southern blot analysis of agouti offsprings. Both targeting vector transfected ES cell clones and the progeny mouse genotypes were determined by digesting genomic DNA with NdeI and SalI followed by probing with an external genomic DNA fragment.

FACS Analysis

Single-cell suspensions were incubated at 4°C for 30 min with appropriate MAb coupled to fluorescent moieties in the presence of Fc block (Pharmingen), washed, and fixed in 1% paraformaldehyde. Samples were analyzed on a FACScan (Becton Dickinson).

Mixed Lymphocyte Reactions

T cells were purified from *catS* +/+, +/−, and −/− splenocytes using nylon columns according to the manufacturer (Polysciences) followed by repeated MHC class II antibody (Pharmingen) and complement (Accurate Chemical) depletions. Such T cells and unfractionated splenocytes from the same mice or from BALB/c mice (I-A^d)

were used as responders at 2×10^5 cells per microtiter well. Single-cell splenocyte suspensions were also prepared from the various mice, irradiated, and used as stimulators (0–8×10^5) in mixed lymphocyte reactions. After 48 hr incubation in RPMI containing 10% fetal bovine serum, 1 μCi of [^{3}H]thymidine was added, and 18 hr later the cultures were harvested (Tomtec) and incorporated thymidine assayed in a Betaplate reader (Wallac). At least five independent experiments in triplicate were performed for each allogeneic or syngeneic mixed lymphocyte reaction.

Pulse–Chase and Immunoprecipitation

Splenocytes were pulsed for 30 min with [^{35}S]methionine (New England Nuclear) followed by incubation in unlabeled medium for the indicated times as described (Villadangos et al., 1997). Cells were then lysed into a buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% NP-40, and 5 mM $MgCl_2$. Protein concentration was normalized to radioactivity of trichloroacetic acid precipitates. Equal radioactivity from each sample was precleared with normal rabbit serum and mouse serum and precipitated with MHC class II antibody (N22, a gift of Dr. Ralph Steinman, New York). An antiserum (JV5) to the NH_2-terminal region of murine Ii, used in some experiments, was provided by Dr. Jean Davoust (Centre d'Immunologie INSERM-CNRS, Marseille, France). Both boiled (B) and nonboiled (NB) samples in sample buffer were separated by 12.5% SDS-PAGE.

Western Blot Analyses of Cells and Tissues

Mouse splenocytes were isolated by mechanically teasing the spleen, followed by red blood cell depletion. Five million cells were lysed into 150 μl of sample buffer and separated onto 12% SDS-PAGE, blotted onto nitrocellulose membrane, and probed with polyclonal antisera against recombinant mouse cathepsin S. The antiserum was raised by immunization of rabbits with a fusion protein comprised of maltose-binding protein (pMAL-cRI, New England Biolabs) and 150 residues of murine cathepsin S extending between the active site cysteine and asparagine residues. Resulting antisera were purified by elution from nitrocellulose-bound fusion protein and verified by Western blotting to react with murine cathepsin S but not other cysteine proteases as described (Pierre and Mellman, 1998; Riese et al., 1998).

Splenocytes, thymocytes, and thymic stromas were frozen in liquid nitrogen. The frozen stromas were pulverized and tissue powders, splenocytes, and thymocytes were lysed in NP-40 lysis buffer with protease inhibitors for 1–2 hr on ice. After centrifugation at 16,000 × g for 10 min, tisssue extracts were collected and the protein concentrations measured. Three hundred micrograms of protein from each sample was dissolved into 100 μl of nonreduced protein sample buffer and either boiled or nonboiled samples separated on 12% SDS-PAGE. Protein gels were transferred onto nitrocellulose filters and immunoblots were then probed with Ii antibody J5V and MHC class II α/β chain antibody Y3P. Horseradish peroxidase–conjugated secondary antibodies were used for signal detection. Filters were developed with ECL (New England Nuclear).

Mouse Dendritic Cell Isolation and Characterization

Spleens were enriched in vivo with dendritic cells by stimulation with flt 3 (Maraskovsky et al., 1996; Shurin et al., 1997). Both *catS* +/+ and −/− mice were subcutaneously injected with 5×10^6 flt3-secreting B16 melanoma cells (C57BL/6 background) prepared by transfection of murine FLT3-ligand (FL) cDNA using the retroviral vector MFG (N. Mach and G. D., unpublished data). Spleens were harvested after the tumors reached 2–3 cm in diameter, at which time harvested spleens were 3- to 5-fold enlarged over that of untreated mice. Whole splenocytes were isolated and resuspended in a high-density BSA solution (1 ml per spleen) containing 10.6 g BSA (Intergen), 18.6 ml PBS, 2.9 ml 1 N NaOH, and 6.5 ml H_2O as described previously (Swiggard et al., 1992). After overlaying 1 ml of ice-cold RPMI, the splenocyte BSA solution was centrifuged for 15 min at 9500 × g. Dendritic cells were recovered from the interface and resuspended into RPMI containing antibodies against B220 and CD3. After incubation for 1 hr at 0°C, complement was added to deplete both B and T cells. The purity of recovered dendritic cells was assessed by FACS analysis using CD11c, CD11b, B220, CD3, and I-A^b antibodies. More than 90% of such cells are $CD11c^+$I-A^b $^{hi}B220^-CD3^-$ (data not shown) and ~5×10^7 cells were recovered from each spleen. Purified mouse dendritic cells were analyzed for cathepsin L expression using Western blot analysis with rabbit anti-mouse cathepsin L polyclonal antiserum (a gift from Dr. M. M. Gottesman, National Cancer Institute). Briefly, 1.5×10^7 cells were lysed into 200 μl 1× reduced protein sample buffer and separated onto 12% SDS-PAGE, followed by blotting onto nitrocellulose filter and staining with cathepsin L antiserum at 1:1000 dilution.

Cell Surface Biotinylation and Immunoprecipitation

Splenocytes were incubated for 20 min in ice-cold PBS containing 0.5 mg/ml NHS-sulfo-biotin (Pierce) at 4°C after cells were pulsed and chased. The biotinylated cells were lysed into NP-40 lysis buffer containing 50 mM Tris–HCl (pH 7.4), 0.5% NP-40, 150 mM NaCl, and 5 mM EDTA, and precipitated with N22 for analysis of MHC class II processing; precipitates were transferred to nitrocellulose filters. Filters were developed with 1:1000 dilutions of peroxidase-conjugated avidin (Sigma) and subsequently exposed to Kodak XOMAT film to visualize metabolically labeled proteins.

Mouse Footpad Immunization and Lymph Node Histology

Ipsilateral and contralateral draining femoral lymph nodes were removed 9 days after footpad immunization with OVA (100 μg) and HEL (100 μg) mixed with complete Freund's adjuvant. The nodes were fixed with 10% formalin, embedded with paraffin, and stained with hematoxylin and eosin. Immunostaining was performed using avidin–biotin complexes (ABC) according to the manufacturer's instructions (Vector). Sections were dewaxed, dehydrated, and nonspecific binding blocked with 10% normal goat serum. Biotinylated peanut lectin (Sigma) (100 μg/ml) was applied to some sections and incubated at 4°C overnight. Endogenous peroxidase activity was quenched using methanol containing 1% hydrogen peroxide. ABC were applied to the samples and incubated at room temperature for 1 hr. Biotinylated tyramide was then added for ~7 min followed by strepavidin-horseradish peroxidase (NEN). Immunopositivity was localized using chromagen diaminobenzidine (0.025%) in PBS and 0.1% hydrogen peroxide. All experiments were performed at least in triplicate.

Lymph Node Apoptosis Assays

Lymph node section apoptosis assays were performed using an Apop-Tag kit (Oncor) according to the manufacturer. Briefly, the lymph node paraffin sections were digested with protease K, incubated with terminal deoxynucleotidyl transferase (TdT) enzyme, stained with anti-digoxigenin-fluorescein, and mounted with a propyiodine (2.5 μg/ml)/glycerol gel (Dako) mixture.

Lymph Node Cell Recall Assay

Lymph node cells were teased from the draining lymph nodes of mice foot pads immunized with OVA and HEL. Cells (8×10^5 per microtiter well) were suspended in RPMI containing 10% FBS and incubated with various amounts of antigen (both OVA and HEL) for 48 hr, followed by addition of 1 μCi of [3H]thymidine. Thymidine incorporation was assessed after another 16 hr of incubation at 37°C.

Immunoglobulin Isotype Switching Analysis

Wild-type (+/+) and *catS*-deficient (−/−) mice were immunized i.p. with 15–100 μg OVA or a mixture of both OVA and HEL (100 μg each) in CFA and boosted with antigens (8 μg) in aluminum hydroxide gel 7 days later. Mouse sera were collected at weekly intervals over 4 weeks. In some experiments, +/+ and −/− mice were immunized i.p. with OVA (8 μg) admixed with alum (1 mg), boosted (8 μg) 5 days later, nebulized with 0.5% OVA in PBS on day 12, and then sacrificed on day 15 for blood collection and bronchoalveolar lavage as described (Riese et al., 1998). In all cases, mouse sera were diluted (2- to 10,000-fold) into PBS containing 1% BSA and 0.05% Tween 20 and analyzed for Ig isotypes by ELISA as previously described (Riese et al., 1998). IgM, IgG1, IgG2a, and IgG3 isotypes were measured as OVA- or HEL-specific antibodies, whereas IgE was measured as total IgE by comparison with a purified IgE standard.

In Vitro Antigen Presentation Assay

Single-cell suspensions were prepared from spleens of *catS* +/+, +/−, and −/− mice. After centrifugation, cells were resuspended in AcK lysis buffer to remove red blood cells, centrifuged, washed, and resuspended in RPMI + HEPES supplemented with 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin. The splenocytes were then plated in 96-well flat-bottom plates (Corning Costar) (5×10^5 cells per well) and γ-irradiated (3300 rads). T-hybridoma cells (10^5) were then added to each well, followed by addition of protein (either OVA or HEL) dissolved in culture media. As controls, $OVA_{323–339}$ and $HEL_{74–88}$ peptides (0–250 μM) were added into wells with the respective hybridomas. Plates were incubated for 18 hr at 37°C, and one-half of the supernatant (100 μl) transferred to 96-well round-bottom plates, frozen, and thawed. The concentration of IL-2 in each well was then determined by incorporation of [3H]thymidine by the IL-2-dependent cell line CTLL2 (ATCC) following standard methods. DO11 (Shimonkevitz et al., 1983) was the gift of Dr. P. Marrack (National Jewish Center). BO4H9 (Miyazaki et al., 1996) was the gift of Dr. D. Mathis (Institut de Chimie Biologique, Strasbourg, France). Hybridomas were maintained in RPMI 1640 + HEPES, 10% FCS, 2 mM L-glutamine, penicillin, and streptomycin.

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