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Regulation of CD1 Function and NK1.1$^+$ T Cell Selection and Maturation by Cathepsin S

Richard J. Riese,[1] Guo-Ping Shi,[2] Jose Villadangos,[3] Daniel Stetson,[2] Christoph Driessen,[3] Ana-Maria Lennon-Dumenil,[3] Ching-Liang Chu,[2] Yuri Naumov,[4] Samuel M. Behar,[1] Hidde Ploegh,[3] Richard Locksley,[2] and Harold A. Chapman[2,5]

[1]Department of Medicine
Brigham and Women's Hospital and
Harvard Medical School
Boston, Massachusetts 02115

[2]Department of Medicine
University of California, San Francisco
San Francisco, California 94943

[3]Department of Pathology
Harvard Medical School

[4]Department of Cancer Immunology and AIDS
Dana Farber Cancer Institute
Boston, Massachusetts 02115

Summary

NK1.1$^+$ T cells develop and function through interactions with cell surface CD1 complexes. In I-A^b mice lacking the invariant chain (Ii) processing enzyme, cathepsin S, NK1.1$^+$ T cell selection and function are impaired. In vitro, thymic dendritic cells (DCs) from cathepsin S$^{-/-}$ mice exhibit defective presentation of the CD1-restricted antigen, α-galactosylceramide (α-GalCer). CD1 dysfunction is secondary to defective trafficking of CD1, which colocalizes with Ii fragments and accumulates within endocytic compartments of cathepsin S$^{-/-}$ DCs. I-A^k, cathepsin S$^{-/-}$ mice do not accumulate class II-associated Ii fragments and accordingly do not display CD1 abnormalities. Thus, function of CD1 is critically linked to processing of Ii, revealing MHC class II haplotype and cathepsin S activity as regulators of NK T cells.

Introduction

CD1 molecules are nonpolymorphic proteins with structural similarities to major histocompatibility complex (MHC) class I molecules (Brossay et al., 1998a; Porcelli and Modlin, 1999; Shinkai and Locksley, 2000). Like MHC class I molecules, CD1 heavy chains associate with β2-microglobulin within the endoplasmic reticulum and present antigens to T cells. In contrast to MHC class I, CD1 proteins are encoded by genes outside the MHC, do not require the transporter associated with antigen processing (TAP) protein, and possess a hydrophobic antigen binding groove that binds lipid and glycolipid antigens (Beckman et al., 1994; Burdin and Kronenberg, 1999; Kawano et al., 1997; Porcelli et al., 1992). Whereas "classical" class I molecules present peptide antigens to CD8$^+$ T cells, CD1 presents lipid antigen to and stimulates a subset of CD4$^+$ and CD4$^-$CD8$^-$ T cells. CD1d, the sole CD1 gene product expressed in mice (Porcelli, 1995), presents lipid antigens to CD4$^+$ or double-negative NK 1.1$^+$ T cells, which express the invariant Vα14Jα281 T cell receptor (TCR) paired with Vβ8, Vβ7, or Vβ2 (Lantz and Bendelac, 1994; Taniguchi et al., 1996). These cells can be characterized functionally by their reactivity to the marine sponge-derived glycolipid, α-GalCer. A second subset of CD1-restricted T cells was identified that expresses a more diverse set of TCRs, does not express the NK complex, and lacks reactivity to α-GalCer (Behar et al., 1999; Cardell et al., 1995; Chiu et al., 1999). The semiinvariant TCR usage by the α-GalCer reactive T cells stands in sharp contrast to the variability of TCRs utilized by class I and class II antigen presentation pathways and suggests a different role in immunity of CD1-restricted T cells.

CD1-restricted T cells are also characterized by their rapid and robust release of cytokines upon stimulation (Hayakawa et al., 1992; Yoshimoto and Paul, 1994), including IFN-γ, IL4, and IL10. This cytokine output influences the balance of the Th1/Th2 adaptive responses during the initial stages of the immune response. The rapid release of cytokines along with a more restricted TCR usage may imply that CD1-restricted T cells play a prominent role in the innate immune response (Bendelac, 1995a; Shinkai and Locksley, 2000; Yoshimoto et al., 1995). Experimentally, CD1-restricted T cells have been implicated in preventing development of diabetes mellitus and protecting against parasitic infection (Schofield et al., 1999; Wilson et al., 1998).

An important characteristic of murine CD1d is its exposure to endocytic compartments during intracellular trafficking (Brossay et al., 1998b; Chiu et al., 1999; Shinkai and Locksley, 2000). CD1d molecules are synthesized within the endoplasmic reticulum and associate with β2-microglobulin shortly after biosynthesis. After traversing the Golgi apparatus, CD1 complexes are transported to the cell surface, whereupon they encounter, bind, and display certain lipid and glycolipid antigens. Surface-displayed CD1 molecules may be internalized to endosomes, and colocalize with MHC class II molecules via a tyrosine-based targeting motif in the cytoplasmic tail of CD1(Jackman et al., 1998). This endosomal trafficking is critical for the presentation of some, but not all, CD1-displayed antigens. Cells expressing mutant CD1 molecules that lack the tyrosine-based internalization motif are unable to activate NK1.1$^+$ Vα14Jα281 T cells but can stimulate non-NK CD1-restricted T cells (Chiu et al., 1999). Thus, "sampling" of the endocytic environment by CD1 molecules exposes CD1 to endosomal antigens as well as to the MHC class II-related machinery. This, in turn, creates a potential for "crosstalk" between the class II and CD1 presentation pathways.

Two cysteine proteases, cathepsins S and L, play prominent roles in invariant chain (Ii) degradation and MHC class II maturation in two different cell types. The activity of cathepsin S is tightly linked to Ii degradation and intracellular trafficking of class II-Ii and class II-peptide complexes in B cells and DCs (Driessen et al., 1999;

[5]Correspondence: halchap@itsa.ucsf.edu

Nakagawa et al., 1999; Riese et al., 1996; Shi et al., 1999). Antigen-presenting cells (APCs) from I-A^b mice that lack cathepsin S activity fail to completely degrade Ii, with attendant accumulation of a class II-associated 10 kDa Ii fragment (lip10 Ii) within endosomes and disruption of normal class II intracellular trafficking (Driessen et al., 1999). This, in turn, impedes class II-peptide complex formation and class II-restricted antigen presentation of some, but not all, peptide antigens. Given these dramatic alterations in class II biochemistry in cathepsin $S^{-/-}$ mice, its phenotype has been ascribed largely to these changes. However, this may be a naive assumption, as the other effects of cathepsin S deficiency on immune function remain to be investigated. Cathepsin L plays a role analogous to cathepsin S, but does so in cortical thymic epithelial cells (Nakagawa et al., 1998). Cathepsin $L^{-/-}$ mice have reduced $CD4^+$ T cells, attributed to aberrant positive selection driven by cortical thymic epithelial cells.

The T cell selection defect found in cathepsin L-deficient mice combined with cathepsin S's similar role in bone marrow-derived APCs, and potentially thymic dendritic cells, led us to hypothesize that the cathepsin $S^{-/-}$ mice might also exhibit abnormal T cell selection. While preliminary analyses showed no overt defect in $CD4^+$ or $CD8^+$ T cell populations, the data reported here show a defect in thymic $NK1.1^+$ T cell selection and maturation and dysfunctional CD1-restricted antigen presentation by thymic DCs. These abnormalities appear to be causally related to accumulation of Ii fragments and MHC class II-Ii complexes, which disrupt normal trafficking of CD1d molecules.

Results

Cathepsin $S^{-/-}$ Mice Exhibit Defective $NK1.1^+$ T Cell Selection

We investigated T cell populations in the cathepsin S-deficient mouse. Peripheral or thymic $CD4^+$ and $CD8^+$ T cell populations appear normal in cathepsin $S^{-/-}$ mice. However, further investigations reveal the surprising finding that in I-A^b, cathepsin $S^{-/-}$ mice $NK1.1^+$ T cells are reduced in thymus and spleen, as demonstrated by decreased staining for the NK1.1 antigen (Figure 1A). The magnitude of this reduction is slightly less than that seen in $CD1^{-/-}$ animals (Figure 1B). Interestingly, $Ii^{-/-}$, but not class $II^{-/-}$, mice exhibit a similar defect in $NK1.1^+$ T cell selection, implicating Ii expression and/or processing as an element that contributes to selection of $NK1.1^+$ T cells (Figure 1A). Two other methods for detecting invariant Vα14Jα281 T cells include double staining for $CD44^{high}/V\beta8^+$ T cells (Figure 1B) and staining with CD1-α-galactosylceramide tetramers (Figure 1C) (Benlagha et al., 2000; Matsuda et al., 2000). Both methods reveal decreases in this cell population similar to that by utilizing an antibody against the NK1.1 T cell antigen, confirming our observations. There are no observable differences in double-negative, single-positive, or double-positive thymocytes between wild-type and cathepsin $S^{-/-}$ mice. Thus, the percentage decrease in $NK1.1^+$ T cell selection in cathepsin $S^{-/-}$ mice is also reflected in absolute numbers.

Mice deficient in CD1d are devoid of $NK1.1^+$ T cells, from lack of positive selection (Chen et al., 1997; Mendiratta et al., 1997) (Figure 1B). Since the cathepsin S and CD1d genes are both located on mouse chromosome 3, one simple explanation for the deficiency in $NK1.1^+$ T cell selection observed in cathepsin S knockout mice would be the absence of CD1 expression, either through inadvertent disruption of the gene with the cathepsin S/neo construct or by another, less obvious, mechanism. However, the finding that cells from cathepsin $S^{-/-}$ mice express CD1 at levels equal to that of wild-type control mice on thymocytes, splenocytes, and flt3 ligand (flt3L)-derived DCs eliminates this explanation (Figure 1D). This prompted us to investigate CD1d function as an alternative explanation for the $NK1.1^+$ T cell defect.

CD1d-Restricted Antigen Presentation Is Defective in Cathepsin $S^{-/-}$ Mice

Can aberrant antigen presentation by CD1 account for defective $NK1.1^+$ T cell selection? To study potential conformational alterations of cell surface CD1d complexes between wild-type and cathepsin $S^{-/-}$ APCs, thy1.2-depleted splenocytes were utilized to stimulate a panel of CD1-restricted T cell hybridomas (Figure 2A). Five of these hybridomas express the invariant Vα14Jα281 TCR (3C3, DN32, 24.7, 24.8, 24.9) and three express a more variable TCR α chain (14S.6, 14S.7, 14S.10) (Behar et al., 1999; Gumperz et al., 2000). Several of these hybridomas, notably 3C3, 24.9, 14S.7, and DN32, recognize CD1d complexes on wild-type APCs much more readily than on cathepsin $S^{-/-}$ cells. Hybridoma 24.8 exhibits no difference in stimulation and IL2 generation between wild-type and cathepsin S-deficient splenocytes. Interestingly, clone 14S.6 consistently demonstrates moderately increased activation when incubated with cathepsin $S^{-/-}$ APCs. These data suggest that there are conformational alterations of CD1d on cathepsin $S^{-/-}$ APC, potentially representing CD1d complexes associated with different antigenic epitopes. Thus, absence of cathepsin S activity alters CD1d function by affecting the cell surface antigenic display.

Do alterations in CD1d function also exist on thymic class II^+ cells from cathepsin $S^{-/-}$ mice, thus providing a possible link between cathepsin S activity and $NK1.1^+$ T cell selection/maturation? Class II^+ APCs comprise about 1%–2% of our thymocyte cell preparations. Since direct stimulation of T cell hybridomas requires at least 7.5×10^6 cells/hybridoma, we needed to amplify the signal in order to detect differences in CD1d function in thymocyte class II^+ APC. To do this, we utilized the CD1 "superantigen," α-GalCer, that simultaneously stimulates all T cells expressing Vα14Jα281 TCR, and engenders a rapid and robust release of IFN-γ and IL4. As shown in Figure 2B, thymocytes from the cathepsin $S^{-/-}$ mice exhibit a marked defect in generation of cytokines in response to α-GalCer, with the generation of IFN-γ affected more than IL4. Thymocyte suspensions deficient in class II^+ cells fail to elicit a normal cytokine response, indicating that class II^+ cells are the primary APCs mediating presentation of α-GalCer and stimulation of $NK1.1^+$ T cells.

Is the blunted IFN-γ and IL4 response observed in these thymocyte preparations a consequence of low

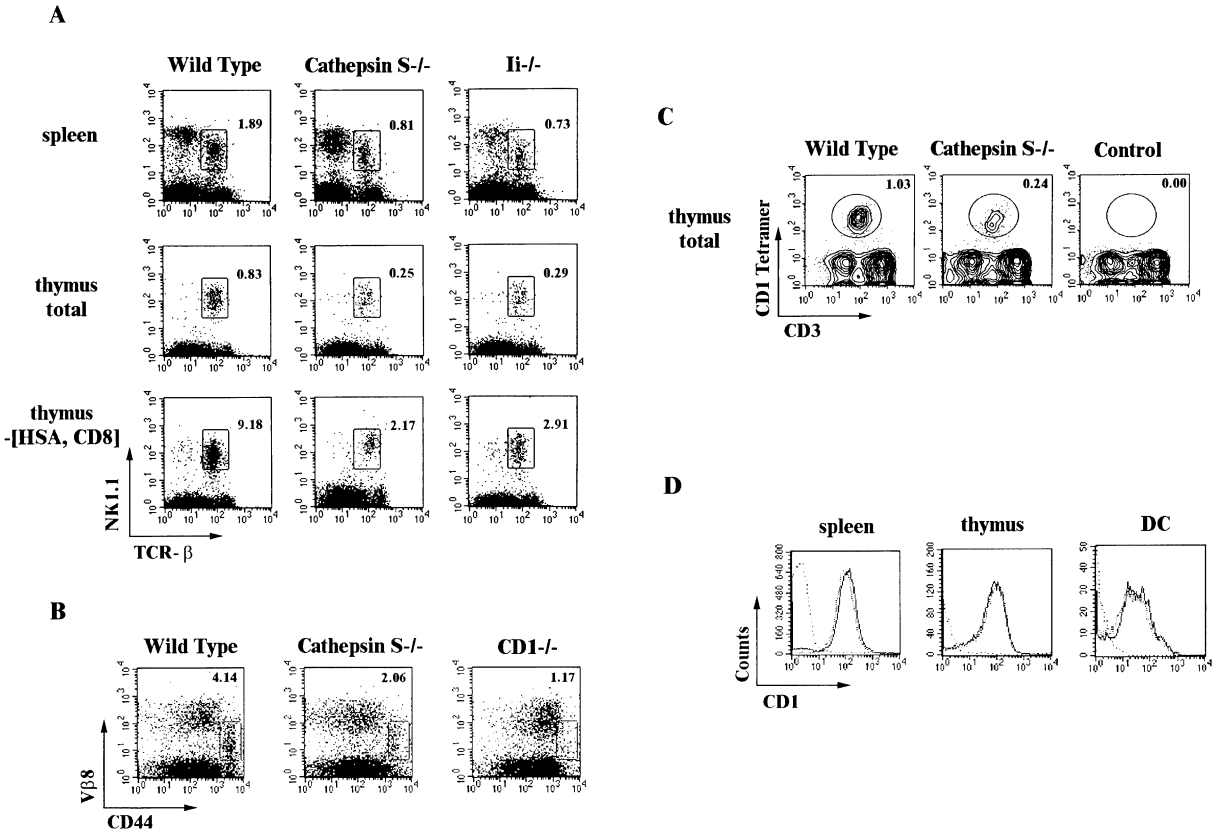

Figure 1. Defective NK1.1+ T Cell Selection in I-Ab, Cathepsin S−/− Mice

(A) Splenocytes, thymocytes, and depleted thymocytes from I-Ab, wild-type, cathepsin S−/−, and Ii−/− mice were stained with TCR-β and NK1.1 antibodies and analyzed by FACS. There are decreased NK1.1+ T cells in both thymocyte and splenocyte cell preparations from cathepsin S−/− and Ii−/− mice, with a more marked decrease in thymocytes.

(B) HSA- and CD8-depleted thymocytes from wild-type, cathepsin S−/−, and CD1−/− mice were stained for the presence of Vβ8+ and CD44high T cells, as a surrogate for NK1.1+ T cells. There are decreased numbers of Vβ8+/CD44high T cells in the thymus of cathepsin S−/− mice, similar to that seen in the CD1−/− thymus.

(C) FACS analysis of splenocytes and thymocytes from wild-type and cathepsin S−/− mice following staining with α-GalCer-CD1 tetramers to identify T cells expressing invariant Vα14Jα281 TCR. As in (A) and (B) above, cathepsin S−/− mice are deficient in this cell population.

(D) Splenocytes, thymocytes, and flt3L-derived DCs from wild-type (continuous line) and cathepsin S−/− (stippled line) mice were stained with anti-CD1 and analyzed by FACS. There is no difference in CD1 expression on these cells. HSA, heat-specific antigen; WT, wild-type.

NK1.1+ T cell numbers alone, or is there an additional antigen presentation defect? To investigate this possibility more directly, thymocytes, depleted of TCR-β+ and NK1.1+ cells, were utilized as APCs in presentation of α-GalCer and Gal(α1→2)α-galactosylceramide (Gal(α1→2)α-GalCer), a digalactose analog of α-GalCer, to the CD1-restricted T cell hybridoma 3C3 (Figure 2C). Gal(α1→2)α-GalCer was used along with the monosaccharide α-GalCer because it has a stringent requirement for endosomal trafficking and processing (Prigozy et al., 2001). Depleted thymocytes from cathepsin S−/− mice are deficient in their ability to present either α-GalCer or Gal(α1→2)α-GalCer to 3C3 cells, as compared with wild-type thymocytes. 3C3 cells alone (without APCs) are capable of low-level autostimulation with both antigens (closed triangles), as they express low levels of CD1. When taking this autostimulation into account, IL2 generation using cathepsin S−/− APC is decreased by >75% and >80% for α-GalCer and Gal(α1→2)α-GalCer, respectively, as compared with wild-type thymocytes. Thus, thymic APCs from cathepsin S−/− mice display impaired CD1d function. Interestingly, Ii−/− thymic APC display no defect in processing and presentation of α-GalCer or Gal(α1→2)α-GalCer, indicating that the defect in NK1.1+ T cell selection seen in these animals potentially occurs via a different mechanism (data not shown).

In an independent set of experiments, thymic DCs (I-Ab+/CD8α+) and NK1.1+ cells were purified by sorting from wild-type and cathepsin S−/− thymi, and CD1-dependent antigen presentation assays performed in the presence of α-GalCer (Figure 2D). These experiments were conducted to pinpoint and certify CD1d dysfunction in cathepsin S−/− thymic DCs, and to look at whether cathepsin S−/− NK1.1+ T cells are able to recognize and respond to CD1d antigens. Cathepsin S−/− DCs (open circles), as compared with wild-type (open squares), generate a markedly reduced IFN-γ response from wild-

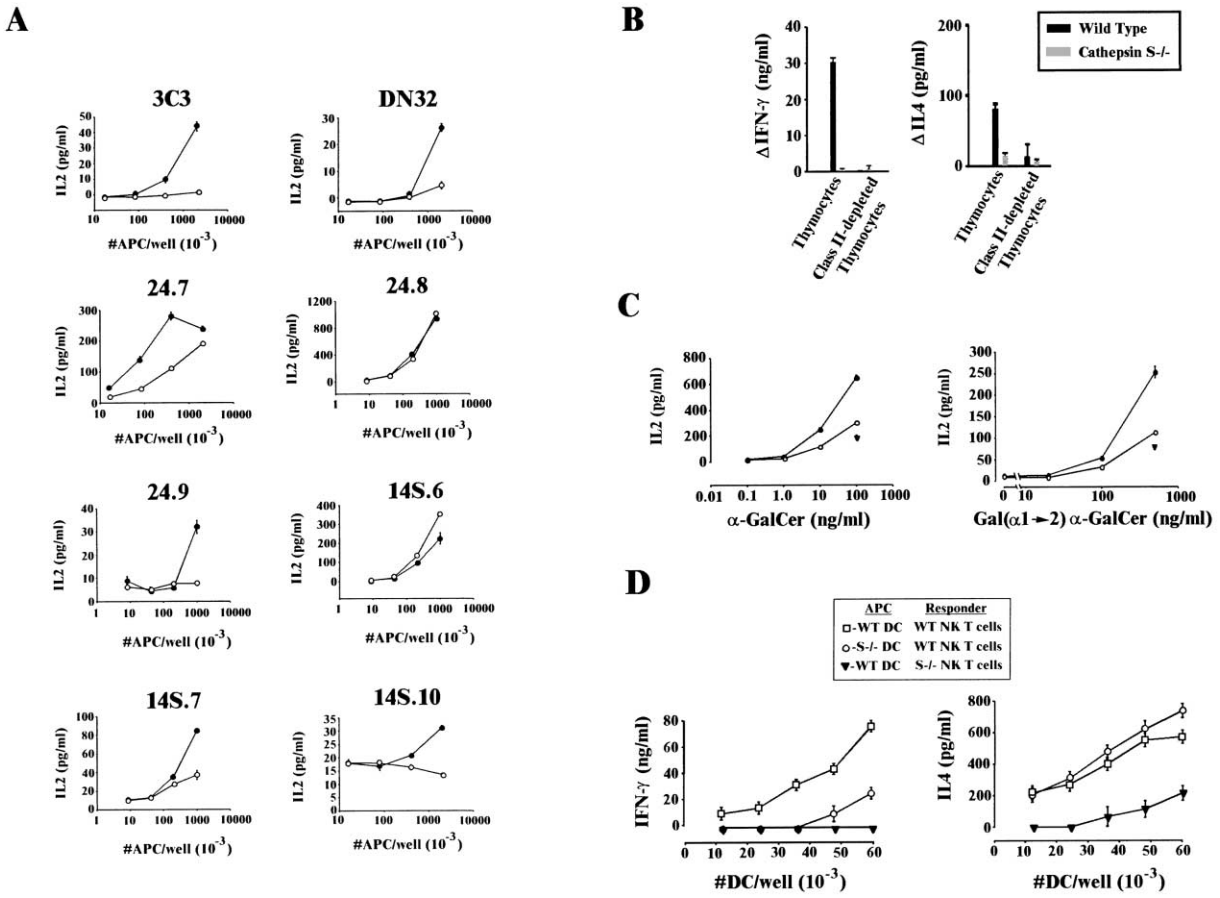

Figure 2. Defective CD1-Dependent Function of Cathepsin $S^{-/-}$ APCs and NK1.1$^+$ T Cells

(A) Thy1.2-depleted splenocytes derived from wild-type (closed circles) and cathepsin $S^{-/-}$ (open circles) animals were incubated with CD1-restricted T cell hybridomas for 24 hr. IL2 levels in the supernatants were measured by ELISA.

(B) Thymocytes (10^6/well) from wild-type and cathepsin $S^{-/-}$ mice were incubated with vehicle and 100 ng/ml of α-GalCer for 48 hr. The differences in cytokine generation between vehicle- and α-GalCer-treated cells were measured by ELISA. Cathepsin $S^{-/-}$ thymocytes show a markedly attenuated cytokine response, IFN-γ more affected than IL4, as compared with wild-type. Thymocytes depleted of class II$^+$ cells fail to elicit normal IFN-γ and IL4 responses. Data (mean ± SEM of triplicates) represent the change in cytokine generation between controls (without α-GalCer) and experimentals. In the absence of α-GalCer control wells generated 0.7 ng/ml IFN-γ and undetectable levels of IL4.

(C) Thymocytes (500 k/well), depleted of TCR-β$^+$ and NK1.1$^+$ cells, were incubated with 3C3 cells (100 k/well) for 24 hr in the presence of α-GalCer or Gal(α1→2)α-GalCer. Cathepsin $S^{-/-}$ thymic APCs (open circles) are defective in their ability to present these CD1-restricted antigens to T cells, as compared with wild-type (closed circles). 3C3 cells alone generate a small amount of IL2 when incubated with maximal concentrations of α-GalCer and Gal(α1→2)α-GalCer (closed triangles). Each data point represents the mean ± SEM of three wells. APCs with antigens alone (no 3C3 cells) failed to generate measurable IL2.

(D) Thymic DCs (class II$^+$/CD8α$^+$) and NK1.1$^+$ T cells from wild-type and cathepsin $S^{-/-}$ mice were isolated by FACS sorting and incubated together in the presence of 100 ng/ml α-GalCer for 48 hr. Cathepsin $S^{-/-}$ DCs (open circles) exhibit a striking defect in stimulating wild-type NK1.1$^+$ T cells (30 k/well) to generate IFN-γ, but not IL4, as compared with wild-type DCs (open squares). Cathepsin $S^{-/-}$ NK1.1$^+$ T cells (30 k/well) are unable to generate either cytokine when stimulated with wild-type DCs (closed triangles). Each data point represents the mean ± SEM from triplicate wells. Control wells (no α-GalCer) failed to generate any measureable IFN-γ or IL4.

type NK1.1$^+$ T cells. Interestingly, the ability of cathepsin $S^{-/-}$ DCs to generate an IL4 response from wild-type NK T cells is not affected. Furthermore, the NK1.1$^+$ T cells from cathepsin $S^{-/-}$ mice are markedly defective in their response to α-GalCer presented by wild-type DC (closed triangles). They fail to generate either IFN-γ or IL4, suggesting that a maturational defect in NK1.1$^+$ T cell development secondary to defective CD1 functioning on thymic DCs may be a contributing factor. Regardless, the abnormal thymic selection and function of NK1.1$^+$ T cells seen in cathepsin $S^{-/-}$ mice is linked to defective CD1-dependent antigen presentation. One potential mechanism for these data, i.e., aberrant intracellular trafficking of CD1 caused by accumulation of Ii fragments and class II-Ii complexes, is investigated in the remainder of this manuscript.

Aberrant CD1 Intracellular Trafficking in Cathepsin $S^{-/-}$ APCs

The processing of Ii and its fragments by cathepsin S regulates the intracellular trafficking of MHC class II molecules in B cells and DCs (Brachet et al., 1997;

Driessen et al., 1999; Pierre and Mellman, 1998). This defect is especially pronounced in APCs from I-A^b, cathepsin S$^{-/-}$ animals. Mouse CD1d, like class II, requires endosomal trafficking for acquisition and subsequent display of some CD1d-restricted antigens (Chiu et al., 1999). To determine if the intracellular trafficking pathway for mouse CD1d is likewise disrupted in APCs from cathepsin S$^{-/-}$ mice, confocal colocalization studies were conducted in cathepsin S$^{-/-}$ flt3L-derived DCs (Figure 3). There is an impressive accumulation of endosomal CD1d molecules in cathepsin S$^{-/-}$ DCs, with increased colocalization of CD1d with class II, Ii, and cathepsin D molecules. In fact, quantitative analysis of these images reveal an almost 100% colocalization of CD1d and the N terminus of the Ii in cathepsin S$^{-/-}$ DCs. Thus, similar to that observed with MHC class II complexes, the intracellular trafficking of CD1d is notably disrupted in I-A^b, cathepsin S$^{-/-}$ mice. In Ii$^{-/-}$ flt3L-derived DCs, there is no colocalization of CD1d with intracellular MHC class II or cathepsin D, again indicating the molecular and cellular bases for defective NK1.1$^+$ T cell selection in Ii$^{-/-}$ and cathepsin S$^{-/-}$ may be distinct (data not shown).

Cathepsin S and lip10 Ii Are Not Expressed in Thymic T Cells

The distribution of cathepsin S and Ii in thymic cells is critical for elucidating the cathepsin S$^{-/-}$ phenotype. To study the distribution of cathepsin S activity in thymic cell preparations, thy1.2$^+$ thymocytes were sorted by FACS and the activity of cysteine proteases determined by labeling cell lysates with ^{125}I-JPM-565, followed by SDS-PAGE and autoradiography (Figure 4A). ^{125}I-JPM-565 irreversibly binds to cysteine proteases in proportion to their activity (Bogyo et al., 2000; Meara and Rich, 1996). Cathepsin S activity is discernable only in the unsorted, and not thy1.2$^+$, thymocytes, consistent with its expression in APCs and not T cells. By immunoblot analysis, expression of Ii and lip10 Ii follows a very similar pattern of distribution, occurring almost exclusively in unsorted thymic cells (Figure 4B). Similar to splenocytes and flt3L-derived DCs, unsorted thymic cells exhibit marked accumulation of class II-lip10 Ii complexes in cathepsin S$^{-/-}$ mice. Low-level Ii expression is seen in CD4$^+$/CD8$^-$ T cells (Figure 4C). However, these CD4$^+$/CD8$^-$ cells from cathepsin S$^{-/-}$ mice fail to accumulate lip10 Ii, consistent with the observation that cathepsin S is not expressed in T cells, and therefore is not involved in degradation of full-length Ii in these cells. Thus, the fact that cathepsin S and lip10 Ii are expressed exclusively in thymic APCs, and not T cells, implies that the NK1.1$^+$ T cell dysfunction observed is mediated through these class II$^+$ thymic APCs.

Abnormal NK1.1$^+$ T Cell Selection Is Haplotype Dependent

The effects of cathepsin S deficiency on accumulation of class II-lip10 Ii complexes, and by implication, its effects on class II trafficking, is strongly haplotype dependent. Villadangos et al. (1997) demonstrated that APCs from mice expressing I-A^b or I-A^d class II molecules exhibited build-up of class II-lip10 Ii complexes upon cathepsin S inhibition, whereas APCs from I-A^k mice did not. This finding can be explained by differences in affinity of the class II molecules for the Ii fragments (Sette et al., 1995). The lower affinity of the I-A^k class II molecules for Ii fragments, compared with I-A^b class II, permits dissociation of class II-lip10 Ii complexes in the absence of complete Ii degradation. One may exploit these haplotypic variations to examine whether the effects of cathepsin S deficiency result directly from loss of enzyme activity or are secondary to incomplete Ii degradation and disrupted class II trafficking within the endosomes of APCs. If the latter explanation is correct, one would expect a far milder phenotype in I-A^k mice.

Homozygous I-A^k cathepsin S$^{-/-}$ mice were generated from C3H parents. To show that indeed APCs from these cathepsin S$^{-/-}$ mice do not accumulate as much class II-lip10 Ii complexes as I-A^b APCs, splenocytes were metabolically labeled with ^{35}S-methionine/cysteine, chased for 4 hr, and class II complexes subsequently immunoprecipitated (Figure 5A). The quantity of class II-lip10 Ii in the I-A^k, cathepsin S$^{-/-}$ splenocytes remains at baseline, whereas there is clear class II-lip10 Ii buildup in the I-A^b, cathepsin S$^{-/-}$ cells. To correlate our biochemical data with the T cell phenotype, thymic cells were stained with CD1-α-galactosylceramide tetramers for detection of T cells expressing the Vα14Jα281 TCR (Figure 5B). Both splenocytes and thymocytes from I-A^b, cathepsin S$^{-/-}$ animals demonstrate a clear reduction in the number of T cells that express Vα14Jα281 TCR, whereas cells from I-A^k, cathepsin S$^{-/-}$ animals show no significant difference as compared with wild-type littermate controls. These findings extend to CD1-stimulated cytokine release. In contrast to I-A^b thymic cells, I-A^k, cathepsin S$^{-/-}$ thymic cells do not exhibit an attenuated IFN-γ or IL4 response to α-GalCer (Figure 5C). Taken together, our data strongly implicate disrupted intracellular trafficking, caused by MHC class II-lip10 Ii accumulation, in defective CD1d function and selection of NK1.1$^+$ T cell in cathepsin S$^{-/-}$ mice.

Discussion

The critical involvement of cathepsin S in Ii degradation and class II trafficking in peripheral APCs is well established. Cathepsin L may play an analogous role in cortical thymic epithelial cells and is required for normal selection of CD4$^+$ T cells. These findings have prompted immunologists to advance the paradigm that differential expression of cysteine proteases, cathepsin S peripherally versus cathepsin L centrally, allows complementary regulation of thymic T cell selection and peripheral T cell stimulation/activation (Cresswell, 1998). The results reported here necessitate a shifting of this theme, that both cathepsins S and L are required for normal T cell selection, but that each may be required for a different thymic T cell population. Thus, the complementary character of cathepsins S and L in development of immunity may not in fact be central versus peripheral, but contained within the thymus itself. Both cathepsins S and L appear to exert their central effects through their involvement in Ii degradation, although each may act in different thymic APC populations.

What are the cells that mediate selection and maturation of thymic NK1.1$^+$ T cells? Previous studies using

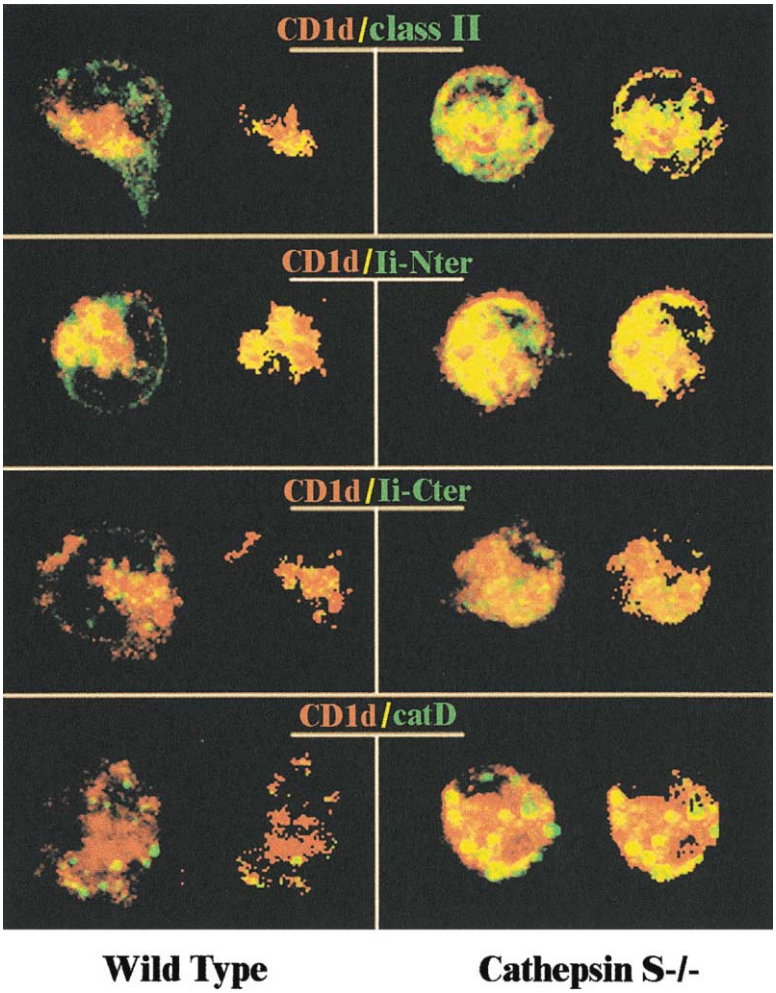

Figure 3. Aberrant CD1d Intracellular Trafficking in Cathepsin $S^{-/-}$ DCs

Flt3L-derived DCs were isolated from wild-type (left panels) and cathepsin $S^{-/-}$ (right panels) spleens and analyzed by confocal microscopy to look for colocalization of CD1d with class II (upper panels), N terminus Ii (second set of panels from top), C terminus Ii (third set of panels from top), and cathepsin D (bottom panels). In cathepsin $S^{-/-}$ DCs, CD1d accumulates within endosomes and colocalizes with class II, Ii, and cathepsin D. The merged image from the green and red fluorescence of one representative cell is displayed (left cell of each panel). A colocalization analysis was performed for the same cell (right cell of each panel), showing the content of all intracellular compartments that display CD1d molecules.

SCID chimeric mice have firmly established $CD4^+CD8^+$ T cells as critical in selection and maturation of $NK1.1^+$ T cells (Bendelac, 1995b; Bendelac et al., 1994). Our data point to thymic DCs as an additional cell type that plays an important role. First, T cells do not express cathepsin S and therefore a deficiency of this enzyme is unlikely to directly alter T cell function. Second, Ii expression is found only on $CD4^+CD8^-$, and not on $CD4^+CD8^+$, or other T cells. Third, $I\text{-}A^b$, cathepsin $S^{-/-}$ mice have normal numbers of thymic $CD4^+CD8^+$ T cells and express a normal level of cell surface CD1d by FACS analysis. Finally, there is a clear abnormality in $NK1.1^+$ T cells derived from cathepsin $S^{-/-}$ animals in their ability to generate IL4 and IFN-γ when stimulated through their TCR.

Based on these data, we propose a model in which both thymic DCs and $CD4^+CD8^+$ T cells play a role in selection and maturation of $NK1.1^+$ T cells. $CD4^+CD8^+$ T cells are necessary, but not sufficient, for normal selection and maturation/differentiation of the full repertoire of $NK1.1^+$ T cells. In this model, $CD4^+CD8^+$ T cells within the thymus are able to mediate differentiation of NK T cells to express CD44 and CD69. Some, but not all, of these CD1d-dependent T cells are also able to express TCR and the NK1.1 antigen, but remain in a less activated state being unable to express large amounts of IL4 and IFN-γ upon stimulation. Thus, these cells are phenotypically distinct from $NK1.1^+$ T cells derived from wild-type animals. DCs appear to augment selection of $NK1.1^+$ T cells within the thymus as well as mediate cytokine activation within these cells to complete the differentiation. The signaling mechanism for this step in $NK1.1^+$ T cell maturation is regulated by class II and Ii within thymic DCs. Thus, use of the cathepsin $S^{-/-}$ animals has revealed a previously unknown interaction among thymic DCs, $CD4^+CD8^+$, and $NK1.1^+$ T cells, and exemplifies the importance of the integrative thymic environment in maturation and development of $NK1.1^+$ T cells.

The attenuation in presentation of the CD1d-restricted epitope Gal(α1→2)α-GalCer as well as the colocalization of intracellular CD1d with MHC class II, Ii, and cathepsin D strongly argue for a CD1d trafficking defect in cathepsin $S^{-/-}$ APCs. Gal(α1→2)α-GalCer is a derivative of α-GalCer and requires endosomal processing by α-galactosidase A prior to recognition by Vα14Jα281 TCR (Prigozy et al., 2001). The data on whether endosomal trafficking of α-GalCer is required for presentation is somewhat equivocal. Presentation of α-GalCer by splenic DCs is completely abrogated by administration of chloroquine or concanamycin A, indicating a require-

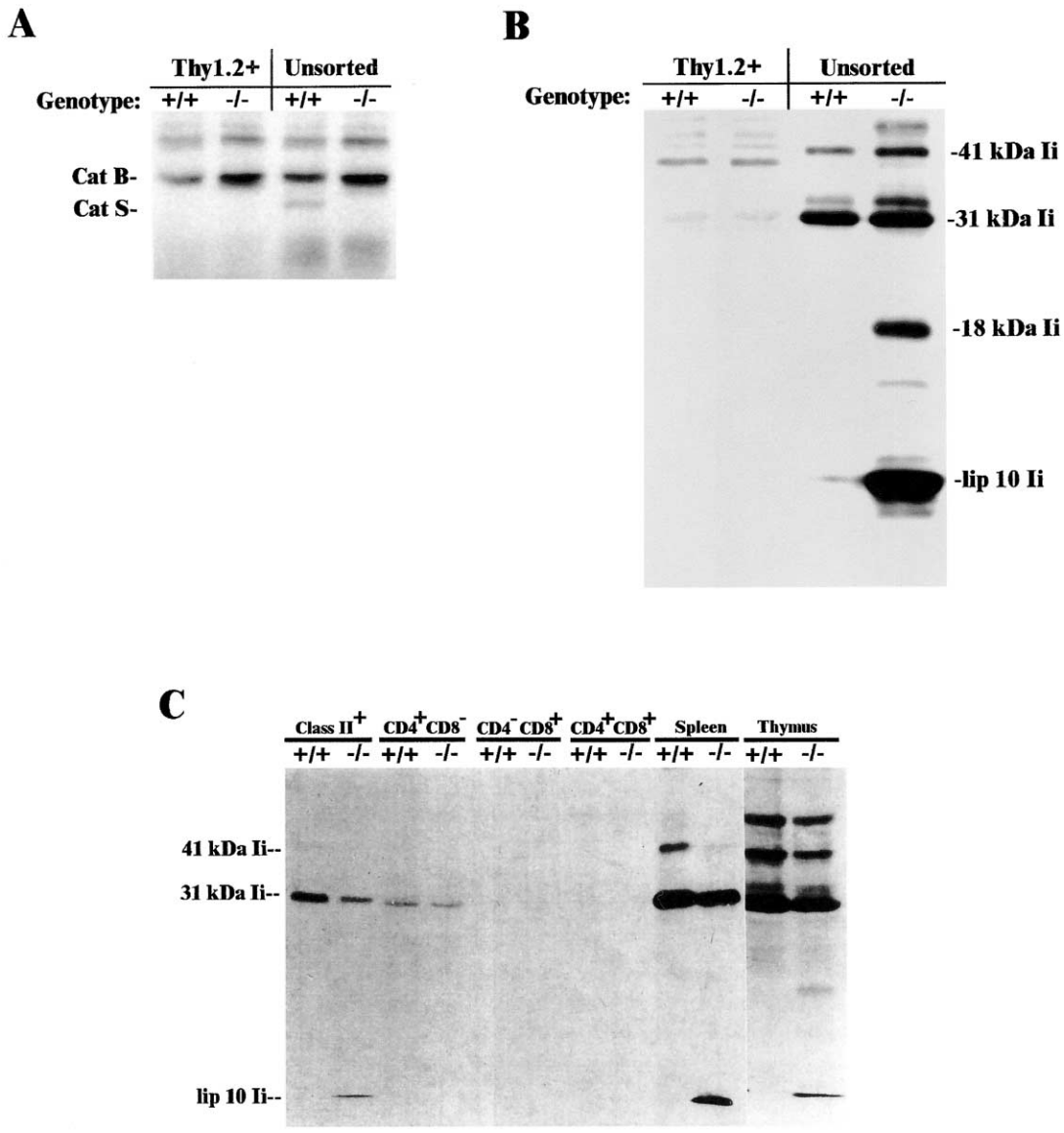

Figure 4. Cathepsin S and lip10 Ii Distributes with Class II$^+$, and Not thy1.2$^+$, Thymic Cells

(A) Thymocytes from wild-type and cathepsin S$^{-/-}$ mice were sorted for thy1.2^{high} surface expression, lysed, and labeled with ^{125}I-JPM-565 (6 million cells/lane). Unsorted thymocytes were run as controls. Cathepsin S is expressed only in the wild-type, unsorted thymocytes.

(B) Thymocytes from wild-type and cathepsin S$^{-/-}$ mice were sorted for thy1.2^{high} surface expression followed by immunoblot analysis for Ii and Ii fragments (In-1 antibody, 5 million cells/lane). Ii has a similar distribution in the thymus as cathepsin S. Cathepsin S$^{-/-}$ thymocytes accumulate lip10 Ii, similar to that seen with peripheral APCs.

(C) Thymocytes were sorted for class II, CD4, and CD8 surface expression, followed by immunoblotting for Ii. Ii and Ii fragments are primarily distributed with class II$^+$ cells, although a small amount of full-length Ii is found in CD4$^+$CD8$^-$ thymocytes.

ment for endosomal function (Kawano et al., 1997). In contrast, CD1-transfected thymocytes are able to effectively present α-GalCer following fixation or treatment with concanamycin A, suggesting that endosomal trafficking is not a requirement for CD1-mediated presentation of α-GalCer in these cells (Burdin and Kronenberg, 1999). Our data that presentation of α-GalCer is blocked to a similar extent as Gal(α1→2)α-GalCer in thymic DCs suggests that endosomal trafficking is required for presentation of this epitope in these cells. Regardless of the case for α-GalCer, CD1-mediated presentation of exogenous ligand(s) required for NK1.1$^+$ T cell selection and maturation might be highly dependent upon endosomal trafficking. This hypothesis is supported by the observation that the reduction in NK1.1$^+$ T cells within the spleen of α-galactosidase A$^{-/-}$ animals is similar in magnitude to the reduction seen in cathepsin S$^{-/-}$ animals (Prigozy et al., 2001).

Although the requirement for endosomal trafficking in presentation of all CD1-restricted antigens is not absolute, disruption of this pathway may have profound effects on CD1d function within the cathepsin S$^{-/-}$ DCs. Experiments utilizing CD1d mutants, which lack the targeting motif in the cytoplasmic tail and hence do not undergo

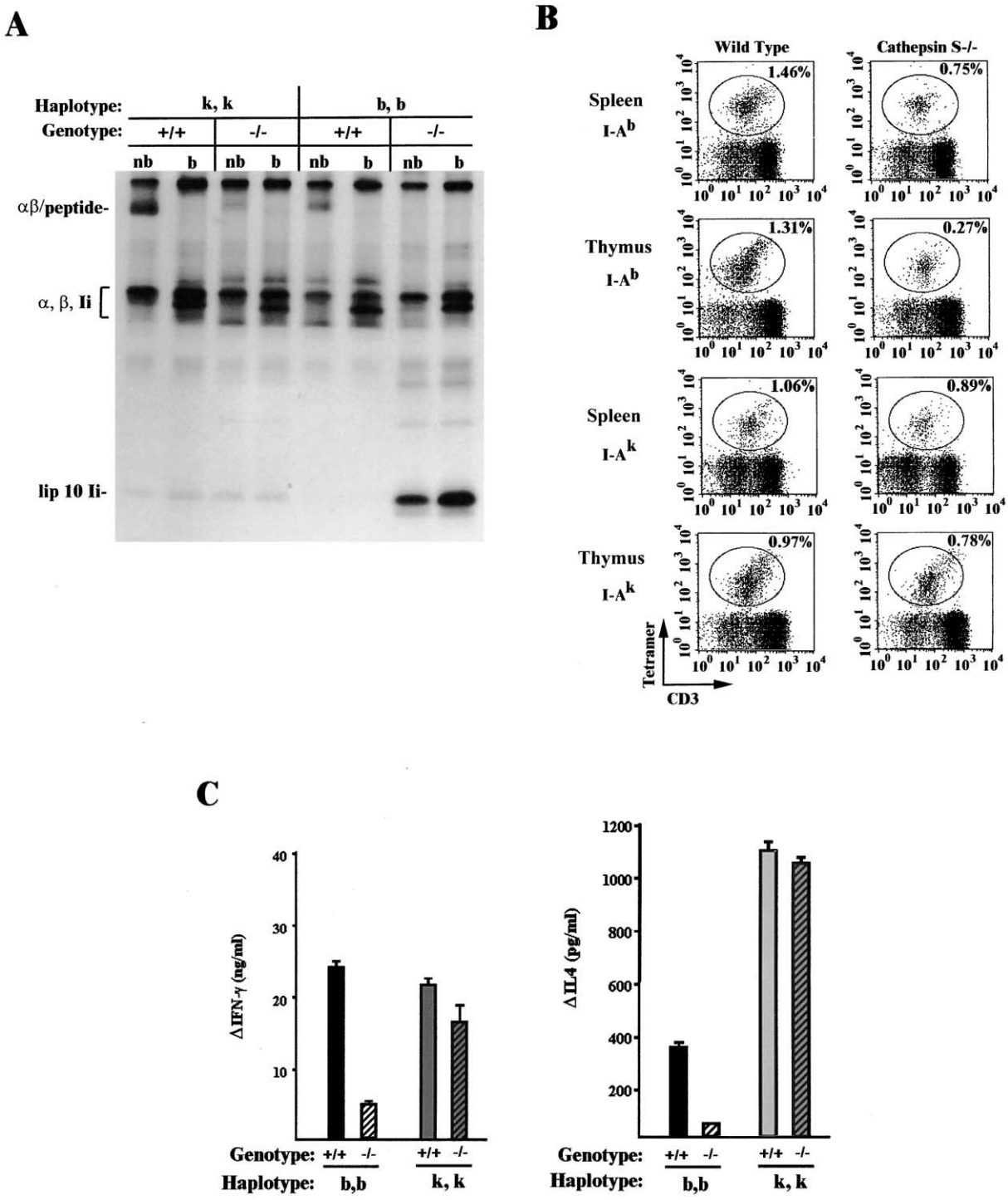

Figure 5. Abnormal NK1.1$^+$ T Cell Selection and CD1 Function Is Haplotype Dependent

(A) Splenocytes from I-A^b and I-A^k, wild-type and cathepsin S$^{-/-}$ mice were pulsed with ^{35}S-methionine, cysteine and chased for 4 hr, followed by immunoprecipitation of class II complexes and SDS-PAGE analysis. Samples were run under both nonboiled (nb) and boiled (b) conditions to delineate the SDS-stable but heat labile class II-peptide ($\alpha\beta$/peptide) complexes.

(B) Splenocytes and thymocytes from I-A^b and I-A^k, wild-type and cathepsin S$^{-/-}$ mice were stained with α-GalCer-CD1 tetramers and analyzed by FACS. There is no significant NK1.1$^+$ T cell selection defect in I-A^k spleen or thymus.

(C) Thymocytes from I-A^b and I-A^k, wild-type and cathepsin S$^{-/-}$ mice were isolated and incubated with 100 ng/ml α-GalCer for 48 hr. Cytokine generation was determined by ELISA. Data represent mean $\pm$ SEM difference in IFN-γ and IL4 generation between control wells (no α-GalCer) and experimentals, each run in triplicate.

intracellular trafficking, demonstrate a defect in their ability to generate an autologous CD1d-bound ligand that stimulates Vα14+NK1.1$^+$ but not Vα14-NK1.1$^-$ CD1-restricted T cell hybridomas (Chiu et al., 1999). Given these findings, one could hypothesize that cathepsin S$^{-/-}$ APC would behave similarly to the CD1d mutants in their ability to stimulate Vα14$^+$ but not Vα14$^-$ CD1-restricted T cells. However, this is not the case. The ability of cathepsin S$^{-/-}$ splenocytes to activate the CD1-restricted T cell hybridomas is dependent on the specific hybridoma and not on the TCR α chain expression (Figure 2A). This makes a certain amount of sense in light of the confocal data showing that CD1d is taken up very well in cathepsin S$^{-/-}$ DCs, thus displaying a distinct trafficking pattern from the CD1d tail mutants that do not sample the intracellular environment at all. It is likely that CD1d molecules trafficking to different intracellular compartments are affected/altered to varying degrees dependent on the quantity of colocalizing class II-Ii complexes in each specific endosome. The alterations in trafficking and CD1d endosomal sampling found in cathepsin S$^{-/-}$ APC are potentially very complex.

These findings also underscore the fine interplay between molecules clearly involved in the MHC class II (Ii and cathepsin S) and the CD1 antigen presentation pathways within APCs. Proper processing of the class II-associated Ii appears to be required not only for normal class II trafficking but also for intracellular routing and function of CD1d. However, the structural interactions between CD1d and Ii are clearly not as direct as between CD1 and β2-microglobulin, or as between Ii and class II. We were unable to demonstrate direct Ii-CD1 association by in vitro translation of their respective mRNAs under conditions where assembly of classical class I products readily occurs (H.P., unpublished data). Instead, the failure to destroy Ii likely produces a "dominant-negative" effect on intracellular trafficking caused by the accumulation of Ii fragments. Ii contains a leucine-based targeting motif in its N terminus, whereas CD1d contains a tyrosine-based motif. Both these motifs can compete for the same intracellular trafficking machinery, as inferred from delayed uptake of the transferrin receptor following overexpression of either Ii or CD1 molecules (Nordeng and Bakke, 1999). Alternatively, inactivation of cathepsin S within APC may disrupt the normal proteolytic environment resulting in both abnormal trafficking of CD1d and processing of CD1d-restricted antigens. For example, a recent study has identified increased levels of mature γ-interferon-inducible lysosomal thiol reductase and cathepsin L in cathepsin S$^{-/-}$ splenocytes (Honey et al., 2001). Also, flt3L-derived DCs from cathepsin S$^{-/-}$ mice exhibit enlarged endocytic compartments (Driessen et al., 1999). However, the distribution of transferrin receptor and the B cell receptor appear unaffected in I-A^b, cathepsin S$^{-/-}$ APC, and argues against a general defect in endocytosis and intracellular trafficking in cathepsin S$^{-/-}$ cells that also express Ii (Driessen et al., 1999) (H.A.C., unpublished data). These data, when taken together, suggest that CD1 "straddles" both the class I and class II antigen presentation pathways by sampling both class I and class II environments and coopting required associated molecules for proper functioning.

Can the abnormalities in NK1.1$^+$ T cell selection and CD1 function help explain the phenotype of the cathepsin S$^{-/-}$ mouse? Cathepsin S$^{-/-}$ mice exhibit an attenuated Th1 type immune response, e.g., decreased IgG2a and IgG3 production following immunization with Freund's adjuvant, while maintaining normal Th2 type immunity, as exemplified by normal IgE levels in a murine model of pulmonary hypersensitivity (Shi et al., 1999, 2000). One possible link between CD1 function and the Th2 bias seen in cathepsin S$^{-/-}$ mice is our finding that the decrease in α-GalCer-stimulated IFN-γ production by cathepsin S$^{-/-}$ thymic DCs is more pronounced than the decrease in IL4 generation. This could potentially influence the adaptive immune response at an early stage. The alterations in CD1 function observed for the cathepsin S$^{-/-}$ animals, as well as abnormalities in the MHC class II biosynthetic pathway, may play an important role in modulating the integrative immune response.

In summary, in mice expressing class II molecules with high affinity for Ii and Ii fragments, inhibition of cathepsin S activity leads to abnormal selection and maturation/differentiation of the CD1-restricted NK1.1$^+$ T cells. This phenotype correlates well with abnormal intracellular CD1d trafficking and defective CD1-restricted antigen presentation, suggesting a causal relationship. Furthermore, the finding that I-A^k do not exhibit this NK1.1$^+$ T selection defect implies that these alterations are mediated through intracellular accumulation of Ii fragments, perhaps as a consequence of a dominant-negative trafficking defect. These findings illustrate the intricate interplay between the innate and adaptive immune responses, and highlight the importance of these interactions in the development of immunity.

Experimental Procedures

Mice

Cathepsin S$^{-/-}$ mice were backcrossed to C57Bl/6 mice for at least ten generations. Wild-type C57Bl/6 mice were used as controls (Jackson Laboratories). All animals were maintained under pathogen free conditions at the animal facilities of Harvard Medical School and the University of California at San Francisco in compliance with institutional guidelines and used at 6–10 weeks of age. Cathepsin S$^{-/-}$ mice expressing homozygous I-A^k were also generated by interbreeding. Littermate I-A^k (C3H), cathepsin S$^{+/+}$ animals were used as controls.

Antibodies

Antibodies against mouse NK1.1, TCR-β, Vβ8, CD44, HSA, CD8α, MHC class II, thy1.2, CD4, Ii (In1), CD1, and CD3, as well as isotype controls for the FACS analysis, were all purchased from PharMingen. JV2 (MHC class II β chain), JV5 (N terminus Ii), and JV11 (C terminus Ii) were generated as previously described (Driessen et al., 1999). Cathepsin D antibody was from Santa Cruz.

Cell Preparation

Whole thymocyte suspensions were enriched for NK1.1$^+$ T cells by complement lysis of HSA- and CD8-positive cells. In brief, cell suspensions were incubated with anti-HSA and anti-CD8 antibodies at 4°C for 30 min. After removal of unbound antibody by washing, rabbit complement (Accurate) was used to lyse HSA$^+$ and CD8$^+$ cells (37°C, 45 min). This resulted in a >95% reduction in total cell number and virtually complete elimination of HSA$^+$ or CD8$^+$ cells. The resulting NK1.1$^+$ T cell-enriched thymocyte-populations (thymocytes [HSA, CD8] were routinely characterized by FACS (CD4, CD8, TCR-β) and were consistent within each experiment as well as between individual experiments. Similar methodology was used to generate class II-deficient thymocytes and thy1.2-depleted splenocytes.

FACS Analysis and CD1-α-galactosylceramide Tetramer Staining

For FACS analysis, single-cell suspensions were incubated for 30 min at 4°C either with the appropriate conjugated antibodies or a nonconjugated primary antibody, washed, and then at least 20,000 cells for each panel analyzed on a FACScan (Becton Dickinson) using Cell Quest software. Isotype controls were included in all experiments.

Soluble murine CD1d molecules were generated, loaded with α-GalCer or a vehicle control, and coupled to fluorescent neutravidin-PE as described (Matsuda et al., 2000). Cell suspensions were stained at 2×10^8/ml for 45 min with predetermined optimal concentrations of CD1d tetramers and cooled on ice before addition of antibodies and staining for an additional 45 min: α-NK1.1-FITC, α-CD8α-TriColor (CalTag), α-CD45R/B220-TriColor (CalTag), and α-CD3-APC. Following washing, cells were resuspended at a final concentration of 2×10^7/ml and analyzed on a dual laser FACSCalibur flow cytometer (Becton Dickinson). Acquired data were analyzed using FlowJo software (Treestar, Inc).

Antigen Presentation Assays

Thymocytes and splenocytes from wild-type and cathepsin $S^{-/-}$ mice were washed and resuspended in DMEM supplemented with 10% heat-inactivated FCS, L-glutamine, nonessential amino acids, HEPES, sodium pyruvate, penicillin, streptomycin, and 50 μM 2-mercaptoethanol (complete medium) at 5 million cells/ml. Cell suspensions were added to each well of a 96-well plate (200 μl/well, 1 million cells/well), and α-GalCer or vehicle added to a final concentration of 100 ng/ml. Samples were incubated for 48 hr and plates were frozen prior to analysis of cytokines in the supernatants. Each sample was run in triplicate.

For the hybridoma experiments, thymocytes and splenocytes were isolated and depleted of thy1.2$^+$ cells, or NK1.1$^+$ cells and TCR-β$^+$ cells. Cells were added to each well of a 96-well plate with antigen and CD1d-restricted T cell hybridomas (100 k/well). Cells were incubated for 24 hr and frozen prior to IL2 supernatant assay. α-GalCer and Gal(α1→2)α-GalCer were gifts of Kirin Pharmaceutical Research Company, Gunma, Japan. The 3C3 Vβ8/Vα14 NK T cell hybridoma was originally generated in the laboratory of Dr. Kyoko Hayakawa, Fox Chase Cancer Research Center (Burdin et al., 2000).

For assays utilizing sorted thymocytes, cells were stained at a concentration of 2×10^8/ml for 45 min on ice with predetermined optimal concentrations of the following antibodies: α-I-A^b-FITC, α-NK1.1-PE, α-CD8α-TriColor (CalTag), α-CD3-APC, α-CD45R/B220-APC-Cy7 (CalTag). Cells were washed and resuspended in PBS/5% FCS at a final volume of 5×10^7/ml, and analyzed and sorted using a MoFlo flow cytometer with CyClone hardware (Cytomation). NKT cells were identified as [CD8α/B220]$^-$, I-A^{b-}, CD3-intermediate, NK1.1$^+$. Thymic lymphoid DCs were identified as CD3$^-$, NK1.1$^-$, I-Ab$^+$, CD8α$^+$, B220$^-$. Cells were sorted into RPMI media/50% FCS, washed, and resuspended in complete medium. Resuspended cells were then plated in triplicate at the indicated concentrations in 96-well round-bottom plates (DCs 12-60 k/well, NK1.1$^+$ T cells 30 k/well) with 100 ng/ml α-GalCer and incubated for 48 hr at 37°C, 5%CO_2. Supernatants were harvested and frozen at −20°C, thawed, and assayed for the presence of IL4 and IFN-γ. As a control for possible activation of NKT cells by the antibodies used for sorting, sorted NKT cells and DCs were incubated at the indicated concentrations without α-GalCer. No proliferation, IFN-γ, or IL4 was detected in any of these control wells (data not shown).

IL2, IL4, and IFN-γ concentrations in the cell supernatants were assayed by sandwich ELISA (PharMingen), using the exact protocol supplied with the product.

Immunofluorescence

Spleens were enriched in vivo with DCs by stimulation with flt3 ligand, and DCs were harvested by BSA-gradient exactly as described (Driessen et al., 1999; Maraskovsky et al., 1996). Freshly isolated DCs were plated in each well of glass chamber slides (Nalge Nunc International) in RPMI medium supplemented with 20% FCS and incubated for 1 hr at 37°C to allow the cells to attach to the slide. Cells were washed once in PBS and fixed for 20 min in a 3.7% solution of paraformaldehyde. After 4 washes in PBS, cells were permeabilized in RPMI medium containing 10% goat serum (GIBCO-BRL) and 0.05% saponin for 15 min. The primary and secondary antibody solutions were prepared in the same medium. Cells were incubated with the antibodies for 30 min, washed 3×, mounted in Aquapoly/Mount solution (Polysciences), and analyzed in a Bio-Rad MRC 1024 confocal laser scanning microscope. The merged images were analyzed for the presence of CD1 in class II, Ii, and cathepsin D positive structures using the colocalization program from Bio-Rad.

Active Site Labeling, Immunoblotting, and Immunoprecipitation

The cysteine protease active site inhibitor JPM-565 (Meara and Rich, 1996) was iodinated via an iodogen-catalyzed reaction. Thymocytes were stained with anti-thy1.2 antibody, washed, and positively stained cells sorted using a MoFlo flow cytometer with CyClone hardware (Cytomation). Sorted and unsorted thymocytes (6 million/sample) were incubated with ^{125}I-JPM-565 and analyzed by SDS-PAGE as described (Shi et al., 2000). To analyze the distribution of Ii, lysates of thymocyte subpopulations were analyzed by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Ii. MHC class II processing was analyzed by pulse-chase analysis of metabolically labeled cells as previously described (Riese et al., 1996).

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